

**PROTECTIVE EFFECTS OF CAFFEINE AND COFFEE CONSTITUENTS IN
INFLAMMATORY MODELS OF DEPRESSION**

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Abstract

Background

Caffeine is the most widely used compound that exerts a pharmacological effect on the central nervous system and is found most predominantly in coffee. It has been well established that coffee has a beneficial effect on numerous disease states including depression.

A number of prospective and cohort studies have shown the benefit of coffee in preventing episodes of depressive symptoms. Studies indicate that the amount of coffee consumed is important with studies showing that moderate intake of caffeinated coffee may be beneficial in decreasing the risk of developing symptoms of clinical depression. It has however been shown that high consumption of coffee increases the risk of depression and suicide. It appears that caffeine in combination with other constituents of coffee may be required to show the antidepressant activity. To date very few studies have been undertaken to evaluate components of coffee for their antidepressant effects and no studies have been undertaken assessing these compounds in combination with caffeine and their subsequent effects on symptoms of depression.

Over the last 50 years there have been countless proposed hypotheses of depression many of which are flawed including the well-accepted monoamine theory of depression. As a result of the shortcomings identified in this theory, numerous newer theories of depression have been proposed in recent times. Evidence strongly supports the cytokine-serotonin theory of depression as a possible and plausible explanation for

the pathophysiology of depression. This theory involves numerous components including increased inflammation, dysregulation of tryptophan catabolism, resulting in the accumulation of the neuroactive compounds known as the kynurenine metabolites and an alteration in the serotonergic system. Due to the complex, heterogeneous nature of depression, this theory may provide a better explanation of the pathophysiology of depression and as a result, this study will evaluate the effects of caffeine and caffeine in combination with other constituents of coffee on the various parameters outlined above.

Hypothesis and aims

It is hypothesised that caffeinated coffee, along with key bioactive coffee constituents will be beneficial in behaviours and biomarkers of depression in *in vivo* and *in vitro* models of depression and inflammation.

There were three aims of the study. The primary aim of this study was to assess the effects of caffeinated coffee, decaffeinated coffee and caffeine on behaviours and biomarkers associated with depression in an *in vivo* inflammatory model of depression. Although traditionally *in vitro* assays are performed prior to *in vivo* assays, in this study this is not the case. This is due to the need to establish the differences in antidepressant-like activities associated with the consumption of caffeine, caffeinated coffee and decaffeinated coffee in order to investigate the compounds causing the possible activity. The secondary aim of this study was to investigate if any key bioactive coffee constituents are lost during the decaffeination process using quantitative HPLC analysis in order to identify possible compounds contributing to the antidepressant effects of

caffeinated coffee. The third aim of this study was to develop a surrogate microglial-like and a surrogate neuronal-like *in vitro* cell-based model and subsequently evaluate the effects of key bioactive coffee constituents, alone and in combination with caffeine, on inflammatory markers and cell death mechanisms.

Methods

The aims of this study were evaluated using a number of different methods. The first aim was evaluated using an *in vivo* mouse model of neuroinflammation where the effects of pre-treatment with caffeine, caffeinated coffee and decaffeinated coffee in LPS-exposed mice was assessed with regards to behaviour, tryptophan catabolism and markers of inflammation.

The second aim of this study was achieved through the development of HPLC methods to quantify the key bioactive coffee constituents. Various brand, bean and roast matched caffeinated and decaffeinated coffee samples were analysed using the developed HPLC methods and differences in constituents identified.

The tertiary aim of this study was achieved through the development of two surrogate cell models. The first, a surrogate microglial cell model and the second a surrogate neuronal cell model. Subsequently, the effects of key bioactive coffee constituents on the parameters of inflammation in the interferon stimulated surrogate microglial cell was assessed along with the neurotoxicity of key bioactive coffee constituents in the developed surrogate models.

Results and discussion

This study showed caffeinated coffee and caffeine to have positive effects on behaviour in animals in the *in vivo* neuroinflammatory model of depression. Furthermore, several of the inflammatory biomarkers associated with depression were also modulated in a positive manner by caffeine and caffeinated coffee. Caffeinated coffee was however shown to have more pronounced effects than that of caffeine alone. The differences in caffeinated coffee and decaffeinated coffee then showed that ferulic acid, one of the key bioactive coffee constituents to be decreased in decaffeinated coffee suggesting that it is lost through the decaffeination process. Successful development of two *in vitro* surrogate cell-based models were then developed representing a microglial-like and a neuronal-like cell. These models were then used to evaluate the effects of coffee constituents on parameters of inflammation in microglial-like cells and neurotoxicity in neuronal-like cells. Coffee constituents alone appeared to produce greater reductions in indoleamine 2,3-dioxygenase (IDO) activity after interferon stimulus in comparison to the constituents used in combination with 100 μ M of caffeine. Furthermore, several coffee constituents were shown to be neurotoxic at high concentrations which may indicate a potential mechanism by which coffee exerts its negative mood effects.

Conclusion

In conclusion, this study highlighted the antidepressant nature of caffeine and caffeinated coffee in an *in vivo* model of inflammatory depression. This evidence provides support to the causal link between caffeine and caffeinated coffee consumption and antidepressant effects. Furthermore, it was shown that ferulic acid, an important

bioactive coffee constituent is lost during the decaffeination process. Investigations, using an *in vitro* cell-based model, have shown that key bioactive coffee constituents may in fact elicit their activities via an anti-inflammatory pathway and through the modulation of IDO activity. Furthermore, the dose dependent negative mood effects associated with coffee may be as a result of certain coffee constituents displaying neurotoxic properties. This study highlights the need for further high quality studies, and in particular, a randomised controlled trial assessing the effects of caffeine and caffeinated coffee consumption on depression.

Statement of originality

This work has not previously been submitted for a degree or diploma at any university.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_____

Susan Hall

Publications during candidature

Hall, Susan, Ben Desbrow, Shailendra Anoopkumar-Dukie, Andrew K. Davey, Devinder Arora, Catherine McDermott, Matthew M. Schubert, Anthony V. Perkins, Milton J. Kiefel, and Gary D. Grant. "A review of the bioactivity of coffee, caffeine and key coffee constituents on inflammatory responses linked to depression." *Food Research International* 76 (2015): 626-636. (Impact Factor = 2.818)

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List of abbreviations

AA	Anthranilic acid
ABC	Avidin-biotin complex
AChE	Acetylcholinesterase
ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
A _{2A} R	A _{2A} receptor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BCG	Bacillus Calmette–Guérin
BChE	Butyrylcholinesterase
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA	Caffeic acid
cAMP	Cyclic adenosine monophosphate
CAPE	Caffeic acid phenethyl ester
CAT	Catalase
CGA	Chlorogenic acid
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CRP	C-reactive protein
CSF	Cerebrospinal fluid

CV	Coefficient of variance
DA	Dopamine
DALYs	Disability-adjusted life years
DAMPs	Damage-associated molecular patterns
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DCF	Dihydrodichlorofluorescein
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
FA	Ferulic acid
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FST	Forced swim test
GABA	gamma-Amino butyric acid
GAC	Glacial acetic acid
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione S-transferase
H ₂ DCF-DA/DCFA-DA	2',7'-dichlorodihydrofluorescein diacetate
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulfuric acid

3-HAA	3-Hydroxyanthranilic acid
HAAO	Hydroxyanthranilic acid oxygenase
HAM-D	Hamilton depression scale
HCA	Hydroxycinnaminic acid
HEPES	4-(2-hydroxyethyl-1- piperazineethanesulfonic acid)
5-HIAA	5-Hydroxyindole acetic acid
3-HK	3-Hydroxykynurenine
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
5-HT	Serotonin
5-HTP	5-Hydroxy-L-tryptophan
5-HTT	Serotonin transporter
ICH	International Conference on Harmonisation
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IS	Internal standard
JAK	Janus kinase
KA	Kynurenic acid

KAT	Kynurenine amino transferase
KCl	Potassium chloride
KH ₂ PO ₄	Potassium phosphate monobasic
KMs	Kynurenine metabolites
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYN	Kynurenine
Kynu	Kynureninase
LC-MS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
L-KYN	L-kynurenine
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LMA	Locomotor activity
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
MADRS	The Montgomery–Åsberg Depression Rating Scale
MAPK	Mitogen-activated protein kinases
MAO	Monoamine oxygenase
MAOI	Monoamine oxygenase inhibitors
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MDD	Major depressive disorder

MHC	Major histocompatibility complex
MnTBAP	Manganese (III) tetrakis(4-benzoic acid)porphyrin chloride
MS	Multiple sclerosis
NA	Noradrenaline
NARI	Noradrenaline reuptake inhibitor
NAD	Nicotinamide adenine dinucleotide
nAChRs	Nicotinic acetylcholine receptors
NaCl	Sodium chloride
Na ₂ HPO ₄	Sodium phosphate dibasic
NaOH	Sodium hydroxide
NET	Norepinephrine transporter
NF-κB	Nuclear factor-kappaB
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OFT	Open field test
OPA	o-Phthalaldehyde
PA	Pyrogalllic acid
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCA	Perchloric acid

PDE3	Phosphodiesterase 3
PGE ₂	Prostaglandin E ₂
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethanesulfonyl fluoride
QA	Quinolinic acid
QC	Quality control
QPRT	Quinolinate phosphoribosyl transferase
RA	Retinoic acid
RIMA	Reversible inhibitor of monoamine oxidase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SERT	Serotonin transporter
SNRI	Selective noradrenaline re-uptake inhibitor
SOD	Superoxide dismutase
SPE	Solid phase extraction
SSRI	Selective serotonin re-uptake inhibitor
STATs	Signal transducer and activator of transcription
TCA	Tricyclic antidepressant
TDO	Tryptophan 2,3-dioxygenase
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
TLR4	Toll-like receptor 4
TMB	3,3',5,5'-Tetramethylbenzidine

TNF- α	Tumour necrosis factor – alpha
Trp	Tryptophan
TRYCAT	Tryptophan catabolite
TST	Tail suspension test
TTBS	Tris buffered saline Tween 20
UV	Ultraviolet
VD ₃	1, 25-dihydroxyvitamin D ₃
WHO	World Health Organisation
YLDs	Years lived with disability
3-NT	3-Nitro-L-tyrosine
α 7nAChR	Alpha-7 nicotinic acetylcholine receptor
ω 3 PUFA	Omega 3 polyunsaturated fatty acids

Chapter One - Literature review

1.1 Introduction

Coffee is a beverage with one of the highest consumption rates in the world with an estimated 16.3 million cups of coffee consumed in Australia alone each day [1, 2]. Extensive studies have been undertaken to assess the biological effects of coffee and its constituents, with numerous health benefits noted including antidepressant activities [3-5]. The antidepressant effect of coffee consumption is of particular interest, as very few studies have investigated these effects with regards to the individual constituents of coffee. To date there have been a limited number of *in vivo* and epidemiological studies assessing the effects of coffee and its key bioactive constituents on depression [3, 4, 6-10].

An inverse correlation between the consumption of caffeinated coffee and depressive symptoms has been shown in a number of large retrospective and prospective human epidemiological studies [3-8, 11, 12]. Interestingly, no association between caffeine consumption alone and antidepressant activity [3] has been shown, suggesting that a combination of caffeine and other bioactive constituents of coffee is required for antidepressant activity. Numerous constituents of coffee have been investigated for their biological effects and have been identified to possess antidepressant and anti-inflammatory properties, although the concentrations tested are much higher than those seen in coffee. Additionally, no studies have assessed the effects of the decaffeination process on the loss of other bioactive coffee constituents to date. Due to these factors, the differences in the bioactive constituent concentrations in caffeinated and decaffeinated coffee will be explored. Furthermore, given the study

limitations identified in the human epidemiological studies, the relationship of caffeine in combination with other constituents of coffee will be evaluated, both *in vivo* and *in vitro*, for their anti-inflammatory and antidepressant effects in inflammatory models of depression.

Over the last 50 years there have been countless proposed hypotheses of depression, the first of which being the monoamine theory of depression. The monoamine theory of depression is the most widely accepted hypothesis of depression. The central theme of the theory is the proposed decrease in the central monoamine neurotransmitters serotonin (5-HT), noradrenaline (NA) and dopamine (DA) [13]. However, a major limiting factor for this theory is that changes in the monoamine level at the synapse occurs within hours of the first dose of an antidepressant medication being given, whereas the antidepressant effects take weeks of continuous therapy to be observed [14]. This subsequently led to the adaptation of the monoamine theory of depression to suggest that the initial increase in the synaptic monoamine levels leads to an adaptive response. The acute increase in monoamine levels serves to desensitize the inhibitory auto- and hetero-receptors, resulting in increased monoaminergic transmission that, time wise, would coincide with the appearance of antidepressant effects [14]. There are still numerous flaws with this theory of depression but it does highlight the importance of 5-HT and NA in the aetiology of depression.

As a result of the shortcomings identified in the monoamine theory of depression, numerous newer theories of depression have been proposed in recent times. The hypothesis that has the most evidence to support it is the cytokine-serotonin theory

of depression [15]. This theory involves numerous components including an increase in inflammation, dysregulation of tryptophan (Trp) catabolism, resulting in the accumulation of the neuroactive compounds known as the kynurenine metabolites (KMs) and an alteration in the serotonergic system [15]. Due to the complex, heterogeneous nature of depression, this theory may provide a better explanation of the pathophysiology of depression. The proposed role of each of the components of the theory, that is inflammation, Trp catabolism and alteration to the serotonergic system, in the pathophysiology of depression will be further explored in greater detail below. Furthermore, the effects of coffee and its key bioactive constituents have on these components will also be explored below.

As outlined above, coffee has been shown, through a number of large studies to exert antidepressant activities, of which it appears caffeine to be an essential component of. This project aims to investigate the combination of caffeine with other constituents of coffee in an *in vitro* model of interferon (IFN) induced inflammation and an *in vivo* model of inflammatory depression for their antidepressant-like activities. Furthermore, their effects on parameters associated with the neuroinflammatory hypotheses of depression including anti-inflammatory and Trp catabolism-altering properties will also be evaluated.

1.2 The antidepressant effects of coffee and its constituents in depression

1.2.1 Coffee

Coffee is a beverage with one of the highest consumption rates worldwide [1] and contains caffeine, the most widely used psychoactive substance [16]. Coffee is well known to have beneficial effects on a number of disease states including type 2 diabetes mellitus, Parkinson's disease, liver disease [17], decreased risk of stroke [18], Alzheimer's disease [19] and depression [3-5].

Human studies

A number of prospective studies have shown a positive association between the consumption of coffee and the prevention of episodes of depressive symptoms. Cross-sectional studies have evaluated the association between caffeine, caffeinated coffee and decaffeinated coffee consumption and the relative risk of depression.

An inverse correlation between the consumption of coffee and the risk of developing depression has been shown [3, 4, 7]. Studies, with male participants, showed a decrease in the risk of depression with moderate consumption by up to 70% in comparison to non-drinkers [3, 4, 7]. No association between caffeine alone or consumption of decaffeinated coffee and the incidence of depression was observed indicating that caffeine in combination with other constituents of coffee may be key in the modulation of biomarkers associated with depression [3]. A smaller study following approximately 500 Japanese men, showed a risk reduction of up to 40% which may be

attributed to either a smaller sample size or ethnicity differences of the two sample populations [11].

Coffee also has beneficial effects in specific patient groups including diabetes. A recent cross-sectional study evaluating the antidepressant effects of coffee in a type 2 diabetic population has shown a strong inverse correlation between coffee consumption (≥ 3 cups per day) and symptoms of depression ($p = 0.023$) [8]. This study highlights the possible antidepressant effects of coffee in a group of known unhealthy individuals however further studies are required to fully investigate if these effects are due to caffeinated or decaffeinated coffee and whether coffee provides prevention or treatment of depressive symptoms.

Given the nature of cross-sectional studies and their short duration of time, in many cases only one time point, limitations exist with regard to the sequence of exposure and disease state and as a result, it is very difficult to infer causality from this type of study [20]. In addition to this, there are limitations with controlling for confounding factors such as the effects of social interactions. This highlights the need to undertake studies that account for and limit the social interaction component.

Numerous prospective cohort studies have evaluated the potential antidepressant effects of coffee and have similarly found a strong inverse correlation between coffee consumption and the risk of clinical depression [4, 12]. In addition, one large study, following over 300,000 older Americans aged 50 to 71 years, found matching

correlations between caffeinated and decaffeinated coffee consumption and the risk of depression suggesting the involvement of other coffee constituents to be evaluated [12]. In contrast to earlier cross-sectional studies, given the large study population, advantages of following individuals over time provides justification for further large studies to investigate the impact of decaffeinated coffee on depression.

A recent systematic review has compiled and analysed the results of the observational studies assessing the correlation between the risk of depression and coffee consumption [6]. A total of 11 observational studies were compiled, with a total of 330, 677 and 38, 223 participants in coffee-depression and caffeine-depression analysis respectively [6]. The analysis showed that both caffeine and coffee were significantly associated with a decreased risk of depression (relative risk of 0.721 and 0.757 respectively). After further dose-response analysis, the association between coffee and depression was linear and the risk of depression decreased by 8% for each cup of coffee consumed per day [6]. In contrast, a non-linear association was found between caffeine consumption and depression. The optimal consumption range of 68 mg per day to 509 mg per day was found in this analysis [6]. The combined results in this meta-analysis provide new insight into the antidepressant effects of caffeine and coffee and further justify the need for high quality controlled studies.

Whilst some coffee consumption appears likely to reduce depression, it is important to recognise that extremely high rates of coffee consumption are correlated with an increased risk of negative mood behaviours and acute health risks associated with depression. Numerous large cohort studies have shown a J-shaped correlation

between coffee consumption and suicide, with a risk reduction of up to 70% in moderate consumers of coffee [4, 5, 21]. It has, however, been shown that heavy consumers of coffee are at higher risk of suicide [21, 22], with people consuming more than 8 cups of coffee per day at an up to 60% higher risk of suicide [22]. Another important factor to consider with the consumption of coffee is its effect on anxiety, another common multifactorial, complex mental health disorder, commonly found to coexist with depression [23]. Coffee has been well reported to cause symptoms of anxiety, a common multifactorial, complex mental health disorder, commonly found to coexist with depression [23]. Coffee has been well reported to cause symptoms of anxiety particularly when consumed excessively or in people with a pre-existing anxiety disorders [24-26] and this has been attributed to the caffeine content of the beverage [27, 28]. Given the co-morbid nature of the two disorders [29], it is imperative that appropriate amounts of coffee are consumed by individuals. These studies highlight the particular importance of the consumption of appropriate quantities of coffee as extremely high rates of coffee consumption are correlated with an increased risk of negative mood behaviours.

The data from the epidemiological studies strongly suggests that coffee possesses antidepressant-like effects. The evidence presented above suggests that combinations of caffeine plus other constituents of coffee, rather than single constituents alone, may be required to show the antidepressant-like activity of caffeinated coffee. It could however be argued that, a psychosocial effect may be the reason for antidepressant-like activity given the evidence to suggest the association between social contact and risk of depression [30]. This suggests that further high quality studies to evaluate the observed effects are appropriate. Studies also highlight

the importance of consumption of appropriate quantities of coffee to avoid the associated negative mood effects. Coffee has a number of other biologically active constituents that may play a role in the proposed antidepressant effects and will be outlined below.

1.2.2 Individual constituents of coffee

Coffee contains numerous biologically active constituents from a number of classes of natural products including polyphenols and alkaloids. The alkaloids found in coffee include caffeine, the most abundant active compound found in brewed coffee and has been shown to have possible anti-inflammatory and immunosuppressant effects [31]. A number of the constituents found in coffee belong to the polyphenol class of natural compounds which broadly categorises compounds comprising of tannins, flavanols, flavonols, flavones, anthocyanins, proanthocyanidins, phenolic acids, hydroxybenzoic acids and hydroxycinnamic acids [32]. Polyphenols have been shown to have numerous biological effects including beneficial effects on cardiovascular and metabolic disorders, inflammation and cancer, oxidative stress, cerebral ischaemia, obesity and functioning of the brain. With specific regards to the brain, polyphenols have been shown to prevent neuroinflammation [33], along with possessing antioxidant properties and the ability to modulate neurotransmitter levels such as 5-HT and NA [32]. All three of these factors have been implicated in the pathophysiology of depression indicating that polyphenols may play an important role in the antidepressant-like activity of coffee. However, as evidenced by the epidemiological studies, caffeine appears to be an important co-factor in the antidepressant-like activity [3, 4, 12].

Caffeine

Caffeine, a methylxanthine (figure 1), is the most widely used compound that exerts a pharmacological effect on the central nervous system (CNS) [1]. There are a number of sources of caffeine most commonly consumed in coffee, tea, energy drinks, soft drinks and chocolate [1]. Caffeine is a well-known antagonist of adenosine receptors and a phosphodiesterase 3 (PDE3) inhibitor [31]. Antagonism of the adenosine A_{2A} receptors ($A_{2A}R$) by caffeine has been shown to produce antidepressant-like activities in *in vivo* animal models of depression [34]. Furthermore, as a result of these pharmacological effects, caffeine has been shown to increase alertness and anxiety [35] and have potential anti-inflammatory and immunosuppressant effects [31]. Numerous parameters of the neuroinflammatory hypotheses of depression are affected by caffeine including Trp catabolism, inflammation and oxidative stress and will be further discussed below.

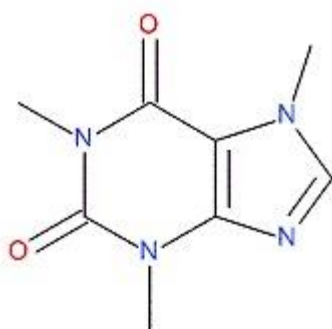


Figure 1 - Chemical structure of caffeine [29]

There is suggestive data that caffeine, an adenosine antagonist, may act via adenosine receptors to elicit its antidepressant-like effects [34]. Studies have shown

caffeine to be both preventative and therapeutic in its antidepressant action in a chronic mild stress animal model of depression [34]. It was shown that these actions were mediated through the antagonistic action of caffeine at adenosine A_{2A}Rs. Furthermore, *in vivo* animal studies have shown that animals administered either adenosine or an agonist at adenosine receptors display depressive-like behaviour [36]. It is hypothesised these changes are via changes in the dopaminergic system [36].

One aspect of the theory of depression currently being investigated is the role of the catabolism of Trp, in particular the role of the KMs, in the pathophysiology of depression. The first metabolite of the kynurenine pathway (KP), kynurenine (KYN), has been implicated in caffeine-induced anxiety. Fifteen patients received a single 2 g dose of caffeine and a statistically significant increase in anxiety was noted at 30 to 60 min post-ingestion. This corresponded to a statistically significant increase in plasma KYN levels [37].

Although depression and anxiety are two separate mental health disorders, the effect of caffeine on KYN levels is critical. Increased KYN levels are associated with increased rates of depression [38] therefore high-dose caffeine ingestion is proposed to be associated with possible depressive symptoms. However, studies assessing the effects of the consumption of normal caffeine concentrations on KYN levels, either *in vivo* or *in vitro* have yet to be undertaken. Increased KYN levels are a key component of the neuroinflammatory hypotheses of depression [13, 15, 38].

In vivo animal studies have been undertaken to assess the antidepressant effects of caffeine. It was found that chronic caffeine administration (8 mg/kg/day), in a chronic unpredictable stress mouse model, produced a positive response not only on symptoms of depression but also on hippocampal 5-HT and DA levels comparable to the tricyclic antidepressant control desimipramine (10 mg/kg/day) [39].

The synergistic effects of caffeine in combination with bupropion or the serotonin noradrenaline re-uptake inhibitor (SNRI) duloxetine have been evaluated in an *in vivo* animal model of depression. It was found that the combination of caffeine with duloxetine had higher levels of the neurotransmitters NA, DA and 5-HT and less observed depressive symptoms than either compound alone [40]. This indicates the relevance of the antidepressant-like effects of caffeine.

Studies have evaluated the effects of caffeine and its major metabolites on parameters associated with inflammation. Caffeine and one of its major metabolites, paraxanthine, have been shown to inhibit the production of tumour necrosis factor alpha (TNF- α) in lipopolysaccharide (LPS) stimulated human whole blood [41], another commonly used model to evaluate the anti-inflammatory effects of compounds. TNF- α has been shown to be an important biomarker of depression in humans in addition to its role in the regulation of Trp catabolism [38, 42-45]. This effect was found to be mediated through the cyclic AMP (cAMP)/protein kinase A pathway [41] which has also been implicated in the pathophysiology of depression [46]. Accelerated degradation of cAMP has been shown in depressed patients [46] but caffeine and one of its other major metabolites, theophylline has been shown to inhibit its degradation [47].

This further implicates caffeine and its metabolites as compounds with antidepressant-like activities.

Caffeine is a well-documented antioxidant with comparable activity to glutathione, a potent endogenous antioxidant that protects against cellular damage caused by free radicals and peroxides [48]. Studies have shown that caffeine and its metabolites theobromine and xanthine, protect against the production of free radicals, such as hydroxyl radical ($\cdot\text{OH}$), peroxy radical ($\text{ROO}\cdot$) and singlet oxygen ($^1\text{O}_2$) resulting in decreased lipid peroxidation *in vitro* [48, 49]. Additional studies have shown that coffee preparations that contained higher concentrations of caffeine displayed higher levels of antioxidant activity [50]. Given the current hypotheses of depression and the proposed role of free radicals, the results of this study may provide, at least in part, an explanation of the possible correlations observed in the human studies outlined earlier. In addition to depression, there is evidence to suggest that oxidative stress plays an important role in the pathophysiology of anxiety [51] indicating the importance of the consumption of appropriate coffee levels to avoid negative mood effects.

Caffeine has been shown to have both beneficial and non-beneficial effects with regard to the aspects of the current neuroinflammatory hypothesis of depression. The non-beneficial effects however appear to be related to high concentrations of caffeine indicating a possible concentration-dependent effect. Given the epidemiological data, it is warranted that further studies are undertaken assessing the effects of caffeine in combination with other coffee constituents on parameters associated with depression.

Chlorogenic acid

Chlorogenic acid (CGA) (figure 2) is a polyphenol found in numerous natural products and foods including coffee [52]. It is the second most abundant component of brewed coffee, after caffeine, and has been shown to possess numerous biological effects including antimutagenic, antiviral, anticarcinogenic [53-55], anti-inflammatory, antioxidant, neuroprotective and neurotrophic activity [52]. This compound has been shown to have a variety of positive effects on inflammation consistent with those outlined in the neuroinflammatory hypotheses of depression and with the effects of current antidepressant therapies.

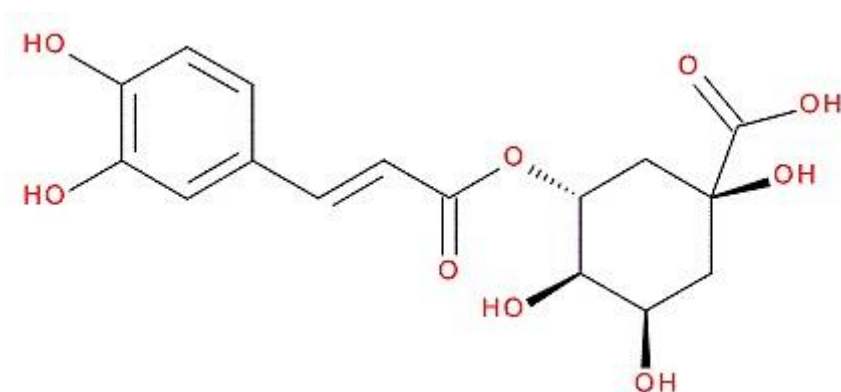


Figure 2 - Chemical structure of chlorogenic acid [56]

As outlined earlier, neuroinflammation plays a key role in the regulation of Trp catabolism and the health and survival of neurons. CGA has been shown to reduce the production of a number of pro-inflammatory mediators in macrophage cells [52] and *Staphylococcus* exotoxin stimulated human peripheral blood mononuclear cells (PMBC), a model of inflammation [57]. Amongst these include TNF- α , interleukin

(IL)-1 β , IL-6 and IFN- γ [52], all of which have been shown to be important biomarkers of depression in humans in addition to their role in the regulation of Trp catabolism [38, 42-45, 58-60]. These pro-inflammatory cytokines have an important role in the neuroinflammatory response in the CNS.

The effects of pre-treatment of LPS-stimulated microglial cells with CGA, in a well-accepted cell-based neuroinflammatory model, found CGA resulted in the concentration-dependent reduction in numerous pro-inflammatory mediators [52]. Nitrite, a general marker of inflammation and TNF- α are shown to be increased in depressed patients [61], however CGA was shown to decrease these markers of inflammation in LPS-stimulated cells [52]. Furthermore, NF- κ B, the transcription factor responsible for regulating immune response and for the activation of microglial cells under inflammatory conditions, was also shown to be decreased by CGA [52, 62]. This in turn results in an imbalance in the Th1/Th2 immune response to the pro-inflammatory side thereby creating the environment for neuroinflammation resulting in neuronal damage and death.

CGA has been shown to decrease numerous other inflammatory markers in hepatic cells. These include toll-like receptor 4 (TLR4), shown to be important in LPS-stimulated microglial induced neuroinflammation, a common neuroinflammatory cell model, [56] and the decrease in the nuclear translocation of NF- κ B [63]. Although these studies were not undertaken in cell lines involved in neuroinflammation and depression the results still further strengthen the evidence towards CGA possessing anti-

inflammatory properties and possible antidepressant properties as seen in the cohort studies outlined above.

The anti-inflammatory effects of CGA are concentration-dependent in nature. At high dose (7 mg/kg), CGA has been shown to have the opposite effects on the inflammatory mediators IL-6 and TNF- α [64], which may in turn increase the risk of the development of depressive symptoms.

The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is a widely accepted treatment of Alzheimer's disease but has been implicated as a possible mechanism of antidepressant activity. This inhibition results in increased acetylcholine in the synaptic cleft and as a result, enhanced communication between neurons occurs, temporarily improving symptoms of Alzheimer's disease [65]. An often over-looked use of AChE inhibitors is in their use for depression. Galantamine, a selective, rapidly-reversible AChE inhibitor has been shown to improve the mood of some depressed patients and decreases manic episodes in bipolar depression patients [66]. It has been proposed that galantamine improves these symptoms by reversing the cholinergic dysfunction involving a decrease in choline acetyltransferase activity and a decrease in nicotinic and muscarinic receptor expression [66]. These results differ from the proposed Cholinergic-Adrenergic theory of depression [67] thereby casting doubt upon parts of it. CGA (0 to 12 μ g/mL) has been shown to inhibit AChE and BChE in a concentration-dependent manner [68]. This suggests that CGA and possibly coffee may be able to improve not only the symptoms of Alzheimer's disease but also improve symptoms of depression or mania through the inhibition of AChE and BChE and/or a

possible decrease in nicotinic and muscarinic receptors. There has been postulation that the neuroprotective KM, kynurenic acid (KA), may have antidepressant activities. KA has been shown to have antagonistic effects on $\alpha 7$ nAChR [69], which are found on numerous cells including inflammatory cells and play an important role in the cholinergic anti-inflammatory pathway [70]. This suggests that KA, at least in part, exerts its antidepressant-like activities via this mechanism. In further support of the role of nicotinic receptors in depression, a number of currently marketed antidepressants including the TCAs imipramine, desimipramine and clomipramine, and the SSRI fluoxetine have been shown to have antagonistic effects at nicotinic receptors [71-73] indicating that their antidepressant activity, may in part, be due to this effect. Nicotine has been shown in numerous studies to be associated with symptoms of depression and has been reviewed extensively by Bertrand [74]. Smoking tobacco, a major source of nicotine, has been shown to have bidirectional effects, and as a result, the mechanistic effects in depression are poorly understood [74]. This again highlights the need for further studies into the role of nicotine and nicotinic receptors in the pathophysiology of depression.

CGA has been shown to have antioxidant properties, however these are significantly less than that of its metabolite and another major constituent of coffee, caffeic acid [75] and as a result the focus of studies has been on the latter.

This evidence indicates that CGA may be an important anti-inflammatory constituent of coffee at certain concentrations. Additionally, due to its CNS effects, it

may play a part in decreasing depressive symptoms but to date there is insufficient evidence to confirm this effect.

Ferulic acid

Ferulic acid (FA), 4-hydroxy-3-methoxy-cinnamic acid, is a polyphenol (figure 3) [76] that has been shown to have antidepressant, antioxidant and anti-inflammatory effects, all critical components of the neuroinflammatory hypotheses of depression.

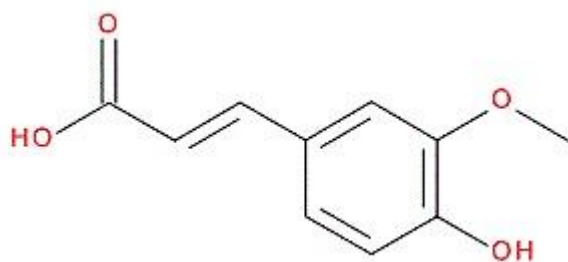


Figure 3 - Chemical structure of ferulic acid [77]

FA has been shown to be comparable to a high dose of the TCA, imipramine with regards to antidepressant activity [9] [9, 76]. FA was evaluated for its antidepressant activities in reserpine-treated mice in two well-accepted animal models of despair – the tail suspension test (TST) and forced swim test (FST) models [76]. Reserpine, an anti-hypertensive agent acting through the depletion of monoamines [78] was found to cause profound symptoms of depression upon administration [76]. The results indicate that FA has a beneficial effect on reserpine-induced depression. It was found that FA induced a significant decrease in immobility time in both the TST and the

FST models, in a concentration-dependent manner, suggesting FA has antidepressant activity comparable to a 10 mg/kg dose of imipramine [76] or the SNRI, milnacipran [79]. FA, in much lower doses, (0.01, 0.1, 1 and 10 mg/kg) was again found to decrease the immobility time in both the TST and FST, again indicating antidepressant activity [9]. In addition to this, the effect of FA on 5-HT levels in various regions in the brain was evaluated. It was found that FA had similar effects on 5-HT levels in both the frontal cortex and hippocampus, increasing levels comparably to a 10 mg/kg dose of imipramine [76]. This evidence clearly shows the antidepressant benefits of FA and shows support towards the current hypothesis of depression with strong evidence on the anti-inflammatory and effects on monoamine levels. The effects of FA on the catabolism of Trp have to date, not been investigated but would be beneficial for future studies.

The synergistic effects of FA in combination with antidepressants have been evaluated by assessing the immobility time in the TST. It was found that FA in combination with the SSRI antidepressants fluoxetine, paroxetine and sertraline resulted in decreased immobility times suggesting a synergistic effect between FA and the respective antidepressants [9]. Another possible avenue of synergism with FA is with caffeine. As outlined in the cohort studies above, overwhelming evidence exists for caffeine in combination with another constituent of coffee to be responsible for the antidepressant effects of coffee. The effects of synergism of FA with caffeine also appears to be a more rational approach due to the relatively high concentrations of this compound needed to exert the antidepressant effects and the relatively low concentrations of 142.8 µg/mL present in coffee [80].

FA has been shown to have numerous effects on inflammation. In the frontal cortex region of the brain, 80 mg/kg of FA decreased IL-1 β production and 40 and 80 mg/kg decreased IL-1 β and TNF- α production in the hippocampus [76]. Both IL-1 β and TNF- α , as outlined earlier, are important biomarkers of depression [43, 44, 58, 59] and play an important role in the catabolism of Trp [81] highlighting the possible importance of FA in the antidepressant-like effects of coffee. FA (20, 40 and 80 mg/kg) also decreased the p65 subunit of NF- κ B in a dose-dependent manner in both regions of the brain in reserpine-treated mice. These results are comparable to imipramine (10 mg/kg) in its anti-inflammatory effects [76]. They are also comparable to current antidepressant therapies as outlined earlier.

FA has been shown to induce proliferation of neural stem cells (NSCs) and neural progenitor cells (NPCs), both of which are precursors to neurons and glia in the foetal and adult CNS [79]. This suggests that FA may be particularly important in the treatment or prevention of depression as two major causes/effects of depression, stress and increased cortisol production, have been shown to reduce NSC/NPC proliferation [82]. Studies to assess the effects that FA have on cortisol levels *in vivo*, however, are yet to be completed. Additionally, an increase in NF- κ B has been shown to be involved in both the onset of depression and the decrease in proliferation of NSCs and NPCs indicating a possible additional mechanism of antidepressant action of FA, although studies to confirm this have yet to be undertaken [61].

Numerous studies have assessed FA for antioxidant properties. FA has been shown to inhibit lipid peroxidation in rat brain homogenates but was 1000-fold lower in activity than caffeic acid, another important biologically active constituent of coffee [83]. In a neuronal cell model, FA was shown to provide antioxidant protection against hydroxyl and peroxy radical exposure [84]. These results are of particular importance in depression given the cell line these results were observed in and the proposed relevance to depression.

There have been numerous studies undertaken assessing the antidepressant and anti-inflammatory effects of FA. To date, FA has been shown to have antidepressant action, but at much higher concentrations than those seen in coffee. This indicates that FA, in combination with caffeine, may possibly be the combination of compounds required to exert the antidepressant effects observed with caffeinated coffee consumption.

Caffeic acid

Caffeic acid (CA) is a phenolic compound, belonging to the hydroxycinnaminic acids (HCAs) group of compounds (figure 4) [85]. It possesses potent antioxidant, anti-inflammatory and free radical scavenging properties [19, 86, 87] in addition to neuroprotective properties [86], all important aspects of the neuroinflammatory hypotheses of depression. Additionally, its derivative, caffeic acid phenethyl ester (CAPE), possesses many similar biological activities including antioxidant, antiviral, anti-inflammatory and immunomodulatory properties [77]. Specifically, CA suppresses the activation of NF- κ B, which is important in the onset of depressive symptoms [61].

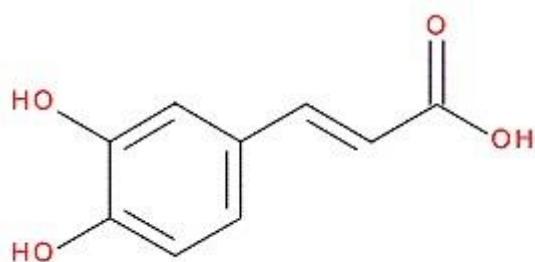


Figure 4 - Chemical structure of caffeic acid [88]

CA possesses antidepressant-like and anxiolytic-like activity [89-91]. Animal studies have demonstrated that CA reduces the duration of immobility and freezing in three well accepted models of depression, the FST, OFT and elevated-plus maze tests [89]. In addition to this, it is thought that the antidepressant and anxiolytic activity may be attributed to indirect modulation of the α_{1A} -adrenoceptor system [91].

The anti-inflammatory properties of CA and its derivatives have been shown in a number of studies. CA (10 $\mu\text{g/mL}$) significantly decreases nitrite concentration, produced in response to inflammatory stimuli, in LPS-stimulated Raw 264.7 macrophage cells [91]. Additionally, CA has an inhibitory effect on LPS-induced NF- κB activity and on the phosphorylation of JNK1/2 and p38 MAPK, signalling molecules involved in inflammation, in the same cell line [91]. The JNK1/2 and p38 MAPK signalling pathways have been shown to be redox sensitive [92, 93], indicating that the anti-inflammatory-like effects may in fact be due to, at least in part, the antioxidant properties of CA. Inhibitory effects of CAPE (1 μM) have been demonstrated towards the synthesis of the pro-inflammatory leukotrienes produced from the 5-lipoxygenase pathway, including leukotriene B₄ (LTB₄) by 85% in human leucocytes, indicating

potent anti-inflammatory properties [94]. Taking into consideration that inflammation is a major regulatory factor of numerous possible components of depression, CA and its derivatives have a potential use as an antidepressant agent.

As described earlier, AChE and BChE are beneficial to symptoms of depression. In addition to CGA, CA is an inhibitor of both of these enzymes (0 to 12 $\mu\text{g/mL}$), in a concentration-dependent manner. However, CA is a more potent inhibitor of these enzymes than CGA [68], suggesting that it has a significant effect on improving the symptoms of depression.

CA, in a concentration-dependent manner, protects against lipid peroxidation in rat brain after exposure to 15 mM quinolinic acid (QA) [68], a catabolic product from Trp, which is implicated in the pathophysiology of depression due to its biosynthesis through the KP. Additionally, CA (5 to 10 mg/kg oral) significantly attenuated increased lipid peroxidation and nitrite concentration and restored superoxide dismutase and catalase activity in response to intrastriatal injection of 300 nM QA [86]. QA is a KM and a known neurotoxin, primarily exerting its toxicity through N-methyl-D-aspartate (NMDA) receptor agonism, resulting in excitotoxicity [95]. It does however produce its secondary toxicity effects through the production of free radicals [95]. Both the KP and oxidative stress are implicated in the neuroinflammatory theories of depression, and the possible protective effects of CA.

CA has potent antioxidant properties that are greater in antioxidant activity than many other important constituents of coffee including CGA and FA [96]. It was proved that the antioxidant activities of CA are similar to or better than that of α -tocopherol, a form of vitamin E and potent antioxidant, using a variety of tests including the Rancimat test, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity of each molecule and the ferric thiocyanate method [88, 97]. CA scavenges hydrogen peroxide [98], superoxide and lipid peroxides [99], all implicated in the pathophysiology of depression [100, 101].

CA and its derivatives have been widely studied for their biological effects. Numerous studies have shown these compounds to possess antidepressant, anti-inflammatory, antioxidant and neuroprotective properties, all of which are important aspects of the neuroinflammatory hypotheses of depression.

Pyrogalllic acid

Pyrogalllic acid (PA), a benzenetriol, polyphenol (figure 5), is another common component of coffee [102]. Studies have shown PA to possess anti-inflammatory, antioxidant and prooxidant activity [103], and antimicrobial activity [104].

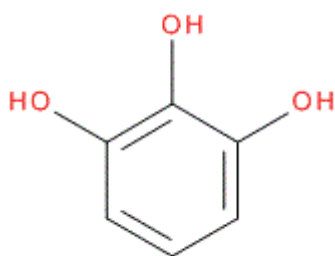


Figure 5 – Chemical structure of pyrogallol acid [105]

PA, at micromolar concentrations, has been shown to decrease the transcription of the inflammatory genes ICAM-1, IL-8, GRO- α , GRO- γ and IL-6 in respiratory cells isolated from a cystic fibrosis patient (IB3-1 cells) and subsequently stimulated with *Pseudomonas aeruginosa* PAO1. In addition, multiplex assays showed a decrease in both IL-6 and IL-8 protein expression further confirming the anti-inflammatory activity of PA [106].

The antioxidant properties of PA occur in a concentration-dependent manner. One study has shown that at lower concentrations of PA (40 to 200 μ M) decreased H₂O₂ levels in comparison to untreated cells. Conversely, at higher concentrations (400 μ M), intracellular H₂O₂ levels were markedly increased [103].

Studies have shown PA to be a non-competitive inhibitor catechol-O-methyltransferase (COMT), an enzyme responsible for the metabolism of DA and NA [107, 108]. Given the proposed roles of DA and NA in depression, PA may provide an improvement in symptoms via this mechanism.

PA has been shown to cause a range of toxicities including oxidative damage, mutagenesis, carcinogenesis and hepatotoxicity. In addition, it has been shown to impair immune responses, including the suppression of proliferation of lymphocytes. The degree of toxicity experienced is due to many factors including age and gender, nutritional status, food habit and obesity, diseases of the gastrointestinal tract, interaction of PA with other drugs, route, dose and duration of exposure, kidney dysfunction/failure, lifestyle factors including alcohol consumption and smoking and environmental factors including chemical exposure [108].

PA exhibits hepatotoxic and mutagenic effects, primarily due to its potential to generate free radicals [108, 109]. Upadhyay *et al* suggested that 183 genes (150 upregulated and 33 down-regulated), either directly or indirectly related to hepatotoxicity, were altered after exposure to PA [108].

To date there have been no studies undertaken to assess the effects of PA on Trp catabolism.

PA has been shown to possess both concentration-dependent antioxidant and anti-inflammatory properties. In addition, it has been shown to have COMT inhibitory effects, therefore potentially important CNS effects. Given this data, further studies assessing its effects in depression are warranted. Further studies assessing the toxicity profile of this compound may be of benefit to assess if toxicity is concentration-dependent.

Trigonelline

Trigonelline, 1-Methylpyridinium-3-carboxylate (figure 6), is a zwitterionic alkaloid found in a number of plant species and foods including coffee [110]. There have been very few studies on the biological effects of this compound, but most noteworthy is its hypoglycaemic effects [110].

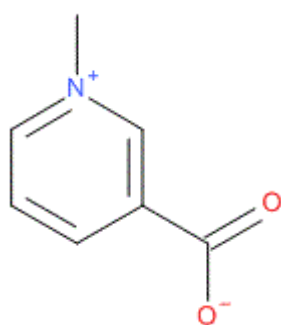


Figure 6 - Chemical structure of trigonelline [111]

A study assessing the effects of trigonelline on the oxidative status of the pancreas in a diabetic rat model found that a 50 mg/kg oral dose returned oxidative parameters back to non-diabetic control levels. Specifically, trigonelline reversed increased malondialdehyde (MDA) levels seen in the diabetic rat controls, indicating that lipid peroxidation was decreased. In addition, trigonelline dosing resulted in the increase of SOD and catalase activities as well as glutathione in the pancreatic tissue, compared to diabetic control rats [112].

Specifically, with regards to the effects in the CNS, one study, using SK-N-SH neuroblastoma cells, a dopaminergic cell-line routinely used in neuron studies, found that trigonelline, isolated from raw coffee beans, promoted neurite outgrowth that was, in part, axonal suggesting possible enhancement of synaptic function [113]. This effect has not been studied in human primary neurons therefore the clinical significance of this occurrence is unknown and requires further evaluation. Additionally, the ability of trigonelline to cross the blood brain barrier to reach the neurons is yet to be determined.

The effect of trigonelline on Trp catabolism has yet to be determined.

Trigonelline has been shown to have many protective effects including antioxidant, and neuroprotective properties [110, 112, 113]. The neuroprotective effects are of particular interest and warrant further investigation into their possible role in the antidepressant activity seen with coffee consumption.

Coffee and a number of its constituents have been shown to have a number of positive effects on behaviours and biomarkers of depression as highlighted in the epidemiological *in vitro* and *in vivo* studies above. The newer inflammatory hypotheses of depression will be discussed below and highlight the potential mechanisms by which they may exert their antidepressant effects and provide details of where further investigation is warranted.

1.3 Depression

1.3.1 What is depression?

Depression is a complex, heterogeneous disorder and to date, the aetiology is yet to be fully elucidated [114]. It is a multifactorial psychiatric disorder that involves a disturbance in emotional, cognitive, immune, autonomic and endocrine functions [115]. Depression is a leading cause of morbidity with approximately 5% of the world's population diagnosed with a mood disorder, including depression, dysthymia and bipolar affective disorder [116, 117]. Furthermore, it has been reported that depression has a lifetime prevalence in excess of 15% [60] and is twice as likely to occur in females as males [13].

Depression has been identified as the fourth leading cause of burden among all diseases, accounting for 3.7% of disability-adjusted life years (DALYs) and 10.7% of years lived with disability (YLDs) [118]. This profound effect is due to the highly recurrent nature of depression, as high as 80% recurrence, and the relatively low rates, less than 60%, of remission achieved with currently marketed antidepressant medications [60].

Based on a study investigating the global burden of depressive disorders undertaken by the World Health Organisation (WHO), the need, firstly, to develop and adapt interventions for reducing the disability associated with depression and secondly, to develop effective strategies to shorten episodes of depression and prevent their reoccurrence has been identified [118]. As a result of the identified burden, depression

has been identified a priority health area [118]. This study proposes to explore the effects of key bioactive coffee constituents, which may have an alternative mechanism to traditional antidepressant therapies, in the hope of decreasing the resistant, recurrent nature of depression.

Numerous hypotheses of depression have been presented over the last 50 years in an attempt to explain the aetiology and pathophysiology of depression and will be outlined further below [15, 116, 119, 120].

1.3.2 Pathophysiology and theories of depression

The first proposed theory of depression, the monoamine theory of depression, was based on the observed depressive effects of reserpine, an anti-hypertensive agent acting by the depletion of catecholamines [78]. The central theme of this theory is the proposed decrease in the central monoamine neurotransmitters 5-HT, NA and DA [13] which are responsible for neurotransmission and indirect control of mood in major depressive disorder [121]. However, a major limiting factor for this theory is that correction of the monoamine levels at the synapse occurs within hours of the first dose of an antidepressant medication being given, whereas the antidepressant effects take weeks of continuous therapy to be observed [14]. This subsequently led to the adaptation of the monoamine theory of depression to suggest that the initial increase in the synaptic monoamine levels leads to an adaptive response. The acute increase in monoamine levels serves to desensitize the inhibitory auto- and hetero-receptors, resulting in increased monoaminergic transmission that, time-wise, would coincide with the appearance of antidepressant effects [14]. However, a number of shortcomings

have been identified with this theory and as a result, there has been an emergence of numerous theories of depression have been proposed in recent times. The current hypotheses of interest are multifactorial and in addition to the principles of the monoamine theory of depression, involve an increase in neuroinflammation, oxidative stress and the dysregulation of Trp catabolism [15, 38, 42, 58-60, 122, 123]. Alteration to Trp catabolism results in the accumulation of the neuroactive compounds known as the KMs and an alteration in the serotonergic system as seen in figure 7 [13, 15, 38]. Each of the components of the neuroinflammatory theories of depression will be explained in further detail below.

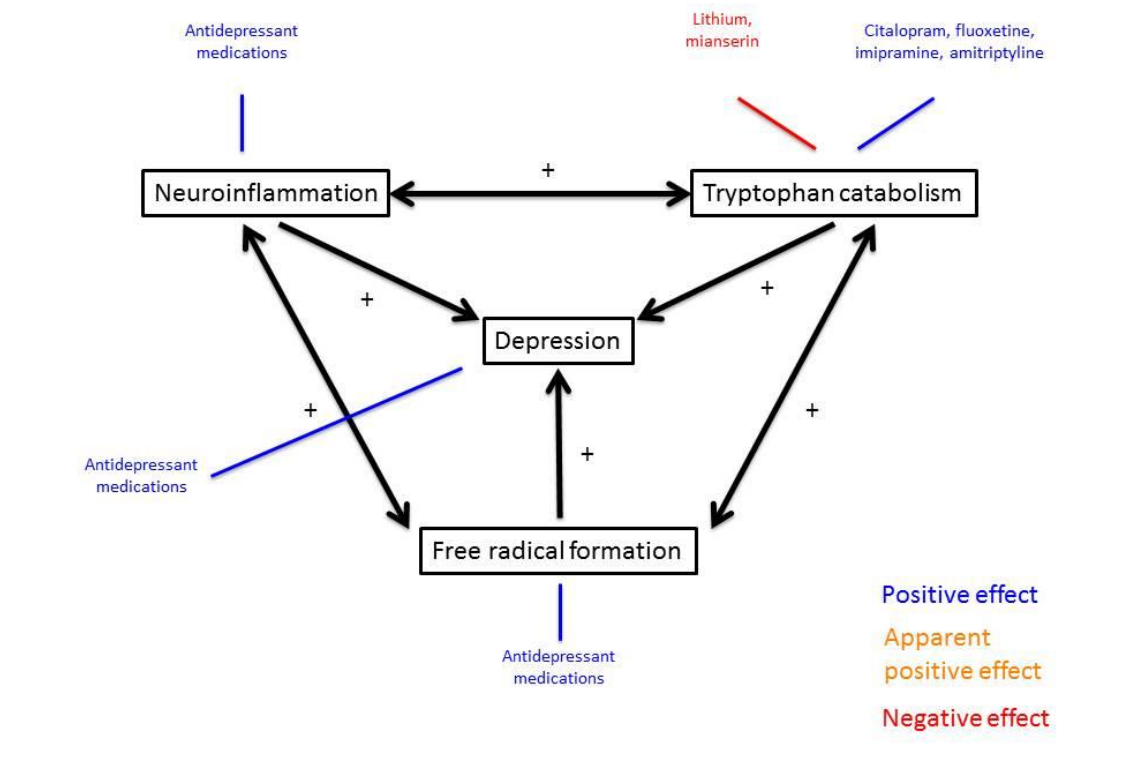


Figure 7 – The neuroinflammatory hypotheses of depression and the action of antidepressant medications [124]

1.4 Neuroinflammatory theories of depression

A number of neuroinflammatory hypotheses of depression have been proposed and are outlined below.

1.4.1 The cytokine hypothesis of depression

The cytokine hypothesis of depression, also known as the monocyte-T-lymphocyte theory of depression was first described in 1995 by Maes *et al* [123]. This theory is based on the evaluation of evidence suggesting the role of activation of peripheral blood monocytes and T lymphocytes in the pathophysiology of major depression [123].

It is hypothesised that major depression is accompanied by an immune response and an abnormal production of cytokines [123]. Additionally, the authors suggest that HPA-axis hyperactivity and abnormalities in the monocytic and T-lymphocytic cell production play an important role in the pathophysiology of depression [123]. They do however state that there is no evidence for a causal relationship between abnormalities in the monocytic and T-lymphocytic cell productions in major depression and further studies are required to investigate this possibility [123].

1.4.2 The inflammatory and neurodegenerative hypothesis of depression

Maes *et al* proposed a number of theories of depression involving inflammation and neurodegeneration including “The inflammatory and neurodegenerative hypothesis of depression” [42]. The central theme of this hypothesis is multifactorial and includes

changes to the secretion of glucocorticoids, free radical formation, Trp catabolite (TRYCAT) production, pro-inflammatory cytokine release and omega 3 polyunsaturated fatty acid (ω 3 PUFA) production [60]. The evidence presented in this theory with regards to the inflammatory and Trp components provides a plausible explanation and suggests that anti-inflammatory compounds may be able to counteract the enhanced neurodegeneration and decreased neurogenesis witnessed [60].

1.4.3 The cytokine-serotonin theory of depression

The cytokine-serotonin theory of depression involves a complex interaction of inflammation and altered Trp catabolism [15]. According to this hypothesis, multiple components are involved in the pathophysiology of depression including neuroinflammation, the increased production of the neuroactive KMs and dysregulation of 5-HT production resulting from a cytokine-serotonin interaction through the enzyme indoleamine 2, 3-dioxygenase (IDO) [15]. It is hypothesised that medical illnesses and chronic psychological stress, which result in increased pro-inflammatory cytokine levels, ultimately lead to the pathophysiology of depression [15].

This theory suggests psychological stress influences depressed mood initially through increased Trp demand resulting in depletion [15]. Once psychological stress becomes chronic, an increase in pro-inflammatory cytokines and onset of sickness behaviour is observed [15]. This is hypothesised to occur due to the pro-inflammatory cytokines up-regulating IDO and subsequently increasing levels of the neuroactive KMs and precipitating an imbalance between neurotoxic and neuroprotective KMs [15]. This imbalance favours towards the “neurotoxic arm” resulting in production of the

neurotoxic metabolites such as 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HA) and QA [15]. The neurotoxic KMs are then thought to cause neurodegeneration, which in turn is proposed to disturb the coping mechanisms of the brain and results in major depression or treatment-resistant depression [122]. Additionally, pro-inflammatory cytokine-induced dysregulation of the serotonergic system is also, at least in part, due to the upregulation of IDO, one of the enzymes responsible for the metabolism of 5-HT [15]. Additionally, the role of physical stress and medical illness is also thought to be a causative factor in the development of depressive symptoms, once again due to the increase in pro-inflammatory cytokines observed and the subsequent effects as described above [15].

The authors of this hypothesis of depression, after analysing the evidence available, suggest that this hypothesis is a plausible and a more complete theory in comparison to the well-accepted monoamine theory of depression. They do however highlight the need for further studies to confirm the involvement of the cytokine-serotonin interaction and possible resultant neurodegeneration in depression [15].

1.5 Components of neuroinflammatory theories of depression

Central to the neuroinflammatory hypotheses of depression, as outlined above, are neuroinflammation, dysregulation of Trp catabolism and monoamine levels and oxidative stress and will be discussed further below.

1.5.1 Neuroinflammation and theories of depression

Neuroinflammation is a relatively new term used to describe chronic, CNS specific, inflammation-like glial responses. These responses do not produce the normal characteristics of inflammation in the periphery but however, promote the occurrence of neurodegenerative effects including dystrophic neurite growth, plaque formation and tau phosphorylation [33]. Neurons, in particular, are extremely vulnerable to this process and hence neurodegeneration occurs [125].

Studies have shown the innate immune system in the CNS as a possible factor in the onset of numerous neurodegenerative diseases. This form of immune activation lacks the prominent features of the adaptive immune response but results in activation of microglia and astrocytes ultimately resulting in neuroinflammation [33]. Recently, neuroinflammation has been suggested to play an important role in a range of neurological disorders including depression and depressive symptoms [126] and has become a central theme to the numerous new theories of depression outlined above.

Genetic polymorphisms of cytokine genes may indirectly affect IDO activity through the altered production of cytokines. Of particular interest in depression are the polymorphisms resulting in overexpression of these cytokine genes particularly the IFNG (+874) [127] and the TNF-alpha (-308A/G) genes which ultimately results in the increase production of the pro-inflammatory cytokines IFN- γ and TNF- α respectively [116]. In particular, it has been shown that high expressers of the TNF-alpha (-308A/G) gene are more likely to be depressed [119].

1.5.2 Monoamines and the neuroinflammatory hypotheses of depression

The current theories of neuroinflammatory depression suggest, as previous theories have, that a dysfunction in the serotonergic system is important in the pathophysiology of depression. As outlined earlier, 5-HT is thought to desensitize the inhibitory auto- and hetero-receptors, resulting in increased monoaminergic transmission [14]. There are numerous proposed mechanisms by which dysfunction to the serotonergic system may occur including a predomination of Trp catabolism through the KP and 5-HT depletion. Studies have shown that under inflammatory conditions, up to 99% of Trp is catabolised through IDO thereby potentially resulting in less 5-HT biosynthesis [122]. To further compound this, one of the metabolic pathways of serotonin is through IDO [128]. Therefore, under inflammatory conditions, 5-HT depletion is likely to be accelerated [122].

1.5.3 Oxidative stress and the neuroinflammatory hypotheses of depression

Oxidative stress is an important component of “The inflammatory and neurodegenerative hypothesis of depression” [42]. It is proposed its involvement is due to the brain and CNS being particularly sensitive to oxidative stress due to its inadequate antioxidant defense systems that ultimately results in neurodegeneration [42]. Studies in humans demonstrate that imbalances in the production of free radicals and the antioxidant defenses are present in depression [129].

1.6 Conclusion

Depression is a complex, heterogeneous disorder with a complex aetiology that is yet to be fully elucidated. Given the prevalence and high rates of morbidity and

mortality associated with depression, it is imperative that a greater understanding of the pathophysiology and aetiology is sought to enable better treatment options. To date numerous studies have demonstrated beneficial effects on depression with the consumption of coffee and with a number of constituents of coffee. Various parameters associated with the neuroinflammatory hypotheses of depression have been affected beneficially in both *in vitro* and *in vivo* studies. A number of these studies have, however, shown that the inverse correlation is between caffeinated coffee and not decaffeinated coffee or caffeine alone indicating the antidepressant-like activity is likely due to a combination of caffeine with one or more biologically active constituents of coffee. The aim of this review was to evaluate the evidence for and against the antidepressant-like effects observed in numerous cohort studies and the *in vitro* and *in vivo* effects of specific constituents of coffee and to explore potential mechanisms by which this may occur. This review provides an insight into compounds that may have positive effects on numerous components of the neuroinflammatory hypotheses of depression highlighting the growing evidence to support the antidepressant-like effects of coffee and a number of its key biologically active constituents however further elucidation of these effects is needed.

The evidence presented above highlights the importance and relevance of the newer neuroinflammatory hypotheses of depression, however, caution must be exercised when evaluating the possible effects seen here given the lack of high quality studies. There are a number of gaps in literature identified and the evidence presented above highlights the need for further *in vitro*, *in vivo* and human studies to assess the potential benefits of coffee in depression, a number of which will be addressed in the current study. The first of these is the lack of studies quantifying the differences of

bioactive coffee constituents between caffeinated and decaffeinated coffee. Given the results of the human epidemiological studies showing caffeinated coffee to reduce the risk of depression whereas decaffeinated coffee appears not to, and the identification of bioactive constituents, in addition to caffeine, that are lost is needed. Furthermore, to date there have been no specific *in vivo* or human studies assessing the causal link between caffeinated coffee consumption and symptoms of depression. This study aimed to address this gap in literature using an *in vivo* model of inflammatory depression. Finally, *in vitro* studies were used to evaluate the toxic effects of coffee constituents on neuronal-like cells along with their effects alone and in combination with caffeine in a surrogate microglial model of inflammation.

1.7 Hypothesis

It is hypothesised that caffeinated coffee, along with key bioactive coffee constituents will be beneficial in behaviours and biomarkers of depression in *in vivo* and *in vitro* models of depression and inflammation.

1.8 Aims and objectives

The primary aim of this study was to assess the effects of caffeinated coffee, decaffeinated coffee and caffeine on behaviours and biomarkers associated with depression in an *in vivo* inflammatory model of depression.

The primary aim of this study will be achieved through the following objectives:

- The development of an LPS-induced *in vivo* mouse model of inflammatory depression; and
- The quantification of plasma and central biomarkers known to be associated with depression using HPLC and ELISA analysis.

The secondary aim of this study was to investigate if any key bioactive coffee constituents are lost during the decaffeination process using quantitative HPLC analysis in order to identify possible compounds contributing to the antidepressant effects of caffeinated coffee.

This aim will be achieved through the following objective:

- The development, optimisation and validation of quantitative HPLC methods for six of the key bioactive coffee constituents, caffeine, CA, CGA, FA, PA and trigonelline.

The third aim of this study was to develop a surrogate microglial-like and a surrogate neuronal-like *in vitro* cell-based model and subsequently evaluate the effects of key bioactive coffee constituents, alone and in combination with caffeine, on inflammatory markers and cell death mechanisms.

This aim will be achieved through the following objectives:

- The development of an *in vitro* surrogate microglial cell model; and
- The development of an *in vitro* surrogate neuronal cell model; and
- The evaluation of the effects of key bioactive coffee constituents, alone and in combination with caffeine on markers of inflammation, including IDO activity and IL-6, IL-1 β and TNF- α concentrations, in the developed *in vitro* microglial cell model; and
- The evaluation of the effects of key bioactive coffee constituents on viability and specific cell death pathways, including oxidative stress, apoptosis and necrosis, in the developed *in vitro* neuronal cell model.

1.9 Significance of the study

Depression is a leading cause of morbidity and mortality in both Australia and the world with 6.2% of the Australian population diagnosed with a mood disorder [116]. In addition to the high prevalence, literature shows there is up to a 65% rate of failure with current antidepressant therapies [130]. Furthermore, the WHO has identified the need for the development of effective strategies to shorten episodes and prevent recurrences of depression and as a result, has identified depression as a priority health area [118].

Recently, increased evidence for both systemic and local CNS inflammation playing an important role in the pathophysiology of depression has been presented [15, 42, 58-60]. In the light of this, the need for more effective antidepressant agents is required and targeting neuroinflammation may provide the solution to the problem.

Previous hypotheses of depression have focused on the monoamine theory of depression however no mechanism by which disturbance of the serotonergic and noradrenergic system has yet been discovered. In addition to this, the regularly occurring non-response rate is approximately 30% for any given antidepressant drug indicating that the monoamine hypothesis may not completely explain the mechanisms of major depression [131]. This highlights the need for expansion of ideas with regard to the pathophysiology and treatment of depressive illnesses. The theory that this research will focus on, the cytokine-serotonin theory of depression incorporates a number of different components including neuroinflammation, disturbances to the balance of the KP and serotonergic system and due to the complex, heterogeneous nature of depression, may provide a better description of this disease state.

As highlighted above, coffee consumption, in human epidemiological studies, has been shown to inversely correlate to a risk reduction in developing depression a trend not observed with the consumption of decaffeinated coffee or caffeine alone [3, 4, 7, 8]. A meta-analysis has however shown caffeine consumption to be beneficial in reducing the risk of depression [6], highlighting the need for further investigations into the effects. Furthermore, a number of key bioactive coffee constituents have shown some beneficial activities towards a number of the inflammatory parameters proposed to be involved in the pathophysiology of depression as outlined above [18, 63, 68, 79, 84, 87, 113]. To date, however, there is still significant information missing with regards to their actions on microglial-like cells and in combination with caffeine. This study aims to fill some of these gaps and provide further details on the actions of these compounds on inflammation associated with depression.

Chapter Two – The antidepressant effects of coffee in an *in vivo* model of neuroinflammatory depression

2.1 Introduction

As outlined in chapter one, numerous human epidemiological studies have been undertaken assessing the relationship between the consumption of coffee and the risk of developing depression [3, 4, 6-8, 12]. These studies have identified that the consumption of caffeinated coffee but not decaffeinated coffee or caffeine alone reduced the risk of depression [3, 4, 6-8, 12]. However, due to the nature of the studies, that is cohort and cross-sectional studies, these trends need to be interpreted with caution. These studies do not allow for investigation into the causal relationship between caffeinated coffee consumption and depression, rather only allow for an association or correlation to be made. This is due to the inability of these studies to control for other influencing factors, such as social interactions, to be taken into account. Due to this, the aim of this study was to use an *in vivo* mouse model of inflammatory depression to evaluate the effects of caffeine, caffeinated coffee and decaffeinated coffee on symptoms and biomarkers of depression. In an animal study, many of the confounding factors including social interactions can be controlled for. Furthermore, the animals study can provide preliminary evidence to support the need of high quality human studies to investigate causality.

In addition to the antidepressant-like activities observed with the consumption of coffee, negative effects of coffee consumption must also be monitored. Caffeine is a well-known CNS stimulant which causes anxiety in susceptible individuals [37, 132,

133]. Furthermore, the anxiogenic effects of caffeine are dose-dependent [134, 135]. In addition to this, chapter one has highlighted the increased risk of suicide at very high levels of consumption of coffee [5, 21, 22]. Given these effects, it is imperative that these factors are accounted for in an animal model of depression.

2.1.1 Animal models in depression

Animal studies are useful in providing a model of inflammatory depression. There are two documented models of inflammatory depression described in literature to date, the LPS model and the Bacillus Calmette–Guérin (BCG) models of inflammatory depression [136-141]. Due to financial, product availability and safety considerations, the LPS model of inflammatory depression was chosen for the current study. Studies have shown that many of the features experienced in human depression are present in models of LPS-induced depression. Such similarities include several behavioural similarities such as depression, anhedonia, weight loss and fatigue along with a number of biomarkers including plasma catecholamines, central monoamines and plasma cytokines such as IL-1 β and IL-6 [142]. In addition to the benefits of using animal models of depression, a number of limitations are also present. This is mainly due to the heterogeneous nature of depression and the difficulty in determining whether an animal has symptoms that constitute a diagnosis of depression in a human. For example, it is almost impossible to ascertain whether the animal has feelings of guilt or sadness [143, 144]. There are however a number of strategies to increase the validity of animal models, namely, to increase face validity, the predictive validity and the construct validity of the model [144].

Face validity refers to the extent in which an animal model captures the anatomical, biochemical, neurophysiological or behavioural features of depression observed in humans [144]. To improve face validity of an animal model it is recommended that multiple parameters are assessed and align with what is observed in human disease [144]. Predictive validity refers to the extent to which various models respond to treatment in a way that corresponds with the effects produced by those same treatments in humans [144]. To ensure predictive validity, it is imperative that strong positive and negative controls are used in the animal study. The third and last strategy is to ensure construct validity. Construct validity is obtained by integrating the most relevant and observable features associated with the onset and progression of depression into the design of an animal model of depression [144]. By incorporating the three principles outlined above into the design of an animal study, many of the limitations can be overcome and an animal model that better represents human disease can be employed in the study of depression.

2.1.2 Animal model of LPS-induced depression

As a result, an LPS model of inflammatory depression was used in the current study. This model has been shown in literature to alter many of the parameters seen to be altered in the human depressive state. The first and most obvious is depressive-like behaviours [136, 137, 145, 146]. Treatment with LPS in mice produces a biphasic change in behaviour. Initially, sickness behaviour predominates and is at its peak at 6 h post-LPS exposure [139]. Subsequently, depressive-like behaviour predominates after 24 h, suggesting that it is appropriate for studies assessing the antidepressant effects of compounds to be measured after 24 h post-LPS exposure [139].

2.1.3 Biomarkers in LPS-induced depression

IDO activity and the kynurenine pathway

The LPS model of depression has also been shown to increase an important biomarker seen in human subjects with depression namely, IDO activation. As outlined in chapter one, IDO is an important, rate-limiting enzyme in the catabolism of Trp [147] under the control of LPS in addition to inflammatory cytokines [138, 147-151]. As outlined earlier, IDO is the first enzyme in the KP. Under inflammatory conditions, the concentration of the neurotoxic 3-HK, 3-HA and QA, are increased and the neuroprotective KA, is decreased [15]. Figure 8 below outlines the KP compounds and enzymes. These compounds and their actions are discussed in detail below.

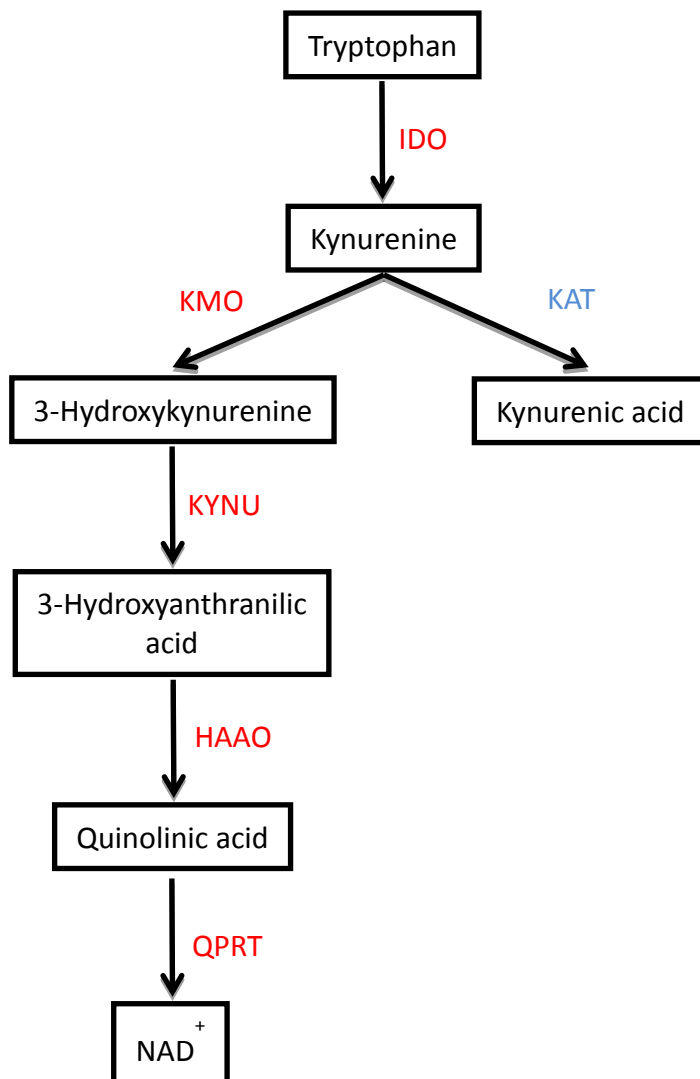


Figure 8 - The catabolism of tryptophan via the kynurenine pathway [69]

Kynurenic acid

KA, a quinoline also known as 4-hydroxyquinoline-2-carboxylic acid (figure 9), is a neuroprotective Trp catabolite [152]. The main biosynthetic pathway of KA involves the enzymatic conversion of KYN through the enzymes kynurenine aminotransferase I/II/III (KATI/II/III), with KATII the primary pathway [153]. A second, non-enzymatic pathway of KA biosynthesis has also been shown. This occurs

through the non-enzymatic oxidation of KYN and Trp via idole-3-pyruvic acid, a reaction increased by oxidative stress [95, 154]. Numerous factors have been identified that regulate the formation of KA including KYN levels, the availability of glucose and the intracellular concentrations of the enzymatic substrates pyruvate, 2-oxoglutarate and oxaloacetate [153].

At physiological concentrations, in the nanomolar range [153, 155], KA has been shown to have neuromodulatory effects [156] and numerous effects on various receptors. Although KA is reported to be in the nanomolar range, it is expected to be significantly higher at neuronal receptor sites but unfortunately cannot be accurately quantified [153].

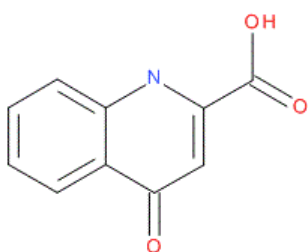


Figure 9 - Chemical structure of kynurenic acid [157]

KA is produced in the CNS by both neurons and astrocytes and causes effects at a number of receptors with varying degrees of affinity. Astrocytes have been shown to decrease KA production in response to the neuronal signal glutamate [158]. KA is produced in the CNS, predominantly by astrocytes, and is not able to cross the blood-brain barrier from the periphery [153]. KA is the only known endogenous antagonist of

the NMDA receptor [69] and displays concentration-dependent activities at this receptor [152]. The NMDA receptor is a subtype of ionotropic glutamate receptors with NMDA selectively binding to the receptor and is involved in memory, an important component in the aetiology of neurotoxicity. The most notable effect is its ability to non-competitively antagonise the GlyB co-agonist site of NMDA receptors [159] at low concentrations and to antagonises the actions of QA, another KM that will be discussed below [152]. At high concentrations, KA shows higher affinity for the glutamate binding site of the NMDA receptor [152]. In addition, KA exerts pharmacological actions at a number of other receptors including the $\alpha 7$ nAChRs, α -amino-3-hydroxymethyl-4-isoxazolepropionic acid (AMPA), kainite and GPR₃₅ receptors [156]. KA exerts antagonistic effects at $\alpha 7$ nAChR with higher affinity than for NMDARs [69].

It has been found that KA exerts its neuroprotective effects through its antagonism of NMDA receptors and as more recently shown through its antioxidant action [160]. In support of the latter, a study undertaken by Lugo-Huitron *et al* has shown a number of important neuroprotective effects of KA [161]. This study showed that KA has $\bullet\text{OH}$, $\text{O}_2^{\bullet-}$ and ONOO^- scavenging properties in a concentration-dependent manner as well as a protective effect on FeSO_4 -induced lipid peroxidation in the forebrain and cerebellum [161]. This research also showed that KA decreased oxidative protein degradation by $\bullet\text{OH}$ in a concentration-dependent manner (100 μM , 150 μM and 500 μM) [161]. It has been postulated that KA exerts its antioxidant effects independently of antagonism of the neurotransmitter receptors [161].

Due to the varying effects of KA, the balance in its concentration is important [153]. Elevated concentrations of KA may result in cognitive impairment [156] whereas low concentrations may result in neurotoxicity and neurodegeneration. This is due to KA's effects on the neurotransmitters DA and glutamate and its effects on cognitive and motor behaviours [153]. Additionally, differences in KA levels have been observed in individuals with different mental health conditions. For example, KA levels are decreased in patients with major depression but increased in patients with schizophrenia [162]. One possible explanation for the antidepressant activity of KA is that NMDA receptor antagonists have been shown to increase 5-HT levels in the brain [163].

3-Hydroxykynurenine

3-HK (figure 10) has been identified as one of the neurotoxic compounds in the KP. It is the first metabolite in the “neurotoxic arm” of the pathway and is synthesised from KYN utilising the enzyme KMO [81]. Due to its neurotoxic nature, it has the potential for playing a role in the pathophysiology of depression.

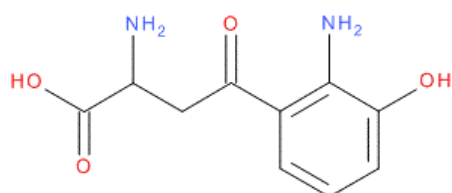


Figure 10 - Chemical structure of 3-hydroxykynurenine [164]

3-HK exerts its neurotoxic effects through its highly redox active nature [95] and undergoes spontaneous auto-oxidation, which as a result, generates free radicals [69]. It has been proposed that different neuronal populations may be selectively affected [165]. In addition to this, the neurotoxic potential has been shown to increase with chronic exposure of up to 72 h in a cell-based *in vitro* assay [165]. In aiding in its cytotoxic effects, 3-HK is readily taken up into cells via the neutral amino acid transport system via a Na⁺-dependent mechanism [166]. Relatively high concentrations of 3-HK (500 to 600 µM) appear to be required to induce toxic effects, as well as a 48 h period of exposure required to show death in PC12 pheochromocytoma cells [166].

The main cause of death identified with 3-HK exposure was apoptosis and additive toxicity with QA, another neurotoxic KM was shown [165]. 3-HK has been shown to induce apoptosis that was potentiated by superoxide dismutase (SOD), heme-like SOD mimetic, manganese (III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and deferoxamine in cerebellar granule cells, indicating free radical involvement in the death process [166].

Due to the nature of the cytotoxicity induced by 3-HK, mainly through the formation of free radicals, and the appearance that the antidepressant effects of caffeinated coffee are independent of its antioxidant properties, indicating that 3-HK alone may only play a minor role, if any in the pathophysiology of depression. This may differ however when combined with the other neurotoxic metabolites – QA and 3-HA and as such the effects of components of caffeinated coffee, require further

investigation into their protective effects against 3-HK toxicity when exposed to neurons alone and in combination.

3-Hydroxyanthranilic acid

3-HA, figure 11, is a highly redox active compound that is biosynthesised either from the hydrolysis of 3-HK through the enzyme kynureninase (KYNU) or the oxidation of anthranilic acid (AA) [152]. 3-HA has been identified as neurotoxic KM and has shown some similarity in toxic effects as QA, with both agents decreasing the activity of choline acetyltransferase [152]. Additionally, like other KMs, 3-HA plays a role in immunoregulation [152] and increased levels have been shown to be correlated with anxiety and depression in an animal model [131].

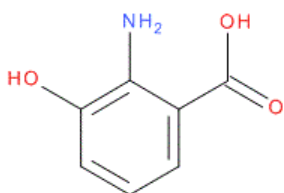


Figure 11 - Chemical structure of 3-hydroxyanthranilic acid [167]

As a result of the redox active nature of this compound, it acts as both a free radical generator [69, 95] and scavenger [152]. In the presence of copper, 3-HA is a generator of the free radicals superoxide and hydrogen peroxide. Conversely, 3-HA also acts as an antioxidant, scavenging peroxy radicals [152].

3-HA has been shown to induce selective apoptosis of TH1 cells, key cells in immunoregulation, through activation of caspase-8 and the release of cytochrome *c* from mitochondria [81] and formation of free radicals and may play an important role in peripheral immunoregulation [152]. 3-HA (200 mM) was found to cause significant apoptosis of U937 and THP-1, human monocytic cell lines [168].

Quinolinic acid

QA, a pyridine dicarboxylic acid/quinoline (figure 12), is an NMDA receptor agonist at the glycine-binding site [131]. QA is synthesised from 3-HA utilising the enzyme 3-hydroxyanthranilic acid oxygenase (3-HAAO) [169]. Activated glial cells and monocytes/macrophages are the only cell lines that possess the full enzymatic pathway for QA synthesis [95]. However, in the presence of inflammation, activated monocytes have been found to be the highest producers of quinolinic acid and until inflammation has subsided, QA production continues [122]. This highlights the importance of QA in the pathophysiology of neuroinflammation and possibly depression.

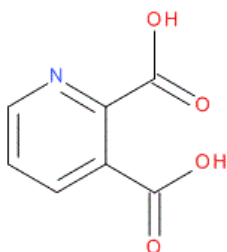


Figure 12 Chemical structure of quinolinic acid [170]

QA has been identified as a neurotoxin and is thought to exert its toxicity primarily through its excitotoxic nature, mediated through agonism at NMDA receptors. Secondary to its excitotoxic effects, QA is also capable of producing (i) free radicals, resulting in oxidative stress [95] and (ii) mitochondrial dysfunction [169]. Studies have shown that death associated with QA toxicity is predominantly necrotic in nature [156] and additive toxicity is observed when in combination with the neurotoxin 3-HK [165]. Additionally, the neurotoxic potential of QA, like 3-HK, has been shown to significantly increase with chronic exposure (up to 72 h) [165].

Studies have shown that endogenous concentrations of QA to be present in brain tissue in the nanomolar range [156] and in blood at less than 50 nM. However, it is thought that QA levels may be much more concentrated around the cells that synthesise it [95] thus potentially have a much more pronounced cytotoxic effect. In particular, it has been shown that some subsets of neurons are particularly sensitive to QA [165], and can be killed by maintaining concentrations around 100 nM [95]. QA levels have been shown to increase dramatically in response to bacterial endotoxin, for example LPS, with concentrations increasing 246-fold in the brain [169]. In addition, inflammatory conditions have been shown to increase the concentration of QA *in vivo*. Such increases have been observed with microbial infections and the resultant immune activation have the ability to increase QA levels to potentially toxic levels in both cerebral spinal fluid (CSF) and blood [95].

QA has been shown to have a number of effects with regard to oxidative stress and free radical formation. It is thought to exert its neurotoxic effects largely through

the formation of free radicals thought to be formed through agonism at the NMDA receptor [171]. This action results in the opening of a Na^+ and Ca^{2+} permeable channel, initiating a cascade of events beginning with an increase in intracellular Ca^{2+} [171]. This in turn results in the activation of nitric oxide synthase (NOS) activity, leading to increased nitric oxide (NO) and free radical formation and resultant mitochondrial dysfunction and deoxyribonucleic acid (DNA) strand breaks [171]. Additionally, QA has been shown to provoke lipid peroxidation and potentiate oxidative stress [156]. The oxidative stress caused by the exposure of QA has been shown to have an effect on immune function. It has been shown to induce selective apoptosis of TH1 cells through activation of caspase-8 and the release of cytochrome *c* from mitochondria and formation of free radicals [81].

Pro-inflammatory cytokines

A number of other pro-inflammatory mediators have been shown to be increased in LPS models of depression. One such study has shown that the concentrations of numerous cytokines including IL-1 β , IL-6, TNF- α and IFN- γ are raised in the plasma or brain after exposure to LPS [146]. These results, however, are time and tissue-dependent [146]. IL-1 β concentrations were increased in the serum of animals taken after 2 h but was returned to baseline at the 6 h and 24 h time points [146]. Furthermore, increases in brain IL-1 β concentrations were not observed [146]. IL-6 concentrations were shown to be significantly increased in plasma 2 h, 6 h and 24 h post-LPS exposure however, in the brain, IL-6 concentrations were increased at the 2 h and 6 h time points but returned to baseline after 24 h [146]. Similarly, TNF- α concentrations were found to be increased in both the plasma and brains of animals at 2 h and 6 h but returned to baseline after 24 h [146]. IFN- γ showed a slightly different

pattern with only detectable concentrations in plasma 6 h post-LPS exposure [146]. Conversely, another study has shown central concentrations of IL-1 β , IL-6 and TNF- α to be significantly raised after 24 h exposure to LPS [172]. This study did however look at the cytokine concentrations in specific regions of the brain, including the hippocampus and hypothalamus rather than full brain homogenates [172].

Cyclooxygenase-1 and -2 both play a central role in neuroinflammation, converting arachidonic acid into numerous prostaglandins and thromboxanes including PGE₂ [173, 174]. Centrally, both cyclooxygenase-1 and -2 are expressed and have differing roles in the inflammatory process [173]. Post-LPS exposure, cyclooxygenase-2 is induced this result in the production of prostaglandins and thromboxanes, in particular PGE₂, which both contribute to the continuing inflammation and ultimately damage [173, 175]. Cyclooxygenase-2, however, in addition to its production of prostaglandins and thromboxanes, is also responsible for anti-inflammatory effects that modulate the neuroinflammatory process [173]. Studies have shown that cyclooxygenase-2 deletion or inhibition of cyclooxygenase-2 with selective agents such as celecoxib increases neuronal damage [173, 176-178]. Furthermore, studies have shown cyclooxygenase-1 inhibitors to suppress neuroinflammatory mediators *in vitro* [179]. This suggests that through blocking cyclooxygenase-1, anti-inflammatory effects and possible antidepressant-like activities may be observed. Furthermore, blocking LPS-induced increases in cyclooxygenase-2 activity may result in a varied response due to decreasing PGE₂ production but also blocking the anti-inflammatory effects.

A number of bioactive coffee constituents including caffeine, CGA, CA and FA have been shown to modulate the cyclooxygenase pathways [180-187]. Caffeine, CGA and FA have all been shown to decrease cyclooxygenase-2 activity in various *in vitro* models, including LPS-induced inflammation [180-182, 184-187]. Furthermore, CA, a polyphenol, was shown to inhibit 12-O-tetradecanoyl-phorbol-13-acetate induced expression of cyclooxygenase-2 *in vivo* [183]. This suggests that further investigations into the effects of coffee are warranted.

Neopterin and biopterin

Neopterin and biopterin are identified biomarkers associated with inflammation and depression [188-190]. Neopterin is formed by macrophages under the control of IFN- γ via GTP-cyclohydrolase I [191, 192] and is then converted to biopterin, an essential co-factor in neurotransmitter synthesis [192]. Biopterin is a required co-factor for tryptophan hydroxylase and tyrosine hydroxylase, both key enzymes in the synthesis of 5-HT and DA and NA, respectively [193]. Given the IFN- γ -dependent control of the production of neopterin, its concentrations strongly correlate with Trp catabolism as measured by IDO [191]. Neopterin concentrations have been found to be raised in depressed patients conversely biopterin concentrations were found to be decreased [194-196], resulting in an increased neopterin to biopterin ratio [189]. Given the role of biopterin in neurotransmitter synthesis, it would be expected that decreased monoamine synthesis is present in depressed patients, consistent with current theories of depression [15, 59, 120].

2.1.4 Monoamines in depression

Further to the components already discussed, it is hypothesised that an alteration in the concentrations of monoamines contributes to the pathophysiology of depression [78]. This is not a new concept, and formed the basis for the original hypothesis of depression [78]. To date, no studies have assessed the effects of caffeine, caffeinated coffee and decaffeinated coffee on central monoamine concentrations.

As a result of the evidence outlined above, this study will evaluate the effects of caffeine, caffeinated coffee and decaffeinated coffee on the various parameters of depression.

2.2 Hypothesis, aims and objectives

2.2.1 Hypothesis

The hypothesis of this study was that caffeinated coffee would show antidepressant-like effects and decrease pro-inflammatory biomarkers associated with depression whereas decaffeinated coffee and caffeine alone would not.

2.2.2 Aims and objectives

The primary aim of this study was to assess the effects of caffeine, caffeinated and decaffeinated coffee on behavioural aspects of depression in an *in vivo* murine model of LPS-induced neuroinflammatory depression.

This aim was achieved through the following objectives:

- The effects of caffeine, caffeinated coffee and decaffeinated coffee on anxiety-like behaviours was assessed using the open field test; and
- The effects of caffeine, caffeinated coffee and decaffeinated coffee on depressive-like behaviours was assessed using the forced swimming test and the tail suspension test.

The secondary aim of this study was to assess the effects of caffeine, caffeinated and decaffeinated coffee on pro-inflammatory biomarkers associated with depression in an *in vivo* murine model of LPS-induced neuroinflammatory depression.

This aim was achieved through the following objectives:

- Plasma biomarkers of inflammation including IDO activity, cortisol concentrations and neopterin and biopterin concentrations were quantified using HPLC; and
- Central biomarkers of inflammation including IFN- γ , IL-6, TNF- α , PGE₂, cortisol and KMs were quantified using either high sensitivity ELISA assay or HPLC analysis.

The tertiary aim of this study was to assess the effects of caffeine, caffeinated and decaffeinated coffee on central monoamine concentrations in an *in vivo* murine model of LPS-induced neuroinflammatory depression.

This aim was achieved through the following objectives:

- The central monoamines 5-HT, NA and DA concentrations were quantified using HPLC.

The final aim of this study was to quantify the concentration of caffeine present in the plasma and brains of animals treated with caffeine and caffeinated coffee.

This aim was achieved through the following objective:

- The plasma and central caffeine concentrations were quantified using HPLC.

2.3 Materials and Methods

2.3.1 Animals

C57BL/6J (male, 8-9 weeks) mice (obtained from Animal Resources Centre, WA, Australia) were used in the *in vivo* animal model of depression according to previous protocols [145, 149, 197, 198], with 5 animals per study group. The sample size was calculated using the power calculation outlined below.

2.3.2 Ethical approvals

Ethical approval to undertake this was obtained from the Griffith University Animal Ethics Committee under the approval "Effect of coffee and its constituents on

LPS-induced neuroinflammation and depression in male mice" (GU Ref No: PHM/01/15/AEC).

2.3.3 Sample size

Power calculations were undertaken, according to previous studies [199] to ensure an adequate sample size of the study. The difference in the outcome was based on previous studies [200, 201]. The required minimum sample size needed was found to be 5 animals per treatment and control group and as a result, 5 animals per group were used in the current study.

2.3.4 Inflammatory stimulus, preparation of inflammatory stimulus and its administration

LPS from *E. coli* O111:B4 was used as the inflammatory stimulus at a dose of 0.83 mg/kg, based on previous studies [136, 145, 202-205], to precipitate depression. Stock solutions (1 mg/mL) were prepared in sterile 0.9% saline (Pfizer, West Ryde, Australia) and were stored at -20°C. Working solutions were further diluted in 0.9% saline and were made up fresh on the day of the experiment. LPS was administered to the animals via intraperitoneal (i.p.) injection.

2.3.5 Pharmacological agents, preparation of agents and their administration

Numerous pharmacological agents were used in this study. These included the positive control imipramine (Sigma Aldrich, MO, USA) and the agents of interest caffeine (PCCA, Matraville, Australia), caffeinated coffee and decaffeinated coffee

(brand, bean and roast matched, sourced locally). Stock solutions of imipramine (10 mg/mL), caffeine (10 mg/mL), caffeinated and decaffeinated coffee stock solutions (140 mg/mL) were prepared in hot water and were further diluted in water prior to dosing.

2.3.6 Summary of protocol

The study was carried out in accordance with the details outlined in figure 13 below. Briefly, animals were allowed to acclimatize to the animal facility conditions for 5 days. Baseline behavioural studies were undertaken using the open field test to assess locomotor activity. Dosing of saline, imipramine, caffeine, caffeinated coffee and decaffeinated coffee began on day 1 of the study and continued daily until day 14 of the study. The open field test, the forced swimming test and the tail suspension test were done on days 1, 7 and 14. After the final dosing and behavioural tests on day 14, LPS was administered. The open field test was then done 6 h post-LPS administration and the open field test, forced swimming test and the tail suspension test repeated at day 15, 24 h after LPS administration. Animals were then sacrificed via cervical dislocation and the plasma and the brains collected and stored at -80°C until further processing.

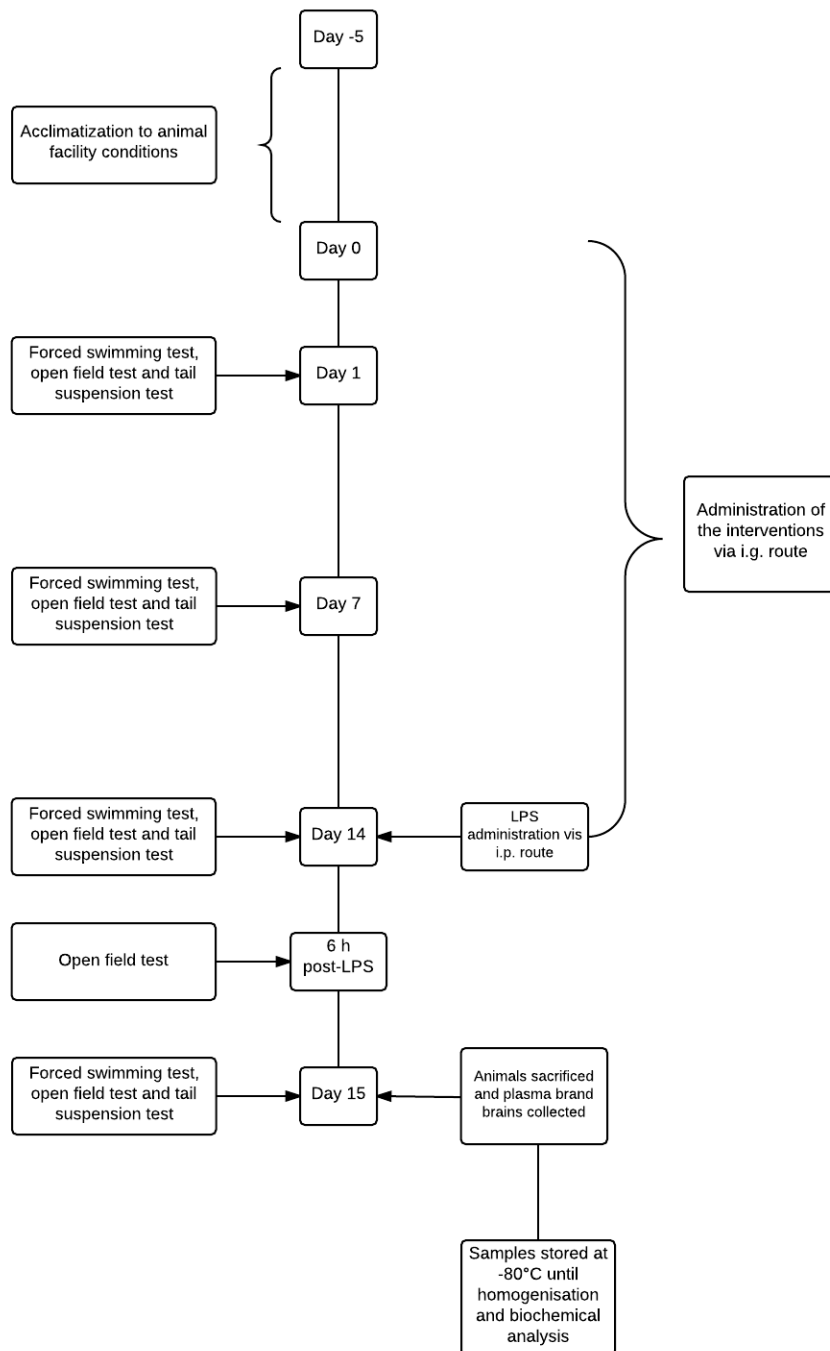


Figure 13 – Flow diagram summarising the animal study protocol

Table 1 – Test groups for *in vivo* animal study

Group number	Control or test group	Intervention
One	Vehicle control group	Intraperitoneal saline injection (0.83 mg/kg) + intragastric saline (10 mL/kg)
Two	Negative control group	Intraperitoneal injection of pro-inflammatory stimulus (LPS) (0.83 mg/kg) + intragastric saline (10 mL/kg)
Three	Positive control group	Intraperitoneal injection of pro-inflammatory stimulus (LPS) (0.83 mg/kg) + intragastric imipramine (10 mg/kg)
Four	Test group – caffeine	Intraperitoneal injection of pro-inflammatory stimulus (LPS) (0.83 mg/kg) + intragastric caffeine (10 mg/kg)
Five	Test group – caffeinated coffee	Intraperitoneal injection of pro-inflammatory stimulus (LPS) (0.83 mg/kg) + intragastric caffeinated coffee (140 mg/kg)
Six	Test group – decaffeinated coffee	Intraperitoneal injection of pro-inflammatory stimulus (LPS) (0.83 mg/kg) + intragastric decaffeinated coffee (140 mg/kg)

2.3.7 Behavioural studies

Numerous behavioural studies were employed to assess the effects of caffeine, caffeinated and decaffeinated coffee on depressive-like and anxiety-like behaviours.

These studies included the OFT to assess for locomotor activity and the FST and TST to assess for despair or depressive-like symptoms. Each of these tests will be explained in further detail below.

Locomotor activity

The OFT was used in this study to assess the effects of the pharmacological compounds of interest on anxiety levels in the animals. This test was performed according to previously described methods [206]. Briefly, The effects of LPS and all the pharmacological treatments on locomotor activity (LMA) were assessed in mice individually placed into a clean, novel cage similar to the home cage, but devoid of bedding or litter. The cage was divided into four virtual quadrants, and LMA was measured by counting the number of line crossings and rearings over a 5-min period. Counting was done by a well-trained observer [136].

Forced swimming test

The FST was employed to study the effect of caffeine, caffeinated coffee and decaffeinated coffee on depressive-like behaviour. The FST involves placing animals in a container of water, which is deep enough so that their tails are unable to rest on the bottom of the container and is assessed by a lack of struggling or increased immobility periods indicating a state of depression [207]. This test was performed according to previously described methods [208]. Briefly, the mice were forced to swim individually in a glass jar (26 x 18 x 16 cm²) containing water at room temperature. The height of the water level was adjusted to 15 cm. After an initial period of vigorous activity the animals assume a type of immobile posture. A mouse is said to be immobile

when it ceases struggling and make minimal movement of limbs to keep the head above water. The total duration of the test was 6 min with the immobility time recorded over the last 5 min of the study [208].

Tail suspension test

The TST, another behavioural test used to assess depressive-like behaviour, was also employed in this study. This test was performed according to previously described methods [209-211]. Briefly, a small piece of medical adhesive tape was placed approximately 2 cm from the tip of the tail. A small hole was then punched in the tape and the mice were hung individually for a period of 5 min on a hook. The duration of immobility was then measured manually by experienced observers during the final 4 min of the test.

2.3.8 Quantification of plasma biomarkers

Numerous plasma biomarkers associated with depression were assessed for changes after exposure to LPS and the pharmacological interventions imipramine, caffeine, caffeinated coffee and decaffeinated coffee. Amongst the biomarkers assessed included plasma IDO activity, plasma neopterin and biopterin concentrations, plasma cortisol concentrations, central KM concentrations, central monoamine concentrations, central PGE₂ and central pro-inflammatory cytokines IFN- γ , IL-6 and TNF- α . The details of each of the assays will be explained in further detail below.

IDO activity

Plasma IDO activity was measured through the quantification of plasma Trp and KYN concentrations and subsequently the ratio of KYN to Trp present. Plasma Trp and KYN were quantified using HPLC according to the protocol outlined below.

Preparation of standard solutions

The stock solutions were composed of 10 mM of Trp (Sigma-Aldrich, St Louis, MO, USA) and KYN (Sigma-Aldrich, St Louis, MO, USA) in water and were stored at -20°C. All working standards were further diluted in water and prepared fresh on the day of analysis.

Apparatus

The HPLC system used consisted of a Shimadzu CBM-20A Prominence communications bus control module, two Shimadzu LC-20AD UFLC liquid chromatograph pumps fitted with a solvent mixer, a Shimadzu DGU-20A3 Prominence degasser, a Shimadzu SIL-20A HT UFLC Prominence chilled autosampler module, a Shimadzu CTO-20AC Prominence column oven, a Shimadzu SPD-M20A Prominence Diode array detector and Labsolutions software. The column used was a Phenomenex Gemini (5 μ m, 250 x 4.6 mm) reverse phase column (Phenomenex, Lane Cove, Australia) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

A previously described trichloroacetic acid (Sigma-Aldrich, St Louis, MO, USA) protein precipitation method [10] was used to isolate the analytes of interest. Briefly, 25 μ L of sample was placed in a tube and diluted with 25 μ L of water. Subsequently, 10 μ L of 100% trichloroacetic acid solution was added to each sample. The sample was vortexed for 30 seconds and then centrifuged at 14000 x g for 30 min. 50 μ L of the supernatant was then placed in glass HPLC vials containing small volume glass inserts and analysed according to the methods outlined below.

Calibration curve

Porcine serum (Gibco, Scoresby, Australia) was used for the calibration and quality control standards. Calibration standards, 100 μ M to 6.25 μ M, were prepared by spiking specific volumes of the working solutions to blank plasma and subsequently serial dilutions undertaken. Quality control samples were prepared at three levels of concentrations, 7.5 μ M, 15 μ M and 30 μ M.

HPLC method

The HPLC method utilised in this study was validated according to the parameters outlined below. Briefly, mobile phase A consisted of a 0.1% glacial acetic acid (GAC) (Merck, Frenchs Forest, Australia) solution and mobile phase B consisted of 100% acetonitrile (ACN) (Scharlau, Barcelona, Spain). An isocratic method, utilising 10% mobile phase B, at 40°C with a flow rate of 1 mL/min and run over 10 min was used to elute the analytes of interest. The UV absorbance of the KYN was

monitored at 360 nm and the fluorescence of Trp was monitored at excitation 275 nm and emission 350 nm based on previously published data [212].

Validation of methods

Each method was validated according to the International Conference on Harmonisation (ICH) guidelines [213] with respect to specificity, linearity, lower limit of detection, lower limit of quantification, accuracy, precision and recovery.

Linearity

The linearity of the respective methods was evaluated using calibration curves prepared according to the methods described above. The calibration curves were constructed using the peak area of the analytes of interest and fitted by least-squares linear regression analysis.

Lower limit of detection and lower limit of quantification

The limits of quantification (signal to noise ratio 10-to-1) and limits of detection (signal-to-noise ratio 3-to-1) were determined using successive dilutions of standards of the analytes of interest.

Precision and accuracy

The precision of the respective methods was measured according to both intra- and inter-day evaluation of triplicate measurements of low, medium and high concentration QC standards on one single day for intra-day evaluation and triplicate measurements on three consecutive days for inter-day evaluation. The coefficient of variance of the measured concentrations was used to report precision, and was accepted if it met the ICH recommendations of less than 10%. The accuracy of the respective methods was determined by comparing the calculated concentration from the standard curves to the theoretical concentration. The limits for the accuracy values were 85 to 115% of expected, according to the ICH recommendations [213].

Cortisol

Plasma cortisol concentrations were determined using an HPLC method as outlined below.

Preparation of standard solutions

The stock solutions were composed of 1 mg/mL of hydrocortisone (Abcam, Melbourne, Australia) in ethanol and were stored at -20°C. All working standards were prepared fresh on the day of analysis.

Apparatus

The HPLC system used was as described in section 2.3.8. The column used was a GraceSmart (5 μ m, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

A trichloroacetic acid protein precipitation method was used to isolate the analytes of interest. Briefly, 25 μ L of sample was placed in a tube and diluted with 25 μ L of water. Subsequently, 10 μ L of 100% trichloroacetic acid solution was added to each sample. The sample was vortexed for 30 seconds and then centrifuged at 14000 x g for 30 min. 50 μ L of the supernatant was then placed in glass HPLC vials containing small volume glass inserts and analysed according to the methods outlined below.

Calibration curve

Porcine serum (Gibco, Scoresby, Australia) was used for the calibration and quality control standards. Calibration standards, 100 μ M to 6.25 μ M, were prepared by spiking specific volumes of the working solutions to blank plasma and subsequently serial dilutions undertaken. Quality control samples were prepared at three levels of concentrations, 7.5 μ M, 15 μ M and 30 μ M.

HPLC method

The HPLC method utilised in this study was validated according to the parameters outlined below. Briefly, mobile phase A consisted of a 0.1% GAC (Merck, Frenchs Forest, Australia) solution and mobile phase B consisted of ACN (Scharlau, Barcelona, Spain). A gradient method of 0 to 10 min, 1% mobile phase B to 90% mobile phase B; 10 to 17 min, 90% to 1%; 17 to 20 min, re-equilibration using 1% mobile phase B, at 40°C with a flow rate of 1 mL/min and run over 20 min was used to elute cortisol. The UV absorbance of the cortisol was monitored at 248 nm based on previously published data [214].

Validation of methods

The HPLC method was validated using the protocol listed in section 2.3.8.

Biopterin/neopterin

The HPLC method described below was used to quantify plasma concentrations of biopterin and neopterin. This method was run as previously described [215] and as a result, a full method validation was not undertaken.

Sample preparation

The sample preparation method for neopterin and biopterin was undertaken as previously described [215]. Briefly, 25 µL of sample was diluted with 25 µL of PBS

and 5 μL of 100% trichloroacetic acid solution added to each tube. The sample was vortexed for 30 seconds and then centrifuged at 14000 $\times g$ for 30 min. 50 μL of the supernatant was then placed in glass HPLC vials containing small volume glass inserts and analysed according to the methods outlined below.

Apparatus

The HPLC system used was as described in section 2.3.8. The column used was a Phenomenex Gemini (5 μm , 250 \times 4.6 mm) reverse phase column (Phenomenex, Lane Cove, Australia) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Method

The HPLC method utilised in this study was a previously described method outlined below. Briefly, mobile phase A consisted of water and mobile phase B consisted of 100% ACN (Scharlau, Barcelona, Spain). An isocratic method, utilising 1% mobile phase B, at 40°C with a flow rate of 1 mL/min and run over 10 min was used to elute the analytes of interest. The fluorescence of biopterin and neopterin was monitored at excitation 353 nm and emission 438 nm based on previously published data [215, 216].

2.3.9 Quantification of central inflammatory biomarkers

Preparation of brain homogenates

Mouse brain homogenates used for analysis of inflammatory biomarkers were prepared according to previously described methods [217]. Briefly, mouse brains were halved and weighed. PBS (Sigma Aldrich, St Louis, MO, USA) containing protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA) was added in a ratio of 15 μ L per 1 mg of wet weight brain. Brains were homogenized using a dounce homogenizer on ice and were centrifuged at 13000 x *g* for 30 min at 4°C. Aliquots of the resultant supernatant was taken and stored at -80°C until further analysis.

Quantification of TNF- α

The high sensitivity TNF- α mouse ELISA kit (eBioscience, San Diego, USA) uses a quantitative sandwich enzyme immunoassay technique, with plates coated in a polyclonal antibody specific for mouse TNF- α . TNF- α present in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for mouse TNF- α , which is then recognised by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added, the reaction stopped and the intensity of colour measured.

The TNF- α mouse ELISA kit contains a TNF- α microplate coated with a monoclonal antibody, TNF- α standard (mouse), biotinylated mouse TNF- α antibody (100x), calibrator diluent, sample diluent, assay buffer concentrate (20x), amplification diluent concentrate (2x), amplification reagent I, amplification reagent II, wash buffer

concentrate (20x), streptavidin-peroxidase conjugate (SP conjugate), chromagen substrate (tetramethylbenzidine) and stop solution (1M phosphoric acid). This assay will be used to assess the effects pharmacological modulators have on TNF- α concentration and were performed according to the manufacturer's instructions.

Briefly, all reagents, working standards and samples were prepared as per instructions. 50 μ L of the standard, sample or calibrator diluent in addition to 50 μ L of sample diluent was added to the relevant wells. 50 μ L of biotin conjugate was then added to each well and was subsequently incubated for 2 h at room temperature on a microplate shaker. After incubation, the wells were washed 6 times with 200 μ L of wash buffer and 100 μ L of streptavidin-HRP was added to each well and the plates incubated for 1 h at room temperature on a microplate shaker. The plate was again washed 6 times with 200 μ L of wash buffer and 100 μ L of the amplification solution 1 was added to each well and was incubated for exactly 15 min at room temperature on a microplate shaker. The plate was then washed 6 times with 200 μ L of wash buffer and 100 μ L of amplification solution 2 was added followed by incubation for exactly 30 min at room temperature on a microplate shaker. The plate was then washed 6 times with 200 μ L of wash buffer and 100 μ L TMB substrate solution was added to each well and the plate was incubated at room temperature in the dark for approximately 20 min. 100 μ L of stop solution was then added to each well and the absorbance was read on a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. Sample values were calculated from the standard curve and will be represented as picogram per millilitre \pm standard deviation.

Quantification of IL-6

The high sensitivity IL-6 mouse ELISA kit (eBioscience, San Diego, USA) uses a quantitative sandwich enzyme immunoassay technique, with plates coated in a polyclonal antibody specific for mouse IL-6. IL-6 present in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for mouse IL-6, which is then recognised by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added, the reaction stopped and the intensity of colour measured.

The high sensitivity IL-6 mouse ELISA kit contains a IL-6 microplate coated with a monoclonal antibody, IL-6 standard (mouse), biotinylated mouse IL-6 antibody (100x), calibrator diluent, sample diluent, assay buffer concentrate (20x), amplification diluent concentrate (2x), amplification reagent I, amplification reagent II, wash buffer concentrate (20x), streptavidin-peroxidase conjugate (SP conjugate), chromagen substrate (tetramethylbenzidine) and stop solution (1 M phosphoric acid). This assay will be used to assess the effects pharmacological modulators have on IL-6 levels and were performed according to the manufacturer's instructions.

Briefly, all reagents, working standards and samples were prepared as per instructions. 50 μ L of the standard, sample or calibrator diluent in addition to 50 μ L of sample diluent was added to the relevant wells. 50 μ L of biotin conjugate was then added to each well and the plates were subsequently incubated for two h at room temperature on a microplate shaker. After incubation, the wells were washed 6 times with 200 μ L of wash buffer and 100 μ L of streptavidin-HRP was added to each well and

the plates incubated for one h at room temperature on a microplate shaker. The plate was again washed 6 times with 200 μ L of wash buffer and 100 μ L of the amplification solution 1 was added to each well followed by incubation for exactly 15 min at room temperature on a microplate shaker. The plate was then washed 6 times with 200 μ L of wash buffer and 100 μ L of amplification solution 2 was added and incubated for exactly 30 min at room temperature on a microplate shaker. The plate was then washed 6 times with 200 μ L of wash buffer and 100 μ L TMB substrate solution was added to each well and the plates were incubated at room temperature in the dark for approximately 20 min. 100 μ L of stop solution was then added to each well and the absorbance was read on a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. Sample values were calculated from the standard curve and will be represented as picogram per millilitre \pm standard deviation.

Quantification of IFN- γ

Central IFN- γ concentrations were measured using the Biosensis Mouse Interferon Gamma ELISA kit (Thebarton, Australia). This assay is a sandwich ELISA using Avidin-Biotin-Peroxidase complex (ABC) enzyme and TMB substrate as a means to measure the concentration of IFN- γ present in the sample. The assay was performed according to the manufacturer's recommendations. Briefly, standards and homogenate samples were added to the appropriate wells pre-coated with monoclonal IFN- γ antibody and incubated. Post-incubation a biotinylated mouse IFN- γ polyclonal antibody was added to each well and subsequently further incubated. The enzyme ABC was then added to each well and after the appropriate incubation the TMB substrate was added to each well and left to develop in the dark. Subsequently, the TMB stop solution

was added to each well and the absorbance of each well read at 450 nm on a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Quantification of PGE₂

Central PGE₂ was quantified using the PGE₂ express EIA (Cayman Chemical, Michigan, USA), a competitive EIA. This assay is based on the competition between PGE₂ and a PGE₂-acetylcholinesterase conjugate for a limited amount of PGE₂ monoclonal antibody. Given the nature of the assay, there is an inversely proportional amount of PGE₂ in the sample in comparison to PGE₂-AChE conjugate able to bind to the monoclonal antibody. Therefore when Ellman's reagent, an AChE substrate, is added to the wells, the colour intensity observed is inversely proportional to the concentration of PGE₂ present in the sample.

The PGE₂ Express ELISA kit used in this study contained prostaglandin E₂ express ELISA monoclonal antibody, prostaglandin E₂ express AChE tracer, prostaglandin E₂ express ELISA standard, ELISA buffer concentrate (10X), wash buffer concentrate (400X), polysorbate 20, goat anti-mouse IgG coated plate, Ellman's reagent, ELISA tracer dye and ELISA antiserum dye.

The assay was performed according to the manufacturer's instructions with samples diluted 1 in 5 using PBS. Briefly, all buffers and wash solutions were made up prior to the commencement of the assay according to the manufacturer's instructions. The appropriate amounts of ELISA buffer, PGE₂ express ELISA standard, samples,

PGE₂ express AChE tracer and PGE₂ express monoclonal antibody were added to the appropriate wells of the pre-coated ELISA plate and incubated for 60 min at room temperature on a plate shaker. After incubation, the plate was developed by adding the appropriate amount of Ellman's reagent and tracer to the appropriate wells of the plate. The plate was then incubated in the dark on a plate shaker until development had occurred and the absorbance of each well read at 405 nm on a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

2.3.10 Quantification of central kynurenine metabolites

HPLC methods described below were used to quantify central concentrations of the KMs KYN, 3-HK and KA.

Kynurenine and 3-hydroxykynurenine

KYN and 3-HK were quantified using the HPLC method outlined below.

Apparatus

The apparatus used for the HPLC analysis of KYN and 3-HK was that described in section 2.3.8. The column used in this analysis was a Phenomenex Gemini C18 (5 µm; 250 mm x 4.6 mm) reverse phase column (Phenomenex, Lane Cove, Australia) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Mouse brain homogenates used for analysis of KYN and 3-HK were prepared according to previously described methods [218]. Briefly, mouse brains were halved and weighed. Trichloroacetic acid (1.6%) (Sigma Aldrich, St Louis, MO, USA) was added in a ratio of 10 μ L per 1 mg of wet weight brain. Brains were homogenized using a dounce homogenizer on ice and were centrifuged at 13000 x *g* for 30 min at 4°C. Aliquots of the resultant supernatant was taken and stored at -80°C until further analysis.

HPLC method

The HPLC method used to analyse KYN and 3-HK was that described in section 2.3.8. Briefly, an isocratic method run over 10 min using 0.1% GAC (solvent A) and ACN (solvent B) (90:10 % v/v) and 10 μ L injections of sample was employed to quantify KYN and 3-HK. The UV absorbance was monitored at 360 nm and quantification was made after comparison to a 10 point standard curve.

Kynurenic acid

KA was quantified using the HPLC methods outlined below.

Apparatus

The apparatus used for the HPLC analysis of KA was that described in section 2.3.8. The column used was a GraceSmart (5 μ m, 250 x 4.6 mm) reverse phase column

(Grace Discovery Science, Columbia, MD, USA) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Mouse brain homogenates used for analysis of KMs were prepared according to previously described methods [218]. Briefly, mouse brains were halved and weighed. Trichloroacetic acid solution (1.6%) (Sigma Aldrich, St Louis, MO, USA) was added in a ratio of 10 μ L per 1 mg of wet weight brain. Brains were homogenized using a dounce homogenizer on ice and were centrifuged at 13000 x *g* for 30 min at 4°C. Aliquots of the resultant supernatant was taken and stored at -80°C until further analysis.

HPLC method

The HPLC method used to analyse KA employed an isocratic method run over 10 min using 0.1% GAC (solvent A) and ACN (solvent B) (90:10 % v/v) and 10 μ L injections of sample was employed to quantify KA. The fluorescence of KA was monitored at excitation 344 nm and emission 398 nm and quantification was made after comparison to a 10 point standard curve.

2.3.11 Quantification of monoamines

HPLC methods described below and sample preparation methods described in section 2.8.3 were used to quantify central concentrations of the monoamines 5-HT, DA and NA.

Apparatus

The apparatus used for the HPLC analysis of the monoamine 5-HT, DA and NA was that described in section 2.3.8 with a GraceSmart C18 (5 μ m, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Mouse brain homogenates used for analysis of central monoamines were prepared according to previously described methods with minor modifications [217]. Briefly, mouse brains were halved and weighed. PBS (Sigma Aldrich, St Louis, MO, USA) containing protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA) was added in a ratio of 15 μ L per 1 mg of wet weight brain. Brains were homogenized using a dounce homogenizer on ice and were centrifuged at 13000 x *g* for 30 min at 4°C. Aliquots (50 μ L) of the resultant supernatant were then taken and 5 μ L of 100% trichloroacetic acid solution added

HPLC method

The HPLC method utilised in this study was validated according to the parameters outlined below. Briefly, mobile phase A consisted of a 0.1% GAC (Merck, Frenchs Forest, Australia) solution and mobile phase B consisted of ACN (Scharlau, Barcelona, Spain). A gradient method of 0 to 10 min, 1% mobile phase B to 90% mobile phase B; 10 to 17 min, 90% to 1%; 17 to 20 min, re-equilibration using 1% mobile phase B, at 40°C with a flow rate of 1 mL/min and run over 20 min was used to elute cortisol. The fluorescence of 5-HT, DA and NA was monitored at excitation 280 nm and emission 320 nm.

2.3.12 Quantification of central cortisol

HPLC methods described in section 2.3.8 and sample preparation methods described in section 2.3.8 were used to quantify central concentrations of cortisol.

2.3.13 Plasma and central concentrations of caffeine

Plasma and central caffeine concentrations were quantified using HPLC according to the conditions outlined below.

Preparation of standard solutions

The stock solutions was composed of 2 mM of caffeine, 1,7-dimethylxanthine, theophylline and theobromine in water and were stored at -20°C. All working solutions were prepared fresh on the day of analysis.

Apparatus

The HPLC system used was the system described in section 2.3.8. The column used was a GraceSmart (5 μm , 250 x 4.6 mm) reverse phase column (Grace Davison Discovery Sciences, Melbourne) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

A standard methanol protein precipitation method was used to isolate the analytes of interest. Briefly, 100 μL of sample was placed in a tube and 300 μL of methanol was added. The sample was vortexed for 30 seconds and then centrifuged at 14000 rpm for 30 min. 200 μL of the supernatant was then placed in glass HPLC vials and analysed according to the methods outlined below.

Calibration curve

Blank porcine serum (Gibco, Scoresby, Australia) was used for the calibration and quality control standards. Calibration standards, 100 μM to 6.25 μM , were prepared by spiking specific volumes of the working solutions to blank plasma and subsequently serial dilutions undertaken. Quality control samples were prepared at three levels of concentrations, 7.5 μM , 15 μM and 30 μM .

HPLC method

The HPLC method utilised in this study is a previously described method with minor alterations [219]. Briefly, mobile phase A consisted of a 25 mM sodium

phosphate monobasic buffer adjusted to pH 5.66 with triethylamine and mobile phase B consisted of 100% methanol. An isocratic method, utilising 30% mobile phase B, at 45°C with a flow rate of 1 mL/min and run over 10 min was used to elute the analytes of interest. The UV absorbance of the analytes of interest was monitored at 272 nm.

Validation of methods

The method was validated using the methods outlined in section 2.3.8.

2.3.14 Statistical analysis

One-way or two-way ANOVA with Tukey's post hoc test (were significance was observed) were used in this study and were performed using GraphPad InStat version 3.06 (2003) with $P < 0.05$ (*,#), $P < 0.01$ (**,##), and $P < 0.001$ (***,###). All graphs were drawn using Graphpad Prism v6 (San Diego, USA). Two-way ANOVA analysis was used for analysing the behavioural studies over the various time points and one-way ANOVA analysis was used for all remaining analyses.

2.4 Results

2.4.1 The effects of caffeine, caffeinated coffee and decaffeinated coffee on behaviour in mice

Locomotor activity day 1

The acute effects of the treatment groups on locomotor activity levels, in the absence of LPS, of the animals was tested 60 min post-administration of the first dose

using the OFT, as seen in figure 14. Animals in the vehicle control group had a mean number of crossings of 47.8 ± 10.1 . In comparison, animals treated with the positive control imipramine ($p < 0.05$), and the two test groups receiving caffeine ($p < 0.001$) and caffeinated coffee ($p < 0.05$) all showed signs of increased activity. The caffeine treatment group's number of crossing increased by almost double and the caffeinated coffee group's number of crossing increased by approximately 30%. There was no observed increase in locomotor activity after treatment with decaffeinated coffee.

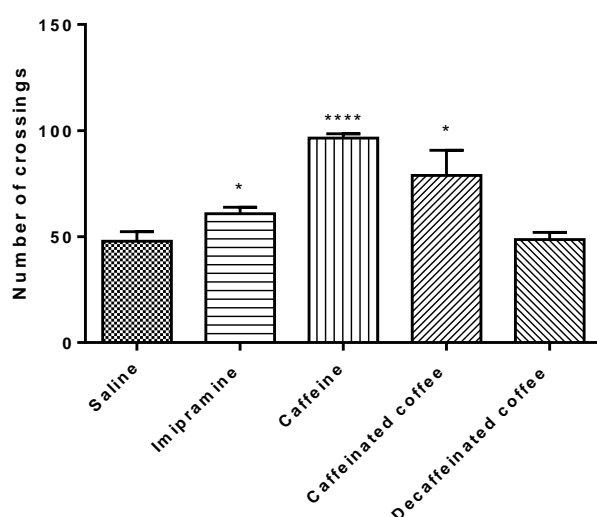


Figure 14 – The acute effects of caffeine, caffeinated coffee and decaffeinated coffee on locomotor activity in C57BL/6J mice measured by the open field test.

Forced swimming test day 1

The acute effects of the treatment groups on depression levels of the animals, in the absence of LPS, was tested 60 min post-administration of the first dose using the forced swimming test as seen in figure 15. The mean immobility time observed in animals in the saline group was 170.2 ± 12.0 seconds. Conversely, animals treated with caffeine ($p < 0.0001$) and caffeinated coffee ($p < 0.001$) both showed significant

decreases in immobility time. The decreases observed in animals treated with caffeine were approximately five-fold lower than saline control and caffeinated coffee approximately 30% lower than saline control.

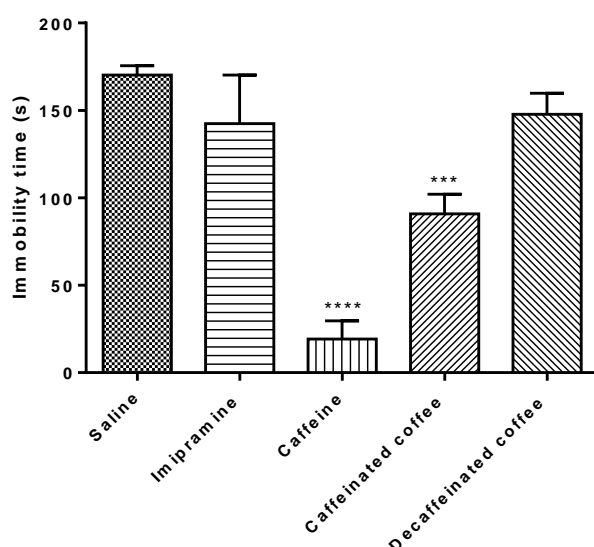


Figure 15 – The effects of caffeine, caffeinated coffee and decaffeinated coffee on immobility time as measured by the forced swimming test.

Tail suspension test day 1

The acute effects of the treatment groups on depression levels of the animals, in the absence of LPS, was tested 60 min post-administration of the first dose using the tail suspension test, as seen in figure 16. The mean immobility time observed in animals in the saline group was 155.2 ± 21.7 seconds. Conversely, animals treated with imipramine ($p < 0.05$), caffeine ($p < 0.001$) and caffeinated coffee ($p < 0.001$) all showed signs of decreased immobility time. The immobility time in both the caffeinated coffee and caffeine treated animals was approximately half of that of

animals in the saline group and was approximately 30% less than the animals in the imipramine treatment group.

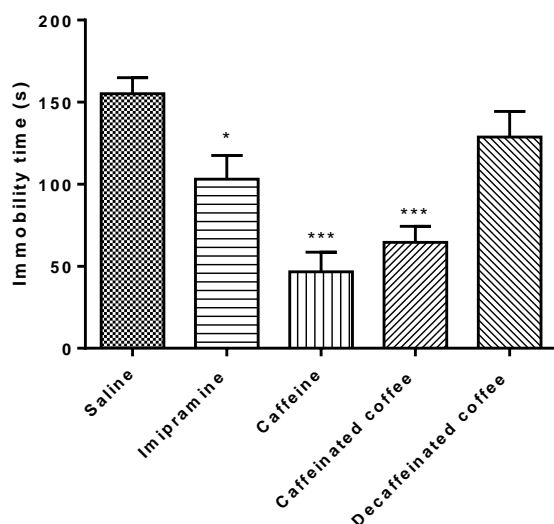


Figure 16 – The effects of caffeine, caffeinated coffee and decaffeinated coffee on immobility time as measured by the tail suspension test.

Locomotor activity

The long term effects of caffeine consumption were observed over the duration of the study to assess if tolerance was occurring. It was shown that chronic dosing of caffeine did not result in any significant changes in locomotor activity for the duration of the study.

Statistically significant increases in locomotor activity were observed at numerous time points in the study using the open field test. The imipramine treatment group at the seven day point showed an increase in locomotor activity in comparison to the vehicle control, saline ($p < 0.05$). The caffeine treatment group showed an increase in locomotor activity at all three time points of day 1, day 7 and day 14 ($p < 0.0001$).

Likewise, the caffeinated coffee treatment group showed an increase in locomotor activity at all three time points of day 1 ($p < 0.05$), day 7 ($p < 0.001$) and day 14 ($p < 0.001$) as seen in figure 17 below. Animals treated with both caffeine and caffeinated coffee both showed increases in locomotor activity at all three time points of the study that were approximately two-fold higher than animals in the saline control group. Furthermore, similar differences were observed between these animals and the animals in the imipramine treatment group. An increase in locomotor activity was observed in animals treated with caffeinated coffee over the duration of the study. An approximate 10% increase in locomotor activity was observed between day 1 and day 14.

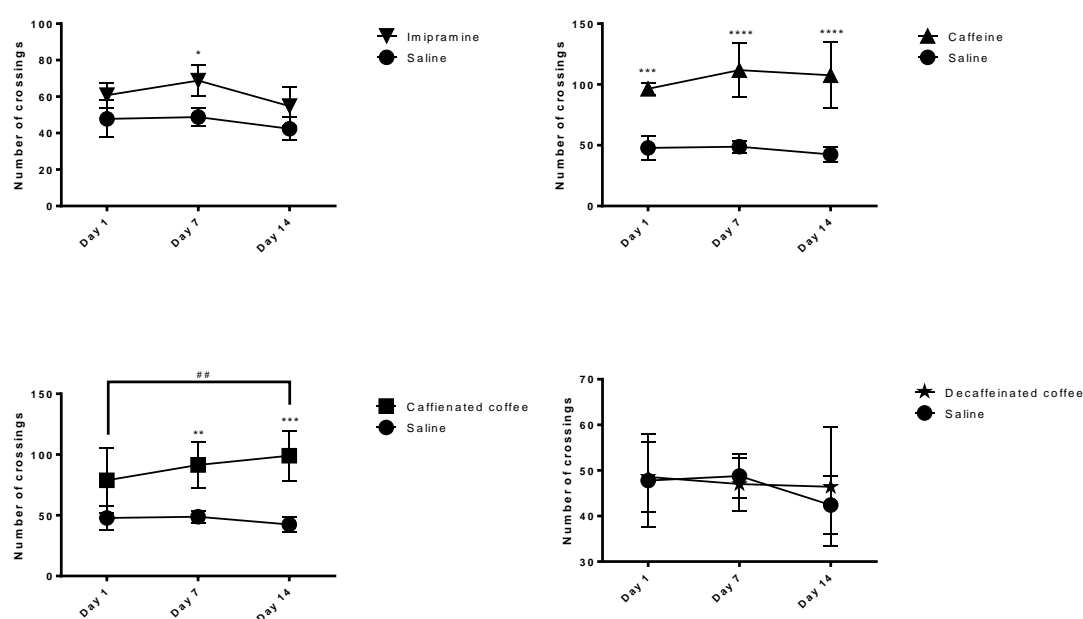


Figure 17 – The chronic effects of saline (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on locomotor activity using the open field test.

Forced swimming test

Statistically significant decreases in immobility time in the forced swimming test were observed at numerous time points in the study. The imipramine treatment group at the 7 ($p < 0.05$) and 14 day ($p < 0.01$) points showed a decrease in immobility time in comparison to the vehicle control, saline. The caffeine treatment group showed a decrease in immobility time at all three time points of day 1, day 7 and day 14 ($p < 0.0001$). Likewise, the caffeinated coffee treatment group showed a decrease in immobility time at all three time points of day 1, day 7 and day 14 ($p < 0.001$) as seen in figure 18 below. Animals treated with both caffeine and caffeinated coffee both showed decreases in immobility time at all three time points of the study that were approximately half that of animals in the saline control group. Furthermore, similar differences were observed between these animals and the animals in the imipramine treatment group. Animals in the caffeinated coffee treatment group showed a progressive decrease in immobility time in the FST over the duration of the study. A significant decrease of approximately 40% was observed in these animals on day 14 in comparison to day 1 ($p < 0.0001$).

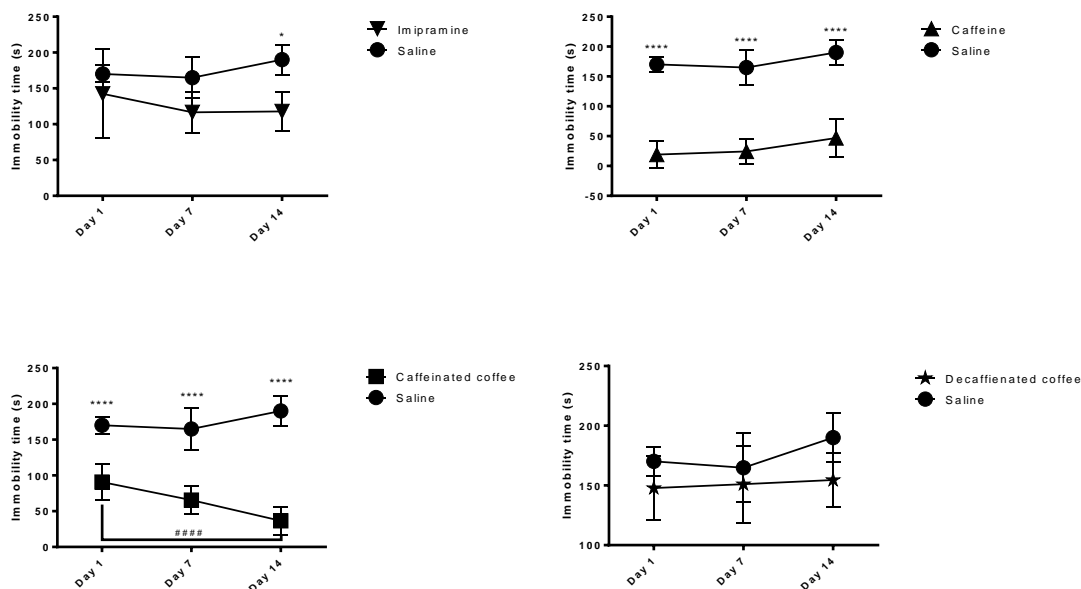


Figure 18 – The chronic effect of saline (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on immobility time using the forced swimming test.

Tail suspension test

Statistically significant decreases in immobility time in the tail suspension test were observed at numerous time points in the study. The imipramine treatment group at the day 1 ($p < 0.05$), 7 ($p < 0.05$) and 14 ($p < 0.01$) points showed a decrease in immobility time in comparison to the vehicle control, saline. The caffeine treatment group showed a decrease in immobility time at all three time points of day 1, day 7 and day 14 ($p < 0.0001$). Likewise, the caffeinated coffee treatment group showed a decrease in immobility time at all three time points of day 1, day 7 and day 14 ($p < 0.001$). Decaffeinated coffee also showed a decrease in immobility time at day 14 ($p < 0.05$) as seen in figure 19 below. Animals treated with both caffeine and caffeinated coffee both showed decreases in immobility at all three time points of the study that

were approximately three-fold lower than animals in the saline control group. Furthermore, similar differences were observed between these animals and the animals in the imipramine treatment group, however the decrease in immobility time was only approximately a two-fold decrease. Animals in the caffeinated coffee treatment group showed a progressive decrease in immobility time in the TST over the duration of the study. A significant decrease of approximately 50% was observed in these animals on day 14 in comparison to day 1 ($p < 0.05$).

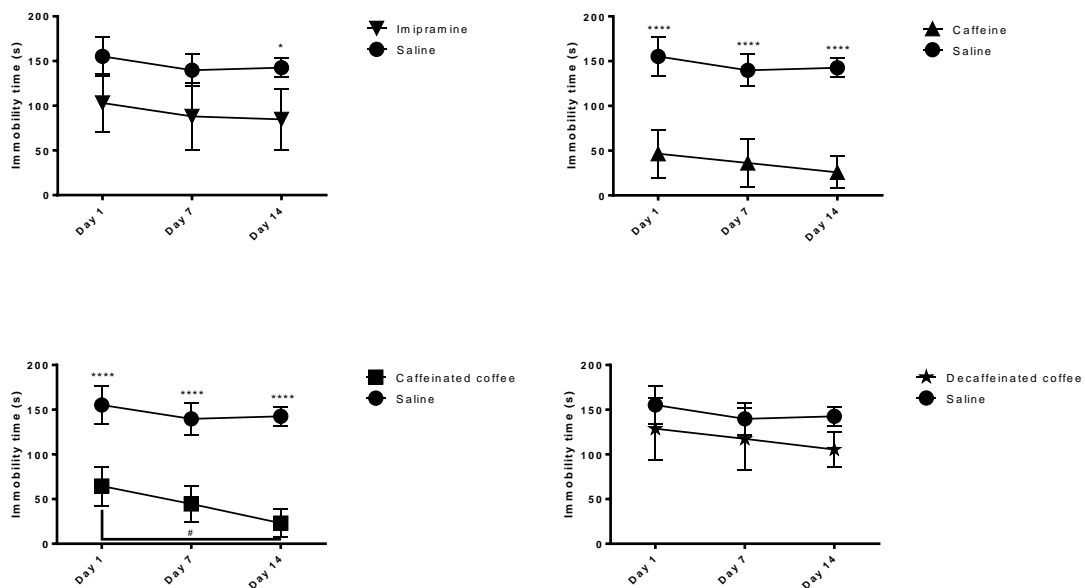


Figure 19 – The chronic effects of saline (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on immobility time using the tail suspension test.

2.4.2 The effects of caffeine, caffeinated coffee and decaffeinated coffee on behaviour in LPS-stimulated animals

Locomotor activity post-LPS

A statistically significant decrease, approximately three-fold in magnitude, in locomotor activity was observed in the LPS alone treatment group at 6 h post-LPS injection. This decrease in locomotor activity however had returned to that of the saline group 24 h post-LPS treatment as seen in figure 20 below.

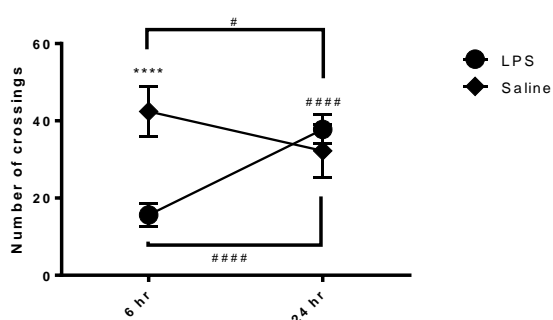


Figure 20 – The effects of LPS (n = 5) on locomotor activity measured using the open field test 6 and 24 h post administration in comparison to saline (n = 5).

Statistically significant increases in locomotor activity were observed in animals treated with imipramine at 6 h ($p < 0.0001$) post-LPS, caffeine treated animals at 6 h ($p < 0.0001$) and 24 h ($p < 0.05$) post-LPS and caffeinated coffee treated animals at 6 h ($p < 0.05$) and 24 h ($p < 0.0001$) post-LPS treatment. Conversely, decaffeinated coffee decreased locomotor activity in treated animals at 6 h ($p < 0.05$) and 24 h ($p < 0.01$) post-LPS treatment as seen in figure 21. At 6 h post-LPS exposure, pre-treatment with

imipramine, caffeine and caffeinated coffee all approximately doubled the locomotor activity of animals in comparison to LPS alone. After 24 h, caffeine and caffeinated coffee pre-treatment increased locomotor activity in comparison to LPS alone by approximately double. Conversely, pre-treatment with decaffeinated coffee results in an approximate two fold decrease in locomotor activity in comparison to LPS alone.

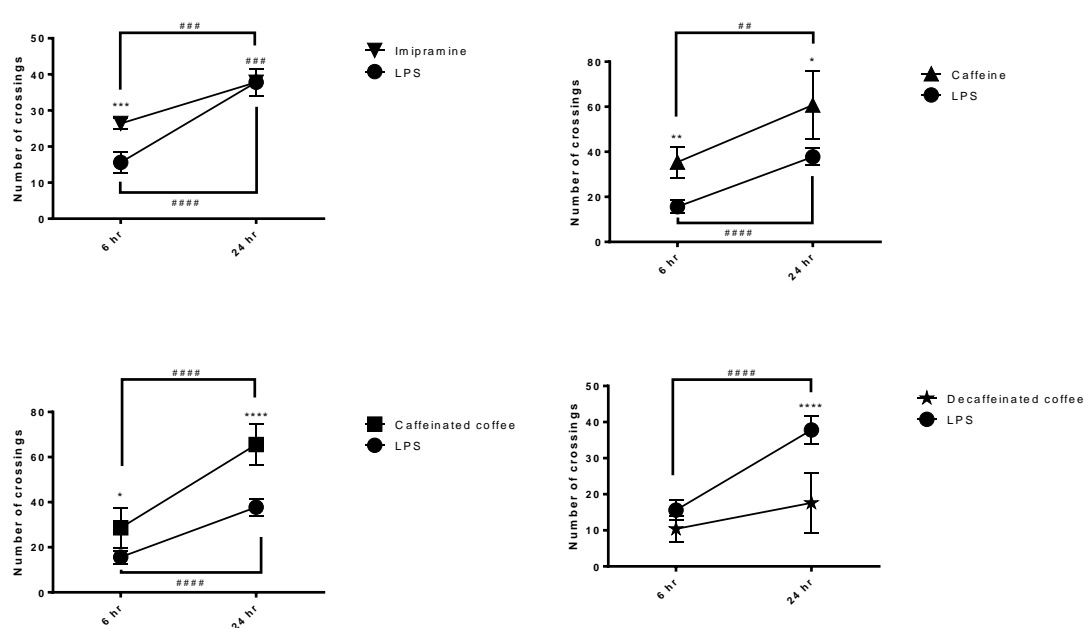


Figure 21 – The effects of LPS (n =5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on locomotor activity using the open field test 6 and 24 h post-LPS administration.

Forced swimming test post-LPS

A statistically significant increase in immobility time was observed in animals treated with LPS 24 h post-exposure ($p < 0.01$). Statistically significant decreases in immobility time were observed in animals pre-treated with imipramine ($p < 0.05$),

caffeine ($p < 0.05$) and caffeinated coffee ($p < 0.001$) as seen in figure 22 below. Immobility time decreased by approximately half in animals pre-treated with imipramine and caffeine and by approximately two thirds in animals pre-treated with caffeinated coffee. Furthermore, pre-treatment with decaffeinated coffee afforded an approximate one third decrease in immobility time in comparison to LPS alone.

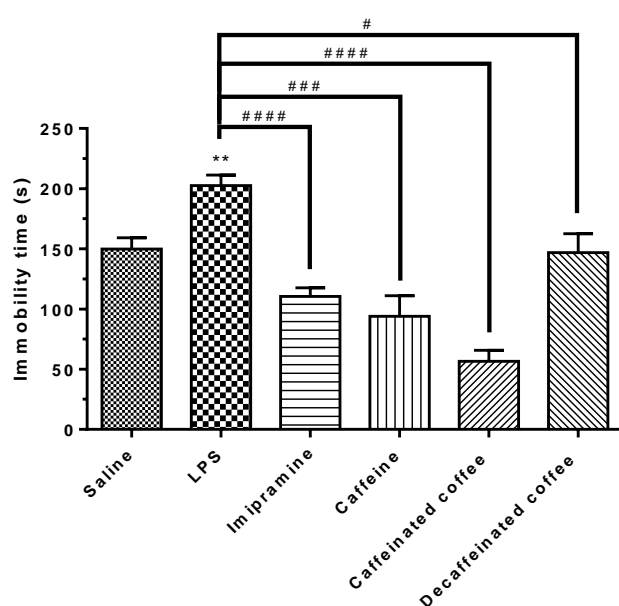


Figure 22 – The effects of saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on the immobility time using the forced swimming test 24 h post-LPS administration.

Tail suspension test post-LPS

A statistically significant increase in immobility time was observed in animals treated with LPS 24 h post-exposure ($p < 0.01$). Statistically significant decreases in immobility time were observed in animals pre-treated with imipramine ($p < 0.05$),

caffeine ($p < 0.0001$) and caffeinated coffee ($p < 0.0001$) as seen in figure 23 below. Decreases in immobility time of approximately 50% were observed in animals pre-treated with imipramine. Furthermore, an approximate 60% decrease and 75% decrease in immobility time was observed in animals pre-treated with caffeine and caffeinated coffee respectively.

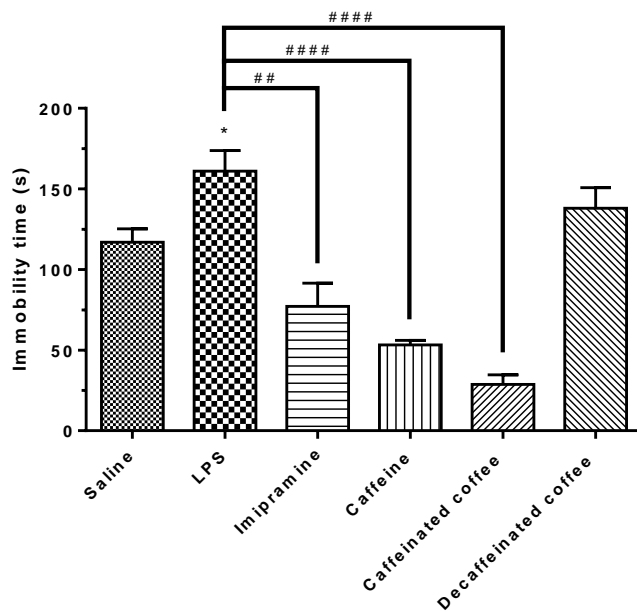


Figure 23 – The effects of saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on immobility time using the tail suspension test 24 h post-LPS exposure.

Weight loss

A statistically significant decrease in weight was seen in all animals receiving LPS regardless of their pre-treatment group in comparison to saline as shown in table 2

below ($p < 0.0001$). Furthermore, there was no significant difference between treatment groups in comparison to LPS alone as seen in table 3 below.

Table 2 – Weight loss of animals treated with LPS and LPS in addition to imipramine, caffeine, caffeinated coffee and decaffeinated coffee in comparison to the saline control

Treatment	Number	Mean \pm SD	P	CI
Saline	5	0.32 ± 0.26	-	-
LPS	5	-2.40 ± 0.255	<0.0001	-3.099 to -2.349
Imipramine	5	-2.13 ± 0.12	<0.0001	-2.744 to -2.156
Caffeine	5	-2.44 ± 0.09	<0.0001	-3.042 to -2.478
Caffeinated coffee	5	-2.41 ± 0.35	<0.0001	-3.182 to -2.278
Decaffeinated coffee	5	-2.41 ± 0.53	<0.0001	-3.342 to -2.118

Table 3 - Weight loss of animals treated with LPS in addition to imipramine, caffeine, caffeinated coffee and decaffeinated coffee in comparison to animals treated with LPS

Treatment	Number	Mean \pm SD	P	CI
LPS	5	-2.40 ± 0.255	-	-
Imipramine	5	-2.13 ± 0.12	NS	-0.01692 to 0.5649
Caffeine	5	-2.44 ± 0.09	NS	-0.3148 to 0.2428
Caffeinated coffee	5	-2.41 ± 0.35	NS	-0.4562 to 0.4442
Decaffeinated coffee	5	-2.41 ± 0.53	NS	-0.6166 to 0.6046

2.4.3 The effects of caffeine, caffeinated and decaffeinated coffee of plasma markers of inflammation in an LPS model of depression

Quantification of plasma IDO activity

Method validation

The HPLC method for the quantification of Trp and KYN in plasma described in section 2.3.8 was shown to be accurate, precise, repeatable and specific. An example HPLC chromatogram utilising the sample preparation and analytical methods in section 2.3.8 is shown below in figure 26. The retention time of Trp and KYN using the abovementioned method was found to be 5.840 ± 0.079 min and 4.404 ± 0.044 (n = 30) respectively.

Linearity

The standard curve was prepared as described in section 2.3.8. The results represented in figure 24 are an average of the area of triplicate injections from 3 separate runs (n = 9) \pm 1 standard deviation of each of the 10 concentrations tested and resulted in a linear curve across the concentration range tested.

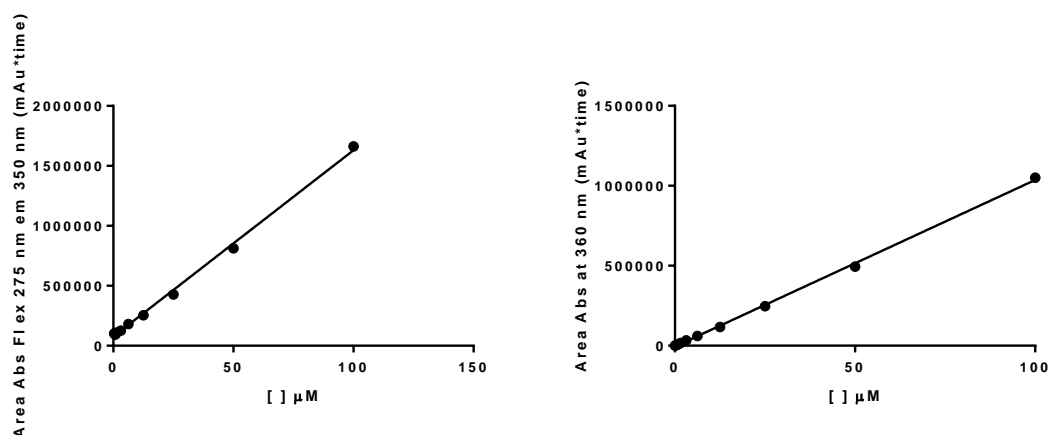


Figure 24 – Calibration curve for the quantification of tryptophan at 275 nm and kynurenine at 360 nm (area) (n =3; $Y = 15539 \cdot X + 77014$ and $Y = 1041 \cdot X - 4984$; $R^2 = 0.9974$ and 0.9991 , under the chromatographic conditions described in section 2.3.8), which produced a linear curve across the concentration range tested (% RSD < 5%).

Accuracy and precision

Figure 25 below demonstrates the accuracy and precision of both the sample preparation method and the HPLC method for the isolation and quantification of Trp and KYN. All three of the concentrations tested, 6.25 μM, 12.5 μM and 25 μM, were within the acceptable range of 85 to 115% of expected. The HPLC method used to quantify Trp and KYN in plasma samples was found to be precise. Figure 25 below shows the precision of the method with regards to the area, height and retention time represented as the coefficient of variance. All results fell within the acceptable range of less than 10%.

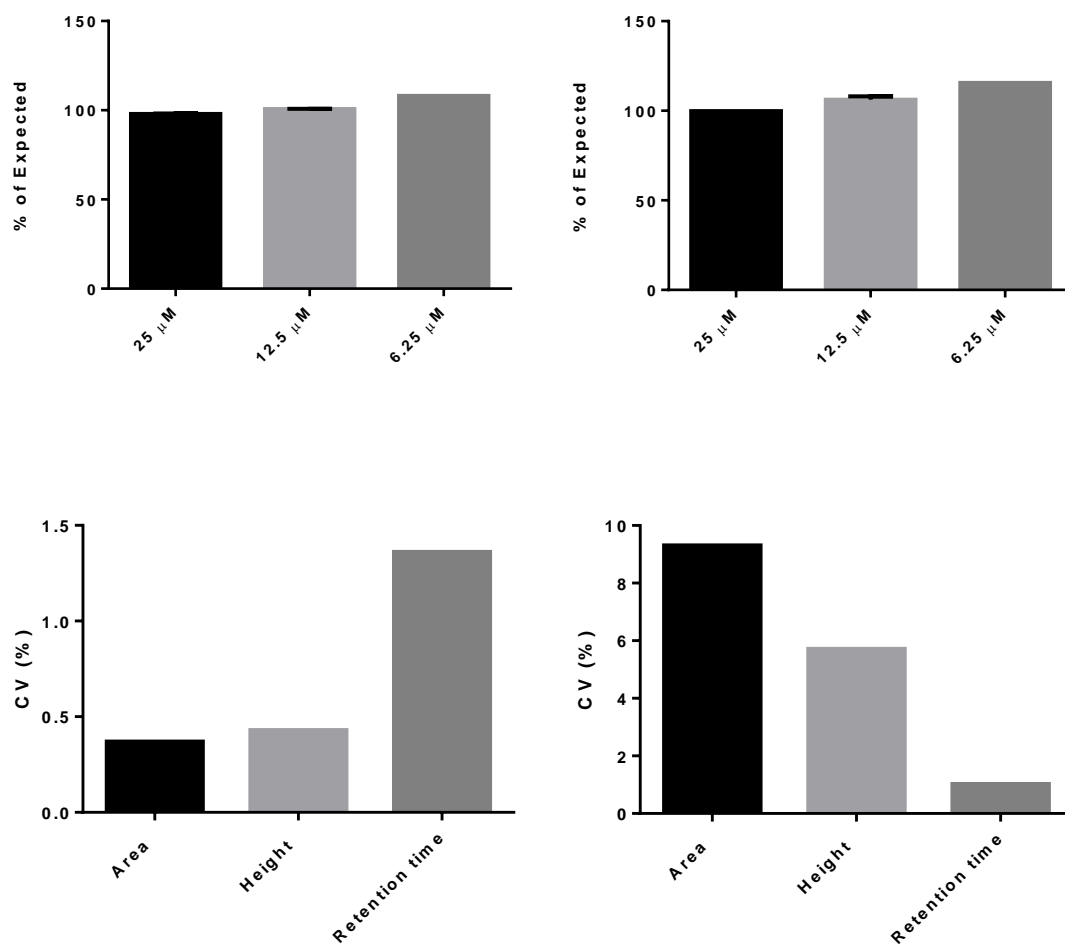


Figure 25 – The a) accuracy of the HPLC method used to quantify tryptophan; b) accuracy of the HPLC method used to quantify kynurenine; c) precision of the HPLC method used to quantify tryptophan; d) precision of the HPLC method to quantify kynurenine (n = 30).

Selectivity

The HPLC method used to quantify Trp and KYN was found to be selective for both analytes of interest. This can be seen in figure 26 below.

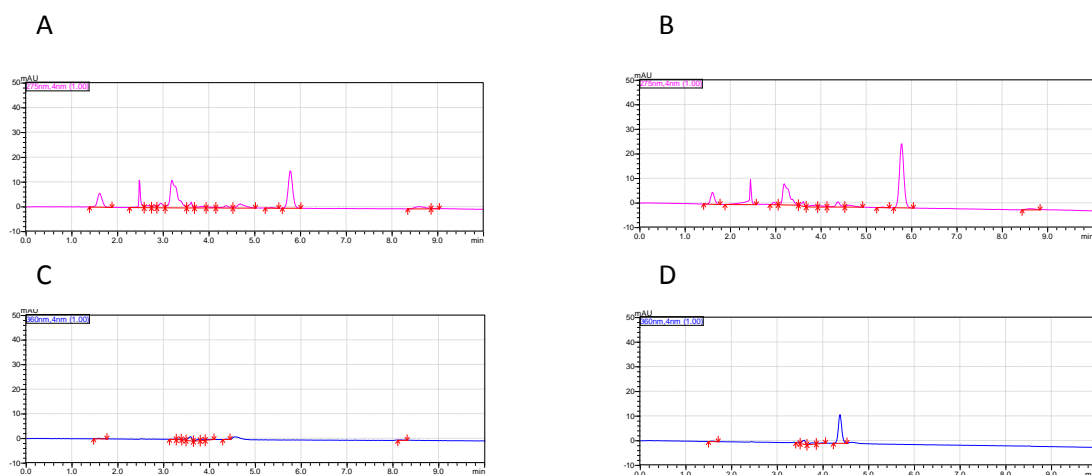


Figure 26 – HPLC chromatograms highlighting the selectivity of the method used to quantify Trp and KYN at fluorescence ex 275 nm and em 350 nm and absorbance 360 nm, respectively a) blank porcine serum sample at ex 275 nm em 350 nm; b) Trp spiked porcine serum sample; c) blank porcine serum sample at 360 nm; d) KYN spiked porcine serum sample.

Lower limits of detection and quantification

The limit of detection (signal to noise ratio 3-to-1) was found to be 0.0003 mM and 0.012 mM and the limit of quantification (signal to noise ratio 10-to-1) was found to be 0.006 mM and 0.019 mM for Trp and KYN, respectively.

Plasma IDO activity

Plasma IDO activity was assessed by the quantification of KYN and Trp and subsequently the KYN to Trp ratio. Statistically significant increases in IDO activity, more than doubled were observed after animals were exposed to LPS for 24 h. Pre-treatment of mice with caffeinated coffee for 14 days resulted in the protection from increased IDO activity after 24 h exposure to LPS ($p < 0.01$) in comparison to the

vehicle control animals exposed to LPS alone similar in magnitude to the results observed with pre-treatment with the trichloroacetic acid and positive control, imipramine ($p < 0.01$). The activity of IDO was approximately 30% lower in animals pre-treated with caffeinated coffee or imipramine after exposure to LPS, however, it did not return to the levels observed in the saline control group.

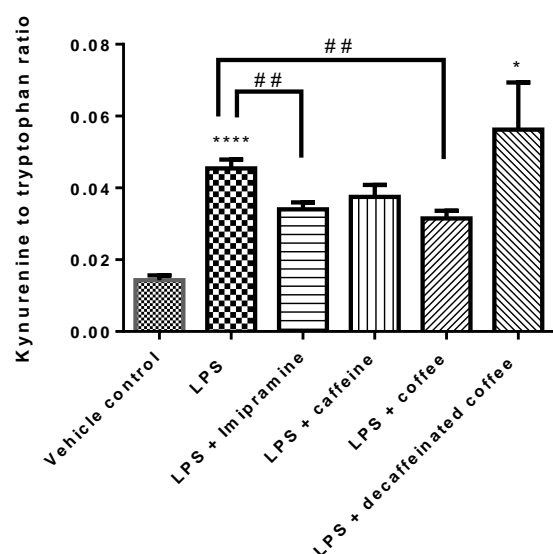


Figure 27 – The effects of chronic exposure of mice to saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on IDO activity 24 h post-LPS exposure where * is in comparison to saline control and # is in comparison to LPS stimulus.

Quantification of plasma cortisol concentrations

Method validation

The HPLC method for the quantification of cortisol in plasma described in section 2.3.8 was shown to be accurate, precise, repeatable and specific. An example

HPLC chromatogram utilising the sample preparation and analytical methods in section 2.3.8 is shown below in figure 30. The retention time of cortisol using the abovementioned method was found to be 8.159 ± 0.007 min ($n = 30$).

Linearity

The standard curve was prepared as described in section 2.3.8. The results represented in figure 28 are an average of the area of triplicate injections from 3 separate runs ($n = 9$) \pm 1 standard deviation of each of the 10 concentrations tested and resulted in a linear curve across the concentration range tested.

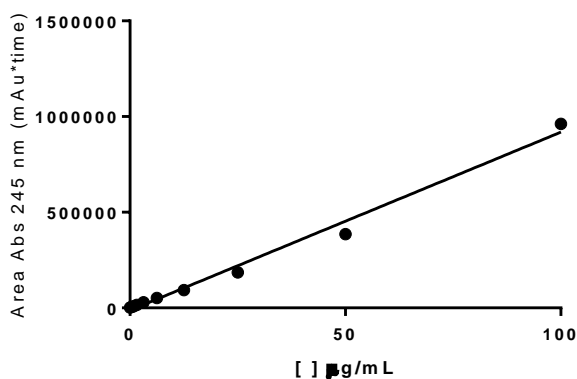


Figure 28 - – Calibration curve for the quantification of cortisol at 248 nm (area) ($n = 3$; $Y = 9309 \cdot X - 12093$; $R^2 = 0.9893$, under the chromatographic conditions described in section 2.3.8), which produced a linear curve across the concentration range tested (% RSD < 5% %)

Accuracy and precision

Figure 29 below demonstrates the accuracy of both the sample preparation method and the HPLC method for the isolation and quantification of cortisol. All three of the concentrations tested, 15 μ M, 35 μ M and 50 μ M, were within the acceptable range of 85 to 115% of expected.

The HPLC method used to quantify cortisol in plasma samples was found to be precise. Figure 29 below shows the precision of the method with regards to the area, height and retention time represented as the coefficient of variance. All results fell within the acceptable range of less than 10%.

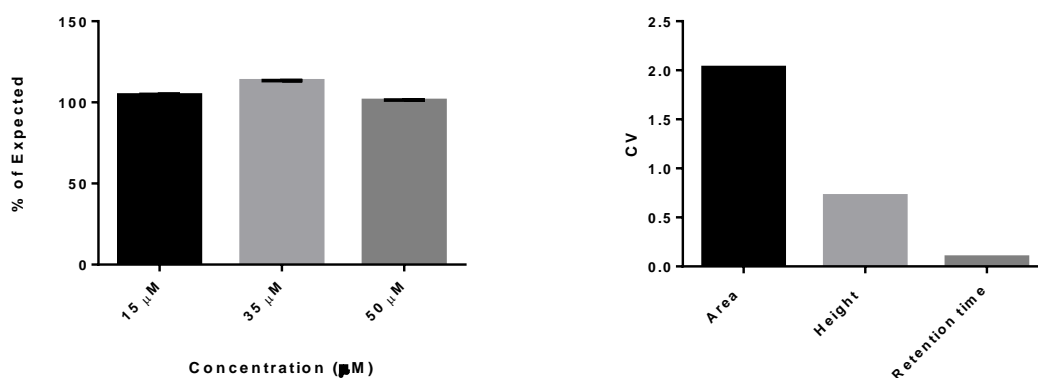


Figure 29 – The a) accuracy; and b) precision of the HPLC method used to quantify cortisol in plasma and brain homogenates (n = 30)

Selectivity

The HPLC method used for the quantification of cortisol in plasma was shown to be selective for the analyte of interest as seen in figure 30 below.

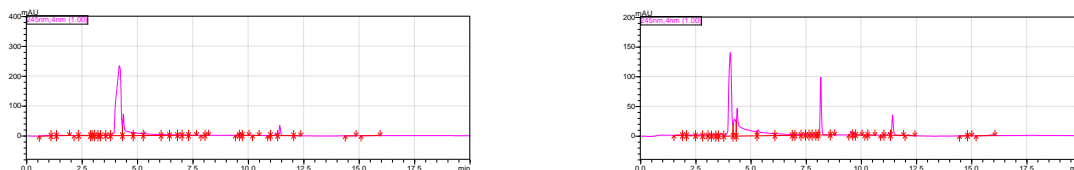


Figure 30 – HPLC chromatograms demonstrating the selectivity of the method to quantify plasma cortisol concentrations a) blank porcine serum; b) spiked porcine serum.

Lower limits of detection and quantification

The limit of detection (signal to noise ratio 3-to-1) was found to be 0.012 mM and the limit of quantification (signal to noise ratio 10-to-1) was found to be 0.097 mM.

Quantification of plasma cortisol

No detectable levels of plasma cortisol were present in the plasma of animals in any of the treatment groups.

Quantification of plasma neopterin and biopterin concentrations

Plasma biopterin concentrations were significantly lower in animals in the LPS alone treatment group in comparison to animals in the saline group ($p < 0.0001$). Concentrations in the LPS treatment decreased this concentration by approximately 50% in comparison to the saline control group. Furthermore, imipramine ($p < 0.001$), caffeine ($p < 0.05$), caffeinated coffee ($p < 0.001$) and decaffeinated coffee ($p < 0.001$)

pre-treatment resulted in significant increases in the plasma concentrations of biopterin. Pre-treatment with imipramine was shown to prevent the decrease of biopterin plasma concentrations with a plasma concentration comparable to that of the saline control group. Furthermore, caffeine, caffeinated coffee and decaffeinated coffee did not protect against the full decrease in plasma biopterin concentrations but were still significantly higher than those observed in the LPS treatment group.

Plasma neopterin concentrations were shown to be significantly increased in animals in the LPS treatment group in comparison to those in the saline treatment group. None of the treatment groups, including the positive control imipramine, were found to be significantly different to the LPS-treated group.

The ratio of neopterin to biopterin was significantly increased in animals in the LPS treatment group. The ratio observed in the LPS group was 4 times higher than that observed in the saline treatment group. Furthermore, significantly lower neopterin to biopterin ratios were observed in all of the treatment groups. Animals receiving the positive control imipramine, displayed ratios that were comparable to the animals in the saline control group. Furthermore, caffeinated coffee was comparable to imipramine in its protective effects, with only a small increase in the ratio in this group compared to imipramine. Pre-treatment with caffeine and decaffeinated coffee also prevented the increase in the ratio but to a lesser degree than that of imipramine and caffeinated coffee as seen in figure in 31.

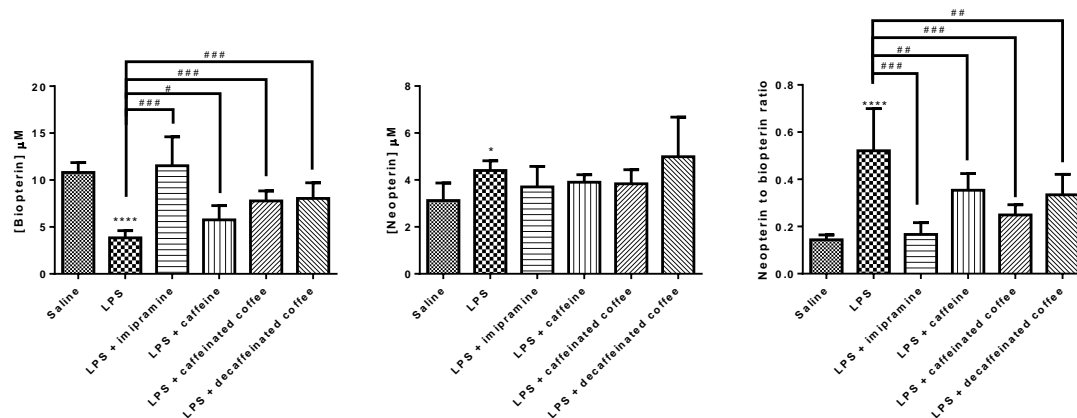


Figure 31 – The effects of saline (n = 5), LPS (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) on a) plasma biopterin concentrations; b) plasma neopterin concentrations; c) neopterin to biopterin ratio.

2.4.4 The effects of caffeine, caffeinated coffee and decaffeinated coffee on central markers of inflammation

Quantification of central interferon gamma concentrations

No change in central IFN- γ concentrations were observed between the vehicle control group and LPS alone or any of the other treatment groups.

Quantification of central interleukin-6 concentrations

Central IL-6 were found to be below the detection limit of the assay used in this study. That is less than 0.21 pg/mL of IL-6 was present in the samples.

Quantification of central TNF- α concentrations

No change in central TNF- α concentrations was observed between the vehicle control group and LPS alone or any of the other treatment groups.

Quantification of central PGE₂ concentrations

PGE₂ concentrations were quantified using EIA. PGE₂ concentrations in LPS-treated animals were found to be approximately twice as high as those observed in the saline treatment group. Furthermore, PGE₂ concentrations were significantly decreased in animals pre-treated with caffeine ($p < 0.05$), caffeinated coffee ($p < 0.01$) and decaffeinated coffee ($p < 0.0001$). PGE₂ concentrations were decreased by approximately 30% after pre-treatment with caffeine and caffeinated coffee and approximately 50% after pre-treatment with decaffeinated coffee.

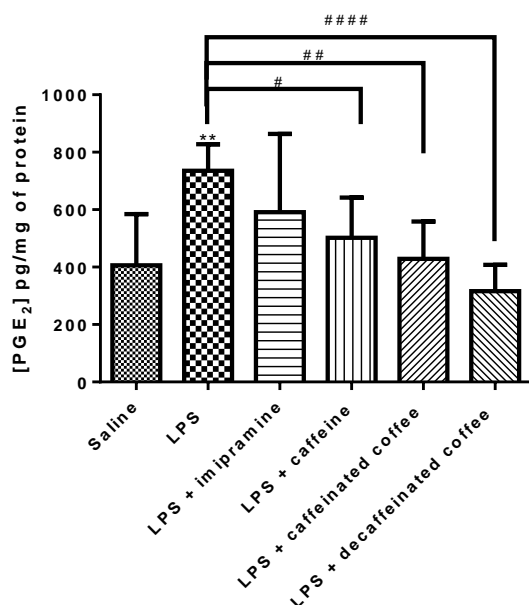


Figure 32 – Central PGE₂ concentrations after 24 h exposure to LPS in animals pre-treated with saline (n = 5), LPS alone (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5) where * is in comparison to saline and # is in comparison to LPS stimulus

Quantification of central kynurenine metabolites

A number of the KMs, including KYN, 3-HK and KA, were quantified in the brains of animals pre-treated with caffeine, caffeinated coffee and decaffeinated coffee 24 h post-LPS exposure. The ratios of KA to KYN, 3-HK to KYN and 3-HK to KA were then calculated.

Kynurenic acid to kynurenine ratio

A statistically significant increase in the KA to KYN ratio was observed in the brains of animals treated with LPS alone ($p < 0.001$). This ratio significantly increased

in the groups where animals were pre-treated with caffeine ($p < 0.001$) and decaffeinated coffee ($p < 0.05$) in comparison to LPS alone.

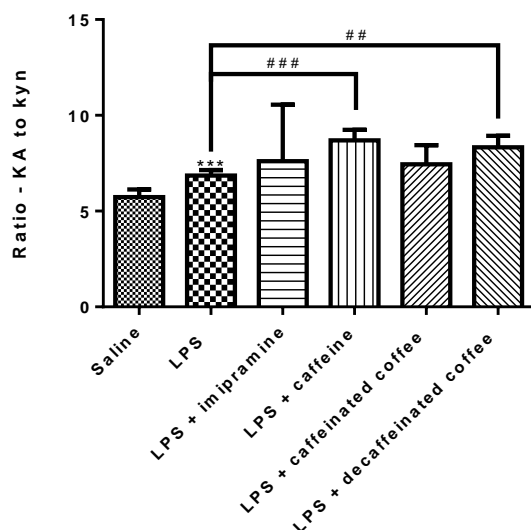


Figure 33 - The changes in the ratio of kynurenic acid to kynurenine in brain homogenate after animals were treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

3-Hydroxykynurenine to kynurenine ratio

No significant change in the brain ratio of 3-HK to KYN was observed 24 h post-LPS exposure. Increases in the ratio of 3-HK to KYN were however increased 24 h post-LPS in animals dosed with imipramine ($p < 0.0001$) and caffeine ($p < 0.0001$). Significant decreases in this ratio was seen in animals treated with decaffeinated coffee ($p < 0.0001$). The observed changes in this ratio are relatively small as seen in figure 34 below.

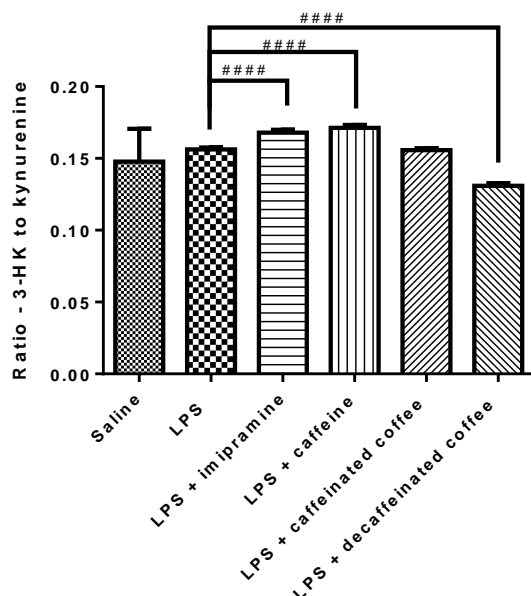


Figure 34 - The changes in the ratio of 3-hydroxykynurenine to kynurenine in brain homogenate after animals were treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

Kynurenic acid to 3-HK ratio

Modest statistically significant increases in the ratio of KA to 3-HK were observed 24 h after animals were treated with LPS ($p < 0.05$). Furthermore, caffeine ($p < 0.0001$), caffeinated coffee ($p < 0.01$) and decaffeinated coffee ($p < 0.01$) all increased the ratio of KA to 3-HK. Increases in the order of approximately 30% were again observed as seen in figure 35 below.

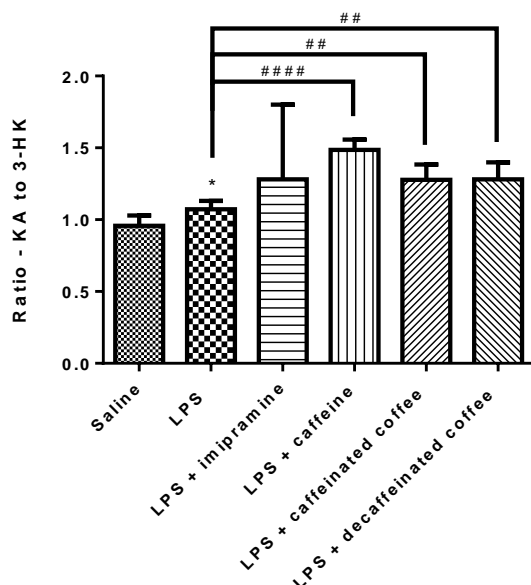


Figure 35 – The changes in the ratio of kynurenic acid to 3-hydroxykynurenine in brain homogenate after animals were treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

2.4.5 The effects of caffeine, caffeinated coffee and decaffeinated coffee on central monoamines in an LPS model of depression

Several monoamines, including 5-HT, NA and DA, were quantified in the brain homogenates using HPLC analysis.

Quantification of central serotonin

No significant differences in central 5-HT concentrations was observed after animals were treated with LPS, caffeine, caffeinated coffee or decaffeinated coffee as seen in figure 36 below.

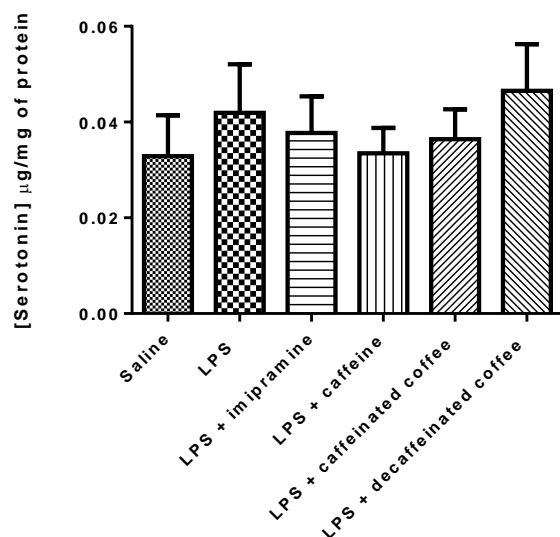


Figure 36 - Central serotonin concentrations per mg of protein in animals treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

Quantification of central noradrenaline

No significant differences in central noradrenaline concentrations was observed after animals were treated with LPS, caffeine, caffeinated coffee or decaffeinated coffee as seen in figure 37 below.

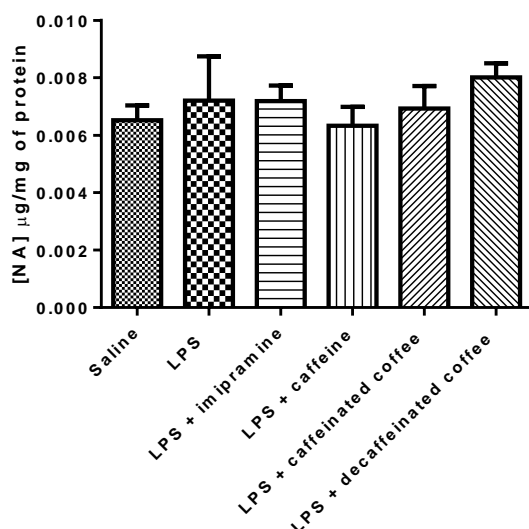


Figure 37 – Central noradrenaline concentrations per mg of protein in animals treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

Quantification of central dopamine

No significant differences in dopamine concentrations were seen in the brains of animals treated with LPS in comparison to the saline control group. Statistically significant decreases in dopamine concentration were however observed in the brains of animals treated with caffeine ($p < 0.05$) as seen in figure 38 below.

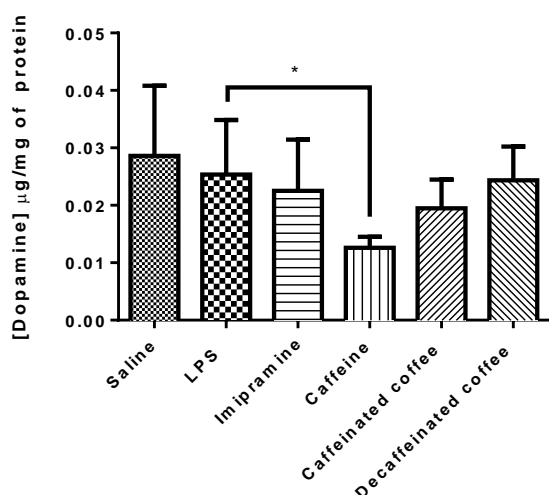


Figure 38 – Central dopamine concentrations after animals were treated with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5)

2.4.6 Quantification of plasma and central caffeine concentrations after treatment with caffeine and caffeinated coffee

Method validation

The HPLC method for the quantification of caffeine in plasma described in section 2.3.8 was shown to be accurate, precise, repeatable and specific. An example HPLC chromatogram utilising the sample preparation and analytical methods in section 2.3.8 is shown below in figure 41. The retention time of caffeine using the abovementioned method was found to be 8.681 ± 0.078 min (n = 30).

Linearity

The standard curve was prepared as described in section 2.3.8. The results represented in figure 39 are an average of the area of triplicate injections from 3 separate runs ($n = 9$) ± 1 standard deviation of each of the 10 concentrations tested and resulted in a linear curve across the concentration range tested.

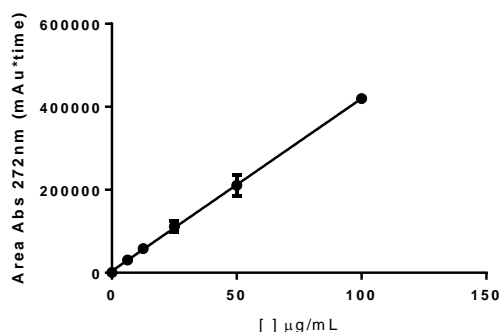


Figure 39 – Calibration curve for the quantification of caffeine at 272 nm (area) ($n = 3$; $Y = 4156 * X + 4409$; $R^2 = 0.9933$, under the chromatographic conditions described in section 2.3.8), which produced a linear curve across the concentration range tested (% RSD < 5% %)

Accuracy and precision

The accuracy of the HPLC method to quantify caffeine was assessed using spiked porcine serum. The results for the accuracy of the method can be seen in figure 40 below. All 3 concentrations of caffeine tested fell within the recommended 85 to 115% of control ($n = 9$).

The precision of the method was assessed using spiked porcine serum and is represented as intermediate precision. The intermediate precision of the method was found to be less than 1% as seen in figure 40 below. This indicates the method is precise for the quantification of caffeine.

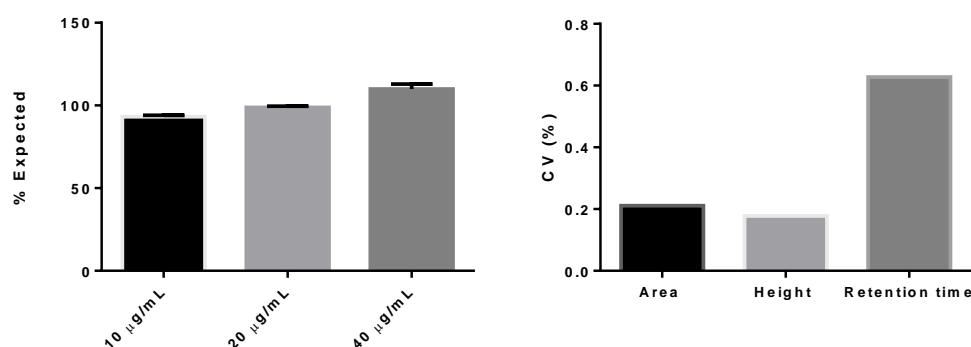


Figure 40 – The a) accuracy and b) precision of the method used to quantify caffeine in the plasma and brain homogenates (n = 30).

Specificity

The HPLC method used was evaluated for the specificity of caffeine. As seen in the chromatograms below in figure 41 the method was shown to be specific with no co-elution of caffeine with 3 of the major metabolites of caffeine namely theophylline, theobromine and 1,7-dimethylxanthine.

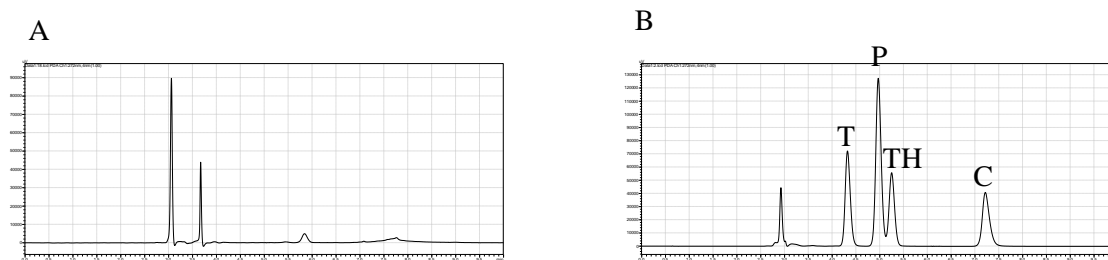


Figure 41 – Chromatograms of theobromine (T), paraxanthine (P), theophylline (TH) and caffeine (C): a – blank plasma; b – caffeine and metabolites isolated and analysed

Lower limits of detection and quantification

The lower limits of detection and quantification of caffeine using the method described in section 2.3.8 was assessed in spiked human plasma. The lower limit of detection was found to be 0.0122 mM and the lower limit of quantification to be 0.0976 mM.

Quantification of plasma caffeine

Plasma caffeine levels were significantly raised in animals pre-treated with caffeinated coffee ($p < 0.0001$). Conversely, no other treatment group, including the caffeine treatment group, had detectable concentrations of plasma caffeine as seen in figure 42 below.

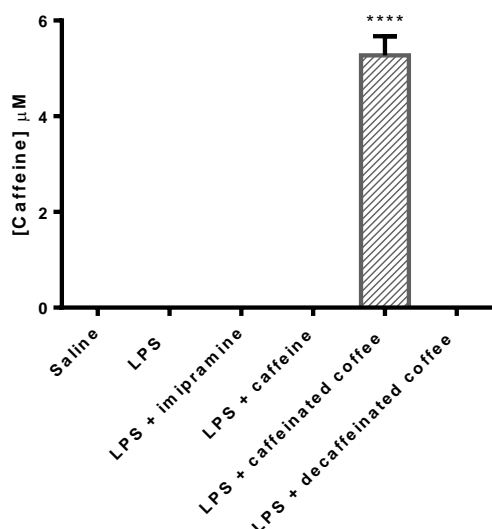


Figure 42 – Plasma caffeine concentrations of animals treated with LPS in combination with saline (n = 5), LPS (n = 5), imipramine (n = 5), caffeine (n = 5), caffeinated coffee (n = 5) and decaffeinated coffee (n = 5).

2.5 Discussion

An LPS model of depression was used in the current study utilizing methods described previously [136, 137, 139, 201]. This model was useful in assessing a number of parameters associated with depression including changes to Trp catabolism and behaviours of depression. The model used has been used extensively in the past to assess the effects of compounds on depression [136, 137, 139, 201]. Furthermore, the various parameters of validity were also addressed to ensure the highest quality model was developed.

The model used in the current study was shown to display many of the features known to be present in human patients suffering from depression. These include

alteration in mood towards depressive-like behaviour and an increase in markers of inflammation including PGE₂, neopterin and IDO activity. Animals in the current study were treated with 0.83 mg/kg of LPS from *E. coli* strain O111:B4 24 h before sacrifice. The immobility times in the FST and TST were both significantly increased in the LPS treatment group in comparison to the saline group. Treatment of mice with LPS has been shown to increase depressive-like behaviours including decreased exploratory behaviour [220], reduced social interaction and increased anhedonia [221]. Furthermore increased immobility times have been shown to be increased after exposure to LPS in both the forced swimming test [136, 142, 222] and the tail suspension test [136, 142]. A combination of three behavioural tests were incorporated into the current study. These include the FST, the TST and the OFT. All three of these tests are routinely used to assess the behavioural effects of compounds of interest. The forced swimming test and the tail suspension test are both behavioural models used to assess depressive-like behaviour [208, 209]. Animals displaying behavioural signs of depression will have higher immobility time in each of these tests in comparison to non-depressed animals. The OFT on the other hand is more closely associated with the activity of the animals or anxiety levels [203, 223, 224]. The OFT uses locomotor activity as a measure and animals with increased activity are likely to be experiencing signs of anxiety [203, 223, 224].

Animals in the LPS group displayed significantly less crossing in comparison to animals in the saline control group at 6 h post-LPS exposure measured using the OFT. Conversely, 24 h post-LPS exposure, the number of crossings in animals in the LPS treatment group did not significantly differ from that observed in the saline control group. Literature shows that there is a biphasic response when mice are exposed to LPS

[139]. In the first 6 h, animals show signs of sickness behaviour, whereas, after 24 h sickness behaviour declines and becomes depressive-like behaviour [139]. To show that this had occurred in the current study, the locomotor activity, using the open field test, of the LPS-treated animals was compared to the saline group at 6 h and 24 h. At 6 h, a statistically significant decrease in the locomotor activity of the animals treated with LPS in comparison to the saline treated groups was observed. At 24 h, the locomotor activity of both groups was found to be the same. This is suggestive of the biphasic response being present. To further confirm the depressive-like behaviour at 24 h, the forced swimming test and the tail suspension test were used. Both of these tests showed that animals treated with LPS were displaying symptoms of depression, with their immobility time significantly increased in comparison to animals in the saline treatment group. This is consistent with what occurred in the current study.

Varying doses and strains of LPS have been reportedly used previously including 0.83 mg/kg of *E. coli* O111:B4 [136, 202, 204, 205, 225] . The alteration in the behaviour in the current study is consistent with literature indicating the appropriateness of this model to assess the antidepressant-like activities of compounds of interest and suggest that an appropriate dose and strain of LPS was used. In further support of the model, the positive control included in the current study, the tricyclic antidepressant imipramine, was shown to decrease the immobility time in the FST and TST and has therefore been shown to improve depressive-like behaviours at a dose of 10 mg/kg. Again, the results observed with imipramine are consistent with literature [9]. Imipramine is commonly used as a positive control in *in vivo* animal models of inflammatory depression and dosages of 10 mg/kg of imipramine have been previously shown to decrease immobility time in the FST and TST in LPS-treated animals [9].

Given the results obtained in the current study align with literature and that imipramine decreases depressive-like behaviour, the appropriateness of this model to assess the antidepressant activity of caffeine and coffee is confirmed.

The current study showed that caffeine and caffeinated coffee had an effect on a number of behavioural parameters associated with depression. First and foremost, the acute effects of caffeine, caffeinated coffee and decaffeinated coffee were assessed using the open field test, the forced swimming test and the tail suspension test and were compared to the positive control, imipramine and the negative control group, saline. Caffeinated coffee and caffeine both increased locomotor activity and decreased immobility time in both the forced swimming test and tail suspension test in comparison to saline treated animals. These results are indicative of acute antidepressant-like activities along with possible stimulatory effects in the absence of LPS stimulus. Furthermore, the positive control, imipramine, was found only to increase the animals locomotor activity in the open field test to decrease immobility time in the tail suspension test in comparison to the saline control. These changes, however, were not as significant as those observed after animals were administered caffeine and caffeinated coffee.

The effects of caffeine on locomotor activity were measured daily throughout the duration of the study. This was done to assess if tolerance to caffeine was being demonstrated by animals due to the actions of caffeine in the brain and the high prevalence of tolerance with its consumption [226, 227]. Animals exposed to caffeine over the duration of the study showed no changes in locomotor activity with exception

between day 0 and day 1, the first day of caffeine administration. These results show that animals in the caffeine treatment group experienced no tolerance to caffeine.

In addition to the acute effects of caffeine, caffeinated coffee and decaffeinated coffee on behaviours associated with depression, the chronic effects of these interventions on these behaviours was assessed using the same three methods. The chronic effects of animals exposed to caffeine, caffeinated coffee and decaffeinated coffee were assessed by comparing the results of the open field test, the forced swimming test and the tail suspension test at days 1, 7 and 14 of the study. Statistically significant increases in locomotor activity were observed in animals treated with caffeine and caffeinated coffee at all three time points. Interestingly, maximum locomotor activity was observed in the caffeine treatment group after 7 days however, there was a linear increase in locomotor activity in the caffeinated coffee group, with maximal locomotor activity observed after 14 days. Conversely, decaffeinated coffee had no effect on locomotor activity. Animals in the imipramine treatment group displayed increased locomotor activity on days 1 and 7 of the study however this activity declined back to that of the saline control group by day 14. Given the nature of imipramine, this is not unexpected. *In vivo* animal studies have shown that treatment with imipramine increased anxiety levels with acute treatment and subsequently decreased these behaviours with chronic administration, that is 21 days of treatment [228]. This is consistent with the results obtained in the current study.

Results displayed by animals in the imipramine, caffeine and caffeinated coffee in both the forced swimming test and the tail suspension test were all similar. Animals

in all three of the groups display decreased immobility time at all three time points during both of the tests. Conversely, decaffeinated coffee had no effect on immobility time in comparison to the saline control group. The forced swimming test and the tail suspension test are both models to assess depressive-like behaviours in animals as outlined above. This suggests that animals chronically treated with caffeine and caffeinated coffee may display a reduction in depressive-like behaviours without inflammatory stimulus. When compared to the group treated with the positive control imipramine, a significant decrease in immobility time was observed in animals treated with caffeine and caffeinated coffee. This suggests that caffeine and caffeinated coffee may provide better antidepressant properties than imipramine at the doses tested.

Caffeine and caffeinated coffee afforded protective effects towards both sickness behaviour and depressive-like behaviour in the current model used, as did the positive control imipramine. Conversely, animals treated with decaffeinated coffee showed a significant decrease in locomotor activity and no significant change in immobility time in the forced swimming test and the tail suspension test. This suggests that caffeine and caffeinated coffee may provide antidepressant-like benefits whereas decaffeinated coffee appears to have no benefits on depressive-like behaviours. The behavioural data supports the human epidemiological data and given that a number of confounding factors have been controlled for, such as social interaction, these results increase the validity of the data.

In addition to behaviour, markers of inflammation were also increased in LPS-treated animals after 24 h. Several changes in the KMs both peripherally and centrally were observed. Peripheral IDO activity was shown to be significantly raised in animals treated with LPS in comparison to the saline control. Furthermore, a significant increase in the ratio of KA to KYN was observed in LPS-treated animals in comparison to saline control and the ratio of KA to 3-HK was slightly increased in comparison to the saline control. As highlighted earlier, the KP and its metabolites are hypothesised to play an important role in the pathophysiology of depression [15, 69, 122, 128, 156, 229, 230]. IDO, the first marker associated with the KP, is raised in patients in a depressive state [229, 230]. Furthermore, IDO increases have been shown to align with the onset of depressive symptoms in patients receiving IFN therapy [229, 230].

The effects of caffeine, caffeinated coffee and decaffeinated coffee in LPS exposed animals on a number of pro-inflammatory cytokines and other markers of inflammation in both mouse plasma and mouse brain homogenates were undertaken. Peripheral IDO activity was assessed via the quantification of plasma Trp and KYN and subsequently taking the ratio of KYN to Trp. In the current study, it was found that caffeinated coffee decreased the activity of IDO after exposure to LPS. A similar decrease in the activity in IDO was observed after animals were pre-treated with the positive control, imipramine. As outlined earlier, IDO is an important enzyme in the catabolism of Trp and is directly under the control of pro-inflammatory cytokines and mediators [122]. In this case, IDO is directly regulated by LPS [136]. IDO activity has been shown to be mediated either through IFN- γ -dependent or independent pathways [138]. In the case of LPS, studies have shown this to be through a predominantly IFN- γ -independent mechanism [138]. This mechanism has been shown to be mediated

through toll-like receptors 4 (TLR4) activation and through the release of TNF- α [149, 231]. Furthermore, other pro-inflammatory cytokines including IL-6 and IL-1 β are also known to upregulate IDO activity [229].

As highlighted earlier, IDO activity has been correlated with symptoms of depression on numerous occasions [15, 60, 229, 230]. In the current study, the change in IDO activity observed corresponds and compliments the results observed in the behavioural studies, suggesting that the behavioural results are a true representation.

IDO, the first enzyme in the KP, is the rate-limiting enzyme responsible for the catabolism of Trp into further KMs [122]. These KMs can either be neurotoxic or neuroprotective in nature [122]. Furthermore, studies have shown that IDO levels in the plasma result in increased KYN concentrations in the CNS. KYN is taken up into the CNS from the periphery via the large neutral amino acid carrier [156] and studies have shown that up to 60% of the KYN in the CNS originates in the periphery [122]. Numerous studies have shown IDO activity to be raised in animals after exposure to LPS [136, 232-234]. Studies have shown that this effect is predominantly independent of an IFN- γ mediated response and is rather through TLR4 activation [235, 236].

In the current study, significantly increased ratios of KA to KYN are observed in the brains of animals treated with LPS. The KA to KYN ratio is used as a measure of the catabolism of KYN to KA [122]. These results suggest that the catabolism of KYN is favouring the neurotoxic arm of the KP. However, when quantified, the ratio of 3-

HK to KYN was not significantly different. Furthermore, the ratio of KA to 3-HK, a measure of the balance between the neurotoxic and the neuroprotective arms of the KP [122], was slightly raised in the LPS alone treatment group in comparison to the saline control group. One possible explanation for the disparity between the results observed relates to the KMs and KP enzymes downstream of 3-HK. It is well known that downstream enzymes are also upregulated under inflammatory conditions [156]. This could result in the conversion of 3-HK to KMs further down the neurotoxic pathway including 3-HA and QA and thus may skew the ratios involving 3-HK. Furthermore, LPS has been shown to increase QA synthesis in the brain 246-fold [169]. This would support the conversion of 3-HK to 3-HA and QA. Further studies are recommended to quantify 3-HA and QA in the future to fully assess the impact of LPS on the KP balance in the CNS.

In the brain homogenates of animals pre-treated with caffeine, caffeinated coffee and decaffeinated coffee, the ratio of KA to 3-HK was significantly increased in all three groups of animals. Furthermore, the KA to KYN ratio was significantly increased in the brains of animals in the caffeine and caffeinated coffee pre-treatment groups. These results strongly indicate that the shift in the catabolism of KYN is towards the neuroprotective arm of the KP and secondly, this is supported by the ratio of KA to 3-HK significantly higher than that seen in the LPS alone treatment group. These results indicate that the balance of the KP in the CNS is shifted from the neurotoxic arm to the neuroprotective arm with the pre-treatment of caffeine and caffeinated coffee.

Another important set of biomarkers in depression related to IFN- γ and Trp catabolism are neopterin, biopterin and the ratio of biopterin to neopterin [188, 190, 192]. The current study has shown that in animals 24 h post-LPS exposure, neopterin concentrations were significantly raised, biopterin concentrations were significantly decreased which resulted in the significant increase in the ratio of neopterin to biopterin, all consistent with the results seen in literature in depressed patients. Furthermore, these results mirrored the effects of LPS on IDO activity in the current study.

Neopterin and biopterin are important markers relating to IFN- γ response, with neopterin concentrations increasing in its presence [192]. Furthermore, biopterin is an important cofactor in the synthesis of monoamines [190]. In the current study, LPS-treated animals showed a significant decrease in plasma biopterin which may result in a significant decrease in 5-HT, DA and NA synthesis in the brain, further adding to the depressive-like nature of the model.

The third important pro-inflammatory marker seen in depressed patients, PGE₂, has been previously assessed after LPS administration *in vivo* and was found to be increased [237]. In the current study, PGE₂ concentrations were increased in animals treated with LPS alone in comparison to the animals in the saline control group, comparable to previous studies. A marker of inflammation, PGE₂, was however increased in the brains of animals treated with LPS alone. Furthermore, decreases in the brain concentrations of PGE₂, was observed in animals pre-treated with caffeine, caffeinated coffee and decaffeinated coffee. These results indicate that caffeine, caffeinated coffee and decaffeinated coffee all display anti-inflammatory properties.

Previous *in vitro* studies undertaken in microglial cells, suggest that LPS-induced ROS increases the production of PGE₂ [238]. Furthermore, a ROS-dependent enzyme, Membrane-bound glutathione-dependent PGE₂ synthase, has been found to be critical in the increase of PGE₂ concentrations post-LPS administration, further supporting the role of ROS in the production of PGE₂. The effects of LPS on free radical production was not assessed in the current study, however, it is recommended that it is investigated in the future.

In the current study, protein levels of the pro-inflammatory cytokine IL-6, TNF- α and IFN- γ were quantified using high sensitivity ELISA assays. It was found that administration of LPS for 24 h did not significantly alter TNF- α or IFN- γ concentrations in the brain. Furthermore, it was found that IL-6 concentrations in the brain were below the limits of quantification for the assay. In addition to its effects on behaviour, LPS also plays an important role in inducing an inflammatory response [146]. Pro-inflammatory response has been shown to play an important role in the pathophysiology of depression with a number of inflammatory markers raised in depression. Amongst these are TNF- α and IL-6, as shown in a meta-analysis [239]. The results from the current study suggest that the increases in the mRNA of pro-inflammatory cytokines post-LPS exposure observed in previous studies may not correlate to increased protein production [136]. Furthermore, studies have shown that the production of pro-inflammatory cytokines is time and tissue specific after exposure to LPS [146]. Given the tissue and time point of sacrifice, that is 24 h post-LPS exposure, it is not expected that the current study would provide results that showed a change in cytokine concentrations [146]. Although changes to the pro-inflammatory cytokine concentrations was not observed in the current study, other markers associated with

inflammation, including IDO, neopterin and PGE₂, were all significantly increased suggesting an increase in inflammation. Further studies assessing the mechanisms by which this inflammation occurs is warranted in the future.

Another important parameter in the pathophysiology of depression, alterations to the monoaminergic system were also evaluated in the current model. No significant change in any of the monoamines, 5-HT, NA and DA, quantified was observed after LPS treatment alone. Furthermore, the only significant change in monoamine concentrations was observed in animals in the caffeine group. The dopamine concentrations in animals in the caffeine treatment group were significantly lower than any of the other treatment or control groups. Given that there was no detectable levels of caffeine in the plasma of these animals and that it had been 24 h since the previous dose, the decrease in dopamine levels suggest these animals may be displaying withdrawal symptoms [240]. Furthermore, the animals in the caffeinated coffee group were protected from this effect given the relatively high concentrations of caffeine remaining in their plasma. The lack of changes in the monoamine concentrations may be due to insufficient time for alterations to occur however further studies are required to further investigate this.

From the data outlined above, it can be seen that the various parameters outlined in section 2.1.1 pertaining to model validity, have been included in the current study to minimise the limitations experienced in the study. Face validity exists in the current model of depression as many of the behavioural and biochemical features of depression observed in human disease are mirrored in the current model. Furthermore, this model

displays predictive validity as the positive control, imipramine, displayed the expected behavioural and biochemical changes consistent with human therapy. The third principle of validity, construct validity, was again a prominent feature of this model. Construct validity was achieved through the incorporation of the relevant features of depression as guided by the current theories of depression.

The plasma and central caffeine concentrations were quantified using HPLC. No detectable levels of caffeine were seen in brain homogenates of any of the treatment groups. However, plasma caffeine levels were quantifiable in animals treated with caffeinated coffee but not caffeine alone. The concentrations of caffeine received by the animals in the caffeinated coffee group were equivalent to those in the caffeine group. The plasma was collected from animals that were not exposed to any form of caffeine in the previous 24 h. Furthermore, the coffee administered to the animals in the caffeinated coffee group had a comparable concentration of caffeine present compared to the caffeine group, as determined by HPLC analysis. Given that the half-life of caffeine in a mouse is 0.7 to 1.2 h [241], we would not expect to see any quantifiable concentrations of caffeine present in the plasma of caffeine or caffeinated coffee treated animals. There are a number of possibilities why caffeine may be seen in the plasma of animals treated with caffeinated coffee.

Caffeine is predominantly metabolised through the cytochrome P450 enzyme, CYP1A2, to a number of active metabolites including paraxanthine, theophylline and theobromine as well as to a number of other enzymes including xanthine oxidase and thiopurine methyltransferase [242]. Bioactive constituents of coffee have been shown

to have a number of different effects on the enzymes responsible for the metabolism of caffeine. CA has been shown to inhibit CYP1A2, thereby potentially increasing the plasma concentrations of caffeine in the plasma [243]. Furthermore, both CGA and CA have been shown to have inhibitory effects on another key enzyme in the metabolism of caffeine, xanthine oxidase [244]. A CA analogue that has undergone oxidation has shown more potent inhibition of xanthine oxidase [245]. Given the nature of preparation of coffee, it is possible that the formation of oxidated compounds occurs however further studies are required to assess this.

Given the high concentrations of caffeine present in the plasma of caffeinated coffee treated animals and the previous studies showing caffeine, via A_{2a}Rs, to possess antidepressant activities [34], it cannot be excluded that this is not the mechanism by which caffeinated coffee exerts its antidepressant-like effects.

There have been a number of limitations identified with the current *in vivo* model of depression however attempts have been made to minimise these. The model is valuable in assessing the effects of compounds on behaviour, the KP and some aspects of inflammation however limitations in its ability to assess for changes to the concentrations of the pro-inflammatory cytokines and the concentrations of monoamines was noted. For future studies, a different inflammatory stimulus may provide a better model of inflammatory depression. One such model is using the BCG vaccine to induce a state of chronic depression. BCG vaccine is a beneficial model of inflammatory depression due to its secondary IFN- γ effects. Additionally, this method of inducing animal models of depression is favourable due to its chronic nature of

depression and closer to clinical depression. It has been shown that, in C57BL/6J mice, 10^7 - 10^8 colony forming units of the vaccine results in an initial acute stress response that further develops into depressive-like behaviour after 7 days [140, 141].

A second limitation identified in the current study is the sections of mouse brains used for cytokine, KM and monoamine analysis. In the current study, half brains were taken and homogenised for further analysis however, in previous studies, specific sections of the brain were isolated, homogenised and analysed [9, 139, 246, 247]. It is suggested that this may be beneficial to identify subtle changes to these parameters, such as cytokines, monoamines and KMs in the brain and as a result should be considered in the future.

2.6 Conclusion

In conclusion, the current study has found that caffeine and caffeinated coffee decreased the depressive-like behaviours observed 24 h post-LPS. Furthermore, similar results were observed in peripheral IDO activity neopterin and PGE₂ concentrations. From these results it would suggest that caffeinated coffee acts, at least in part, via anti-inflammatory pathways to reduce the symptoms and biomarkers of depression. Furthermore, caffeine was shown to have similar effects however, these were not as pronounced as those observed with caffeinated coffee. The results obtained suggest that caffeine, in combination with other coffee constituents were responsible for the antidepressant activities of caffeinated coffee. This study complements the human epidemiological data outlined in chapter 1 and provides preliminary evidence of causality. Furthermore, these results suggest that it is appropriate for a high quality

randomised controlled trial be undertaken to fully determine the causal link between the consumption of caffeinated coffee and the risk of depression.

Chapter Three: Quantitative analysis of key bioactive coffee constituents in caffeinated and decaffeinated coffee

3.1 Introduction

As outlined in chapter one, epidemiological studies have shown an inverse correlation between the consumption of caffeinated coffee and risk of depression [3, 4, 6-8]. Further evidence to support this was observed in the *in vivo* animal study undertaken as a part of chapter two. As seen in chapter two, caffeinated but not decaffeinated coffee protected against depressive-like symptoms produced by exposure to LPS. The aim of this study was to investigate if there were any differences in the presence of bioactive coffee constituents.

As outlined earlier, coffee contains many bioactive constituents that may contribute to the antidepressant-like effects of coffee including anti-inflammatory activities [41, 52, 87, 94], antioxidant activities [49, 50, 55, 88, 97] and positive effects on depressive behaviour in *in vivo* animal models of inflammatory depression [9, 76, 248] as outlined in section 1.2. Given the differences in antidepressant-like activity observed between caffeinated and decaffeinated coffee in both the epidemiological and the animal studies presented in chapter two [3, 4], it is possible that key bioactive coffee constituents are lost in the decaffeination process. It is well known that the decaffeination process is used to remove caffeine, however, to date, there have been no studies investigating the effects of this process on other bioactive coffee constituents.

The decaffeination process is the method by which coffee beans are treated to remove the caffeine present in the bean [249]. This process is undertaken on green beans before any further processing or roasting occurs [249, 250]. There has been a number of decaffeination processes outlined to date. Amongst these include the Swiss water process, the direct method, the indirect method, both chemical methods, and the CO₂ method [249, 250]. Of these, the Swiss water process is one of the most common method of decaffeination as it is a “chemical free” method. This method uses water saturated with coffee constituents, referred to as a green coffee extract, to soak the green coffee beans in. The immersion process is repeated several times until the beans are 99.9% caffeine free by mass [250], a required standard for a product to be classified as decaffeinated [251]. Given the methods by which caffeine is removed, it is plausible that other bioactive coffee constituents may be removed by the same process.

This study aimed to evaluate the effects of the decaffeination process on the quantities of a number of bioactive compounds found in coffee. The six most abundant bioactive constituents, caffeine, CA, CGA, FA, PA and trigonelline, were chosen for this study due to their biological activities as outlined in section 1.2. The concentration of these constituents was quantified using HPLC analysis in a variety of commercially available preparations.

3.2 Hypothesis, aims and objectives

3.2.1 Hypothesis

The preparation and decaffeination process affects the quantity of key biologically active constituents present in coffee.

3.2.2 Aims and objectives

The aim of this project was to evaluate the effects of the decaffeination process on key biologically active constituents of coffee including caffeine, CGA, CA, FA, PA, and trigonelline.

This was achieved through the following objectives.

- Quantitative assessment of the key bioactive coffee constituents including caffeine, CA, CGA, FA, PA and trigonelline, in bean and roast matched samples in caffeinated and decaffeinated coffee using analytical HPLC.

3.3 Materials and methods

3.3.1 Sample preparation

The coffee samples were all prepared in the same manner to ensure accuracy when comparisons were made according to a previous method with minor modifications [252]. Briefly, 50 mg of the coffee samples to be tested was dissolved in 1 mL of hot distilled water. Samples were then further diluted 1 in 20 or analysed undiluted before HPLC analysis was undertaken, respectively [252].

3.3.2 Preparation of working solutions

Stock solutions of caffeine, CA, CGA, FA, PA and trigonelline were made up to a concentration of 1 mg/mL in distilled water and stored at -20°C. Standard curves were made up fresh on the day of the analysis and further diluted in distilled water.

3.3.3 HPLC analysis – caffeine and trigonelline

Caffeine concentrations in coffee were quantified using HPLC. The HPLC method used was a developed, optimised and validated ion-pairing method which is described in detail below. A number of methods were tried; however, this method was found to be the optimal method and was chosen for use in this study.

Apparatus

The HPLC used was that outlined in section 2.3.8 above. The column used was GraceSmart C18 (5 µm, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

HPLC Method

The mobile phase consisted of 25 mM NaH₂PO₄ and methanol (70:30, v/v) and was delivered at a flow rate of 1 mL/min throughout the 10 minute isocratic run. The column was heated to 45°C and UV was monitored at 272 nm.

3.3.4 HPLC analysis – Chlorogenic acid and caffeic acid

HPLC analysis of CGA and CA was performed according to previously reported procedures with slight modifications [253]. The method used is outlined in detail below.

Apparatus

The HPLC used was that outlined in section 2.3.8 above. The column used was GraceSmart C18 (5 μ m, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

HPLC Method

A gradient elution method, run at 1 mL/min was employed for the elution of CGA using a 10 mM sodium phosphate monobasic buffer with the pH unadjusted as mobile phase A and acetonitrile as mobile phase B. The gradient program used was as follows: 0-5.0 min, 5-12% (v/v) B; 5.0-10.0 min, 12% (v/v) B; 10.-15.0 min, 12-15% (v/v) B; 15.0-25.0 min, 15-30% (v/v) B; 25.0-30.0 min, 30% (v/v) B; 30.0-30.1 min, 30-90% (v/v) B; 30.1-35.0 min, 90% (v/v) B; 35.0-35.1 min, 90-5% (v/v) B; 35.1-45.0 min 5% (v/v) B The column was heated to 40°C and UV was monitored at 325 nm.

3.3.5 HPLC analysis – Ferulic acid and pyrogalllic acid

FA and PA concentrations in coffee were quantified using HPLC. The HPLC method used was a developed, optimised and validated method and will be described in

detail below. A number of methods were tried; however, this method was found to be the optimal method and was chosen for use.

Apparatus

The HPLC used was that outlined in section 2.3.8 above. The column used was GraceSmart C18 (5 μ m, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

HPLC method

A gradient elution method, run at 1 mL/min was employed for the elution of FA and PA using a 0.1% glacial acetic acid solution as mobile phase A and acetonitrile as mobile phase B. The gradient program used was as follows: 0-10.0 min, 1-90% (v/v) B; 10.0-17.0 min, 90-1% (v/v) B; 17.0-20.0 min, 1% re-equilibration using solvent B. The column was heated to 40°C and UV was monitored at 320 nm.

3.3.6 Method validation

Method validation of each of the methods was performed according to the ICH guidelines summarised in section 2.3.8 [213].

3.3.7 Analysis of results

Quantification of the coffee constituents caffeine, CA, CGA, FA, PA and trigonelline was performed using the peak area of the analyte compared to 10 point standard curves.

3.4 Results

3.4.1 Quantitative analysis of bioactive coffee constituents

Validation of HPLC methods

The HPLC methods outlined in section 3.3 above were used to quantify caffeine, CA, CGA, FA, PA and trigonelline. These methods were validated using the methods outlined in section 2.3.8 and was found to be an accurate, precise and selective method for the quantification of the analytes of interest.

Linearity

The standard curves for each analyte of interest were prepared as described in section 3.3. The results represented in table 4 show the linearity of the 10 concentrations tested. These results show that a linear curve across the concentration range tested was found.

Accuracy

Table 4 below summarises the accuracy of the HPLC methods for the quantification of the analytes of interest. All three of the concentrations tested, 15 μ M,

20 μM and 35 μM , were within the acceptable range of 85 to 115% of expected in all methods with exception of CGA and CA.

Precision

The HPLC methods used to quantify the analytes of interest were found to be precise. Table 4 below summarises the precision of the methods with regards to the area, height and retention time represented as the coefficient of variance. All results fell within the acceptable range of less than 10% with exception of CGA (area) and the methods are therefore precise for the quantification of the analytes of interest.

Table 4 - Validation parameters for the HPLC methods used in the current study

Analyte	Linearity (R^2)	Accuracy	Reproducibility (Area, height & retention time)	Lower limits of detection and quantification
Caffeine	0.9998	90 to 104% of expected	CV < 1%	LOD – 0.012 LOQ – 0.097
Caffeic acid	0.9691	83 to 98% of expected	CV < 9%	LOD – 0.012 LOQ – 0.097
Chlorogenic acid	0.9992	80 to 99% of expected	CV < 11%	LOD – 0.012 LOQ – 0.097
Ferulic acid	0.9877	94 to 102% of expected	CV < 2%	LOD – 0.012 LOQ – 0.097
Pyrogalllic acid	0.9986	94 to 105% of expected	CV < 5%	LOD – 0.012 LOQ – 0.097
Trigonelline	0.9996	90 to 103% of expected	CV < 2%	LOD – 0.012 LOQ – 0.097

Selectivity

The HPLC methods used to quantify caffeine, CA, CGA, FA, PA and trigonelline were found to be selective for the analytes of interest in both a water matrix and a complex coffee matrix sample as shown below in figures 43.

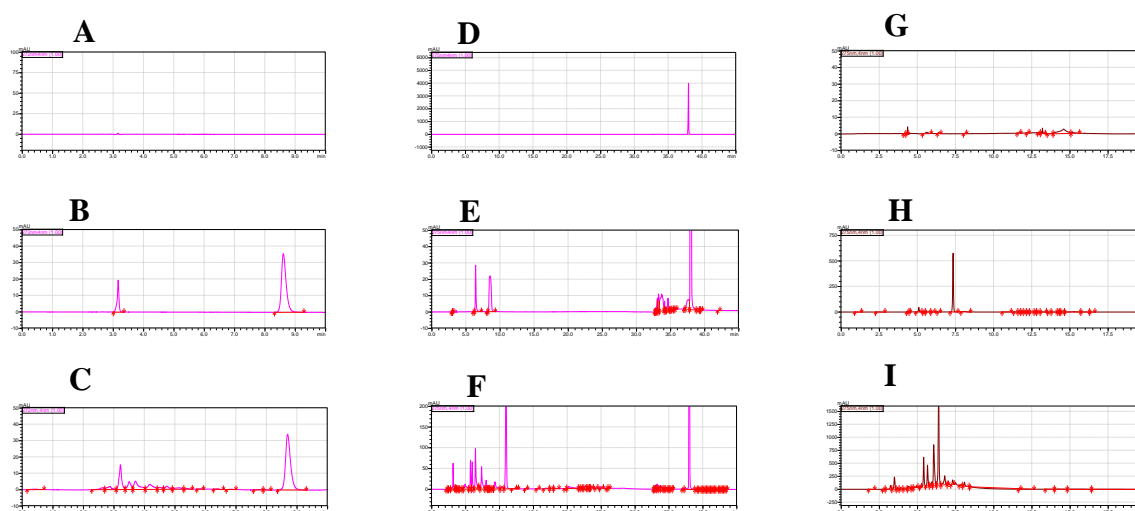


Figure 43 – HPLC chromatograms showing the specificity for the analytes of interest a) water blank; b) water spiked with trigonelline and caffeine; c) trigonelline and caffeine in complex coffee matrix; d) blank water; e) water spiked with caffeic acid and chlorogenic acid; f) caffeic acid and chlorogenic acid in complex coffee matrix; g) blank water; h) water spiked with ferulic acid and pyrogalllic acid; i) ferulic acid and pyrogalllic acid in complex coffee matrix.

3.4.2 Analysis of caffeinated and decaffeinated coffee

Table 5 below outlines the differences in coffee constituents between brand, bean and roast matched caffeinated and decaffeinated coffee. Three batches of three brands of instant coffee and one brand of espresso were evaluated for differences in key bioactive coffee constituents between caffeinated and decaffeinated coffee. This study has shown that key bioactive coffee constituents were lost in the decaffeination process. It was confirmed in all of the decaffeinated samples that statistically significant decreases in caffeine concentration were observed in comparison to their relative caffeinated counterpart. Furthermore, in a number of samples, FA content was

significantly decreased in decaffeinated samples. FA was decreased in samples 2 ($p < 0.01$) and sample 4 ($p < 0.001$).

In addition to the differences observed between bean and roast matched caffeinated and decaffeinated coffee, analysis has shown that there is variability in the concentration of the coffee constituents present in the various coffee samples analysed. Furthermore, variability in concentrations of constituents was observed between the various batches tested of each of the brands of coffee.

Table 5 – Summary of the concentrations of key bioactive coffee constituents in various brand, bean and roast matched coffee samples

Coffee sample	Caffeine (µg/50 mg)	Caffeic acid (µg/50 mg)	Chlorogenic acid (µg/50 mg)	Ferulic acid (µg/50 mg)	Pyrogalllic acid (µg/50 mg)	Trigonelline (µg/50 mg)
Caffeinated coffee brand 1						
Batch 1	3498.59	183.47	3683.63	40.26	67.23	3880.13
Batch 2	3309.74	80.62	1784.28	11.78	50.93	3630.34
Batch 3	3190.41	166.78	3396.42	30.39	69.82	3477.85
<i>Average</i>	3332.91 ± 155.39 (n = 3)	143.62 ± 55.20 (n = 3)	2954.78 ± 1023.80 (n = 3)	27.48 ± 14.46 (n = 3)	62.66 ± 10.24 (n = 3)	3662.77 ± 203.09 (n = 3)
Caffeinated coffee brand 2						
Batch 1	1876.73	72.23	1870.25	87.63	104.49	1460.89
Batch 2	2723.63	87.84	2284.74	93.10	104.95	2143.1
Batch 3	3831.10	72.56	2102.27	97.10	91.70	2665.84
<i>Average</i>	2810.49 ± 980.08 (n = 3)	77.54 ± 8.92 (n = 3)	2085.75 ± 207.74 (n = 3)	92.61 ± 4.75 (n = 3)	100.38 ± 7.52 (n = 3)	2089.94 ± 604.23 (n = 3)

Caffeinated coffee brand 3						
Batch 1	1896.97	145.22	1989.59	90.50	126.93	1427.98
Batch 2	2440.93	104.75	1508.55	60.86	118.20	1832.35
Batch 3	1426.08	118.97	1542.69	56.87	98.20	1116.24
<i>Average</i>	1921.32 ± 507.86	122.98 ± 20.53	1680.28 ± 268.42	69.41 ± 18.37	114.44 ± 14.73	1458.86 ± 359.05
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
Caffeinated coffee brand 4						
Batch 1	1250.10	85.84	1920.21	75.97	123.76	1170.09
Batch 2	2476.82	81.29	1827.06	97.28	122.10	2328.94
Batch 3	1271.03	112.37	2474.40	83.994	130.85	1186.22
<i>Average</i>	1665.98 ± 702.28	93.17 ± 16.79	2073.89 ± 349.96	85.75 ± 10.76	125.57 ± 4.65	1561.75 ± 664.46
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
Decaffeinated coffee brand 1						
Batch 1	486.09	209.27	3074.21	27.10	76.21	3745.88
Batch 2	549.20	223.97	3178.12	24.58	84.55	4088.55

Batch 3	605.45	264.87	3758.41	3.34	52.80	4502.86
<i>Average</i>	546.91 ± 59.71	232.70 ± 28.81	3336.91 ± 368.71	18.34 ± 13.05	71.19 ± 16.46	4112.43 ± 379.05
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
Decaffeinated coffee brand 2						
Batch 1	95.73	81.25	1663.05	56.61	120.99	1626.9
Batch 2	104.40	94.25	2067.16	56.92	124.34	1973.20
Batch 3	96.30	87.89	1753.82	71.73	154.57	1555.31
<i>Average</i>	98.81 ± 4.85	87.80 ± 6.50	1828.01 ± 212.02	61.75 ± 8.64	133.3 ± 18.50	1718.47 ± 223.49
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
Decaffeinated coffee brand 3						
Batch 1	86.89	122.70	1936.11	93.29	144.85	1568.97
Batch 2	68.67	157.03	2302.43	107.25	162.42	1206.85
Batch 3	101.08	141.01	2030.73	88.25	138.54	1904.85
<i>Average</i>	85.55 ± 16.25	140.25 ± 17.18	2089.76 ± 190.16	96.26 ± 9.84	148.60 ± 12.37	1560.22 ± 349.08
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)

Decaffeinated coffee brand 4						
Batch 1	45.42	98.11	714.94	7.99	74.53	727.55
Batch 2	73.18	98.01	650.27	13.32	100.62	892.06
Batch 3	24.64	124.98	883.86	31.62	176.48	1020.31
<i>Average</i>	47.75 ± 24.35	107.73 ± 15.54	749.69 ± 120.61	17.64 ± 12.39	117.21 ± 52.96	879.97 ± 146.75
	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)

3.5 Discussion

The aim of this study was to assess and evaluate the concentrations of several of the highly prevalent bioactive coffee constituents in brand, bean and roast matched caffeinated and decaffeinated coffee samples. This was done in order to identify bioactive coffee constituents present in lower concentrations in decaffeinated coffee in comparison to caffeinated coffee and ultimately identify compounds that may be contributing to the apparent antidepressant effects observed in the epidemiological studies and the *in vivo* studies presented in chapter two. Furthermore, this study investigated the differences in coffee constituents in different brands and the variability of the concentrations of coffee constituents between batches of the same brand of coffee.

Numerous key bioactive coffee constituents, including caffeine, CA, CGA, FA, PA and trigonelline were all quantified using HPLC analysis. All three HPLC methods used were shown to be specific, accurate, precise and linear across the concentration range tested according to the parameters outlined in the ICH guidelines [213]. The six compounds listed above were chosen for the current study as they are the six most prevalent bioactive compounds present in coffee and have been shown to modulate parameters associated with depression [9, 76, 79, 84, 88, 96, 97, 113, 124, 254].

Variability in the quantity of all bioactive coffee constituents analysed was observed between the four brands of coffee investigated. There are a number of possible reasons for this including the coffee bean used in the coffee preparation along

with the roast of the bean used. Coffee is prepared from a variety of beans belonging to the *Coffea* species [255]. The most common of these beans are *Coffea arabica* and *Coffea canephora*, also known as *Coffea robusta* [255]. Previous studies have shown that different beans contain varying quantities of some of the bioactive coffee constituents [256]. For example, quantities of CGA are significantly lower in *Coffea arabica* than those seen in *Coffea canephora* [257]. This is also true of caffeine levels [258]. Furthermore, studies have shown that the quantity of CGA present in a final coffee preparation is dependent on the roast of the coffee and decrease as the roast increases [258-260]. Additionally, the quantity of caffeine present has been shown to increase up to 21% after coffee beans are dark roasted [258]. This provides a justification as to why different brands of coffee vary so greatly in their composition.

In addition to the variability between brands, variability between different batches within the same brand was also observed. This variability may be due to climatic factors. Studies have shown that rainfall and the mean air temperature during seed development are important factors for the quantities of bioactive constituents, particularly the CGAs [261, 262]. This means that beans grown at different times of the year or those that have experienced a different level of rainfall will potentially have varying quantities of bioactive coffee constituents.

As expected, caffeine concentrations in all of the decaffeinated samples was significantly lower than their respective caffeinated counterparts. As outlined above, the decaffeination process is a method by which coffee beans are treated to remove the caffeine present in the bean [249]. Another important bioactive coffee constituent, FA,

was shown to be present in significantly lower concentrations in two of the four coffee decaffeinated coffee samples tested in comparison to their caffeinated counterparts. This suggests that FA may be an important bioactive coffee constituent in the antidepressant-like activities of caffeinated coffee. This is supported by numerous *in vitro* and *in vivo* studies assessing FA on parameters associated with depression and showing beneficial effects [9, 76].

Varying results have been identified with regards to the effects of the decaffeination process on the concentration of CGA in decaffeinated coffee. On one hand, there have been a number of studies where CGA concentrations did not significantly differ between caffeinated and decaffeinated coffee samples [249, 263], which is consistent with the results obtained in the current study. Conversely, another study has shown that CGA concentrations are significantly lower in decaffeinated coffee in comparison to caffeinated coffee [256]. One such explanation for this may be differences in the decaffeination method used. As outlined earlier, numerous different decaffeination methods are used commercially which may have a significant effect on the loss of other additional bioactive coffee constituents in addition to caffeine. This may also provide a justification as to why all brands of decaffeinated coffee do not show decreased concentrations of FA. This also provides a justification for a future study assessing the effects of different decaffeination processes on the quantities of bioactive coffee constituents in decaffeinated coffee.

As previously described in section 1.2, *in vitro* and *in vivo* studies have shown FA to have beneficial effects on depression. Given the results obtained in the *in vivo*

study undertaken in chapter two, it is plausible that FA, in combination with caffeine, may provide the changes to behaviour consistent with antidepressant effects. Furthermore, this combination of constituents may be responsible for decreasing other pro-inflammatory biomarkers associated with depression including IDO activity. This highlights the need to further investigate these compounds on parameters associated with depression. This will be done in the current study using optimised *in vitro* models of inflammation.

There are a number of future avenues of research to follow on from the study. One such study, is given that only a small number of coffee samples and brands have been sampled to date, a larger sample of coffee should be sampled in the future. Furthermore, the popularity of the brand and ultimately the consumption rates of each of the coffees were not assessed in analysis of these results. These factors were not assessed in this study as the aim of this study was to simply identify if differences in bioactive coffee constituents existed between caffeinated and decaffeinated samples. The current study does however provide a basis for future studies assessing the parameters outlined above.

A second possible further study identified was the analysis of the decaffeination process were not taken into account when analysing the data. Once again, this was not an aim of the current study, however, the results obtained provide a justification for future studies assessing the effects of different decaffeination processes on the loss of other key bioactive coffee constituents in addition to caffeine.

3.6 Conclusion

This study has highlighted the differences in coffee constituents between caffeinated and decaffeinated coffee samples. Two key bioactive coffee constituents, caffeine and FA, were shown to be significantly decreased in decaffeinated coffee samples, suggesting that these two compounds may play a role in the antidepressant-like actions of caffeinated coffee. Further studies specifically assessing the effects of these two compounds on parameters associated with depression is required. Furthermore, given the conflicting nature of the results pertaining to the effects of the decaffeination process on CGA concentrations and the variety of decaffeination processes used commercially, further studies investigating the effects of each of the individual constituents and each of the constituents in combination with coffee will be investigated for their neurotoxic and anti-inflammatory effects in optimised *in vitro* models.

Chapter 4 – Development of *in vitro* surrogate microglial and neuronal-like cell models

4.1 Introduction

As discussed in the literature review, numerous epidemiological studies have assessed the effects of coffee on depression and found coffee to have beneficial effects in decreasing the risk of depression [3, 4, 7, 8, 12]. The *in vivo* animal model of depression outlined in chapter two has provided preliminary evidence to support the findings of the epidemiological studies. Numerous parameters of depression including behavioural and relevant biomarkers were found to be altered after the administration of all three of the treatment groups but in animals treated with caffeinated coffee to the greatest degree. Furthermore, chapter three highlighted the loss of key bioactive coffee constituents in the decaffeination process that may play a role in the differences in antidepressant-like activity observed with the consumption of caffeinated coffee in comparison to decaffeinated coffee. With this in mind, the aim of this study was to develop *in vitro* cell-based models to, firstly, assess the effects of key bioactive coffee constituents on inflammatory mediators in microglial-like cells. This provides a means to assess the potential use of these compounds as antidepressant agents and further investigate the mechanism through which they may elicit their effects. Secondly, the development of an *in vitro* surrogate neuronal cell model to assess the toxicity of key bioactive coffee constituents on neuronal-like cells.

4.1.1 Cell types in the CNS

Two major cell types present in the CNS are microglial and neuronal cells [264]. Both of these cell types are critical in the neuroinflammatory process and are thought to play an integral role in the pathophysiology of depression [128, 264, 265]. Ideally, human primary *in vitro* cell models are the optimal model for assessing the pharmacological and toxicological effects of compounds of interest however this is not always possible given the cost and ethical restraints on using these cells.

Microglial cells are the resident macrophage and ultimately inflammatory cells in the CNS [62, 266]. Microglial cells are found to be either in their resting quiescent state or in an activated state [266]. There are numerous stimuli that are known to activate microglial cells including immune challenge and psychological stressors [266]. Microglial activation can occur in response to infection pathogen-associated molecular pattern (PAMPs), danger-associated molecular patterns (DAMPs), inflammatory cytokines and mediators and neurotransmitters [266]. Furthermore, these activators may originate in either the periphery or in the CNS [266]. As a result of activation, microglial cells increase their production of cytokines, chemokines KMs and glutamate [266].

4.1.2 Surrogate models of microglial-like and neuronal-like cells

Numerous models of macrophage or microglial-like cells have been described previously. One such cell-line is the THP-1 human monocytic cells [267, 268]. THP-1 cells have been shown to be cytotoxic to SH-SY5Y neuroblastoma cells in a similar manner to that of human microglial cells [269, 270]. Furthermore, studies have shown

that these cells can be further differentiated into macrophage- and microglial-like cells using a number of chemical stimuli [268]. Differentiating agents include phorbol 12-myristate-13-acetate (PMA) [268, 271], 1,25-dihydroxyvitamin D3 [268], retinoic acid [272] or the cytokines TNF- α and IFN- γ [273] and have been shown to induce differentiation to varying degrees [268].

It was shown that PMA differentiated THP-1 cells have induced morphological changes along with altered cell surface markers and expressed cytokine profiles that are consistent with primary macrophages [271]. Furthermore, these cells have been shown to be more resistant to apoptosis than their undifferentiated counterpart [271]. Enzymes and metabolites present in the KP have also been shown to be present when these cells are exposed to pro-inflammatory stimulus [274]. Given the outlined features of the PMA differentiated THP-1 monocytes and similarity in phenotype to macrophages and microglial cells, this model was chosen for the current study.

Numerous immortal neuron-like cell lines have been used in pharmacological and toxicological studies in the past. One such cell line is the SH-SY5Y neuroblastoma cells [275-277]. The SH-SY5Y neuroblastoma cell resembles human foetal sympathetic neurons [278] and shows expression of many of the receptors expressed by primary human neurons. Amongst the receptors expressed, ionotropic and metabotropic NMDA receptors [279], nicotinic acetylcholine receptors (nAChRs), 5-HT receptors, muscarinic receptors, adrenergic receptors and opioid receptors have been identified [278]. Additionally, various subunits of nAChRs have been identified as being natively expressed including the $\alpha 3$ and $\alpha 7$ nAChRs [278].

In addition to the receptors outlined above there are numerous other features these cells display including the expression of the biosynthetic enzymes required for the biosynthesis of noradrenaline, neuronal characteristics of expression of neurofilament proteins, neuron specific enolase, gamma-aminobutyric acid (GABA) and norepinephrine uptake, glutamic acid decarboxylase and muscarinic acetylcholine receptors positively coupled to inositol phosphate turnover [280].

Guillemin *et al* characterised the differences in the enzymatic pathway of the KYN metabolism between primary human neurons and the SK-N-SH neuroblastoma cell line [281]. Observations of SK-N-SH cells are of importance in this project as the neuroblastoma cell line used, SH-SY5Y, is thrice cloned from the SK-N-SH cells and bares high similarity in characteristics [281].

In this study, cells were stimulated with IFN- γ and subsequently the differences in levels of the KP enzymes between the two cell lines were measured using Western blotting. Key differences in the enzymatic pathways are shown in table 4. The main difference in the enzymatic pathway of KYN metabolism is the slightly higher expression of 3-hydroxyanthranilate 3,4-dioxygenase (HAAO) and QPRT in the SK-N-SH cells in comparison to the primary cells [281].

Table 6 – Comparison of the kynurenine pathway enzymes in neuroblastoma cell line SK-N-SH and primary human neurons [281]

Stimulus	Enzyme	SK-N-SH cells	Primary human neurons
IFN- γ	IDO	Increased expression	Increased expression
Unstimulated	IDO	No expression	No expression
IFN- γ	TDO	Decreased expression	Decreased expression
Unstimulated	TDO	Expressed	Expressed
IFN- γ	KAT I	Low levels of expression	Low levels of expression
Unstimulated	KAT I	Low levels of expression	Low levels of expression
IFN- γ	KAT II	Extremely low levels of expression	Extremely low levels of expression
Unstimulated	KAT II	Extremely low levels of expression	Extremely low levels of expression
IFN- γ	KYNU	Low levels of expression	Low levels of expression
Unstimulated	KYNU	Low levels of expression	Low levels of expression
IFN- γ	KMO	Low levels of expression	Low levels of expression
Unstimulated	KMO	Low levels of expression	Low levels of expression
IFN- γ	HAAO	Expressed slightly higher than primary cells	Expressed
Unstimulated	HAAO	Expressed slightly higher than primary cells	Expressed
IFN- γ	QPRT	Expressed slightly higher than primary cells	Expressed
Unstimulated	QPRT	Expressed slightly higher than primary cells	Expressed

Other major differences this study identified pertained to the levels of KMs produced by the primary cells and the SK-N-SH cells. It was observed that SK-N-SH cells catabolise Trp at a rate of 3.6 times faster than primary human neurons when stimulated with IFN- γ [281]. However, it is interesting to note that SK-N-SH cells

produced lower levels of KYN than primary cells, with 17.66 and 19.75 μM produced respectively. In addition to this the levels of KA produced in response to IFN- γ stimulus increased in both cell lines although production was higher in primary neurons. Of particular interest is the paradoxical effects observed in the SK-N-SH cells with regards to the toxicity profiles of picolinic acid and QA. Picolinic acid, usually a neuroprotective agent, was found to decrease the proliferation of SK-N-SH cells at 1 and 5 μM concentrations whereas QA, a known neurotoxin, increased proliferation of SK-N-SH cells at 5 μM [281]. There are, however, a number of similarities between the two cell lines with 3-HK detectable in spent supernatant of both cell lines in the nanomolar range and both cell lines have the ability to degrade QA present in media.

To date there have been no studies undertaken assessing the effects of the various differentiation protocols described for SH-SY5Y neuroblastoma cells on the expression of the various enzymes involved in the KP.

In addition to the similarities of SH-SY5Y neuroblastoma cells to primary human neurons, there have been numerous differentiation protocols described that result in a phenotype that closer resembles that of a primary neuron [282-284]. Common differentiation agents include dibutyryl cAMP (dbcAMP), brain-derived neurotrophic factor (BDNF) and retinoic acid (RA) [282-284].

Kume *et al* evaluated the effects of dbcAMP and RA on differentiating SH-SY5Y neuroblastoma cells. It was found that exposure of these cells to 1 mM dbcAMP

produced morphological changes consistent with change into neuron-like phenotypes. In addition to this, tyrosine hydroxylase (TH) expression in combination with HPLC analysis confirming NA in spent supernatant confirmed a noradrenergic phenotype was formed. In contrast, RA (10 μ M) did not produce a noradrenergic phenotype although forming a neuron-like morphology and expressing TH [284]. As depression is thought to be a partially noradrenergic-dependent disorder, dbcAMP appears to be a promising differentiation agent for SH-SY5Y neuroblastoma cells.

4.1.3 Neuroinflammation in depression

In addition to neuronal cells, inflammatory cells such as microglial cells are also thought to play an important role in depression. It is proposed that this is through their role in the pro-inflammatory immune response [266]. Numerous cells are involved in the immune system of the CNS and subsequent neuroinflammatory responses, primarily microglial cells and astrocytes. Microglial cells release an array of inflammatory cytokines resulting in reactive gliosis [33], a process of accumulation of enlarged glial cells, specifically microglial and astrocytes [33]. Activation of the CNS immune system occurs in response to a large number of disease states including brain inflammation, trauma, ischaemia and stroke, brain infections and neurodegenerative diseases [125].

Evidence to support neurotoxicity in neuroinflammation has been shown through activated microglia cells and infiltrating macrophages causing neuronal death through numerous mechanisms of action including quinolinic acid (QA) [125], a well-documented neurotoxin biosynthesised through the KP [95, 165, 169]. Another

important endogenous neurotoxin is the pro-inflammatory cytokine IL-1 [285]. Its neurotoxic effects are dependent on the activation of the IL-1 type 1 receptors on astrocytes which mediates the initiation of caspase-dependent neuronal cell death, via the release of reactive oxygen species (ROS) [285].

Neuroinflammation plays an important role in the health and survival of neurons and the integrity of the BBB [286]. In the CNS there is a balance between the pro-inflammatory Th1 immune response, regulated by the activated microglial cells [287], and the anti-inflammatory Th2 immune response, regulated by the astroglial cells [287], that is critical to CNS health. When the Th1/Th2 balance is disturbed favouring the Th1 response neuronal damage and death occurs [81, 288, 289]. The role of each of these responses and the cells responsible are shown in figure 44 below.

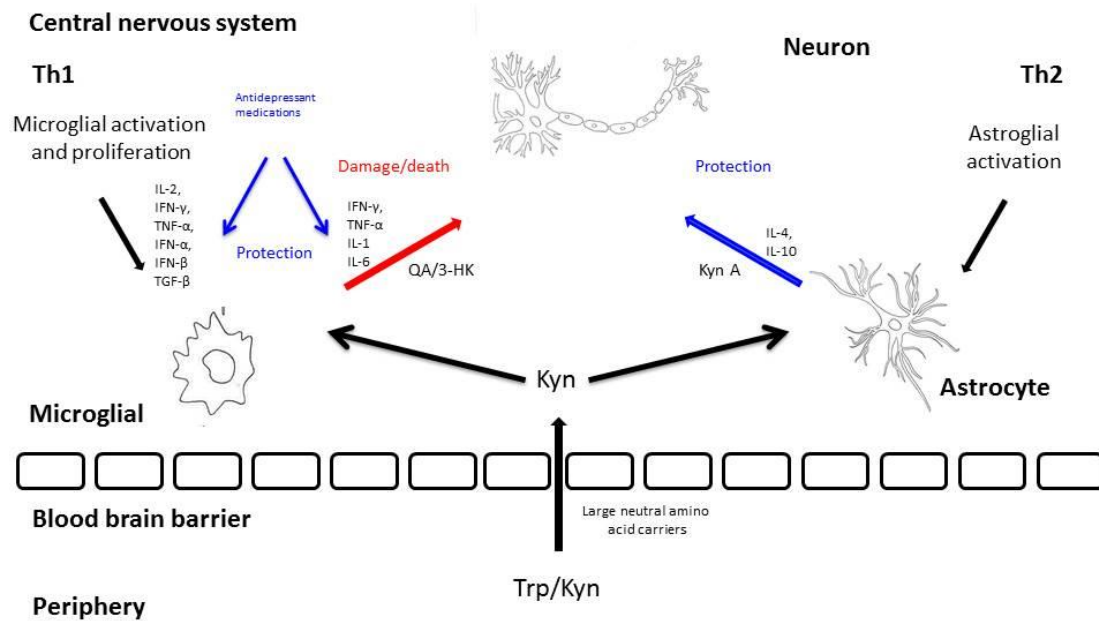


Figure 44 – Neuroinflammation in the central nervous system and the known action of antidepressant medications [124].

The catabolism of Trp is highly regulated by numerous inflammatory mediators and as a result is altered in neuroinflammatory states. KYN, the first Trp catabolite, is biosynthesised utilising the enzyme IDO [13, 15, 38]. IDO is highly regulated by the immune system with numerous inflammatory mediators up-regulating the enzyme and as a result is upregulated in neuroinflammation shown in figure 45 below [81]. During neuroinflammation, IDO is upregulated in activated microglial cells which in turn results in increased production and accumulation of the KMs, a number of which are neuroactive [159, 290]. Activated microglial cells play an integral role in neuroinflammation and are usually the first cellular response under inflammatory conditions [62]. Microglial cells are the main cells responsible for the production of cytokines in the CNS, with these being Th1 cytokines [115]. Additionally, activated microglial cells produce PGE₂, a biomarker observed in depressed patients [288],

further implicating inflammation and microglial activation in the pathophysiology of depression. Figure 45 below shows the effects of inflammatory mediators and compounds on the enzymes and metabolites of the KP. QA, 3-HK, and 3-HAA are known KMs with neurotoxic properties. QA is an excitotoxin through its N-methyl-D-aspartate receptor (NMDA) agonism and secondary free radical production [95, 156]. 3-HK and 3-HA are free radical generators and elicit their cytotoxicity through this mechanism [69, 95].

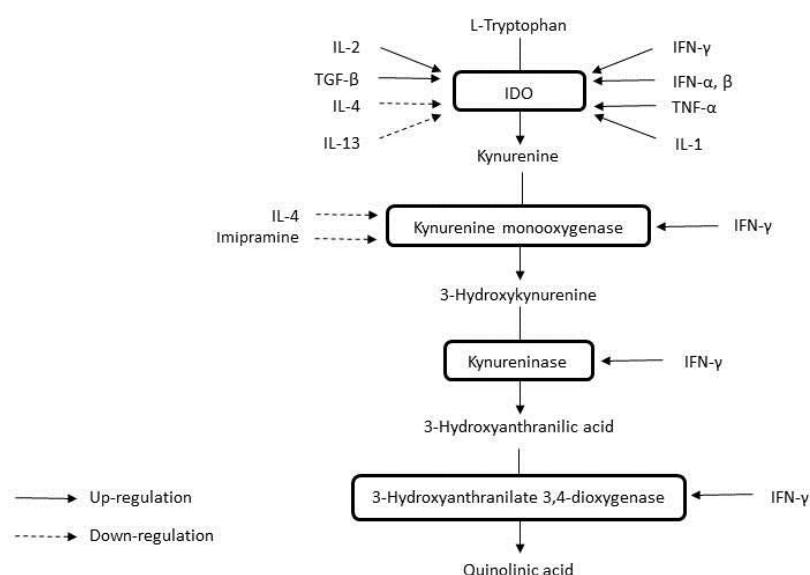


Figure 45 - Effects of cytokines on the enzymes in the kynurenine pathway [81]

4.1.4 Role of endogenous and exogenous interferons in depression

What are interferons?

IFNs are a group of class II cytokines that have pleiotropic effects in addition to their antiviral properties [291]. All IFNs share a degree of structural homology

however this does not confer to similar biological activity [292-294]. This is highlighted with two of the well-documented IFNs – IFN- γ and IL-10. IFN- γ and IL-10 are structurally related, as evidenced by their similar α -helical structure, however, they display opposite biological effects. IFN- γ is a potent pro-inflammatory cytokine and IL-10, a potent anti-inflammatory cytokine [295, 296].

IFNs are produced endogenously in response to viral infections, tumours and are responsible for immune stimulation [297, 298]. IFNs are typically produced by a number of cells throughout the body [298, 299]. However, type II IFNs are only produced by immune cells, primarily Th1 cells. With regards to IFN- γ , numerous other immune cells, including CD8⁺ cytotoxic lymphocytes, natural killer cells, B cells and professional antigen presenting cells, secrete IFN- γ [297].

In addition to being produced endogenously, IFNs are administered exogenously for their responses against viral infections, their inflammatory processes and their anti-tumour activities [300-304]. This encompasses a variety of disease states including hepatitis C [305, 306], multiple sclerosis (MS) and various cancers, such as melanoma [307]. Endogenously, IFNs play a role in both the innate and the adaptive immune response [308, 309].

IFNs exert their activities through binding to the class II cytokine heterodimeric receptors initiating signalling pathways [310]. When IFNs bind to their respective receptors they activate cell-signalling pathways [311-313]. Initially, all IFNs activate

the signal transducers and activators of transcription (STATs) and Janus tyrosine kinases (JAKs) [311, 313]. When IFNs bind to their respective receptor, the associated JAK protein is activated and leads to the phosphorylation of the receptor and ultimate docking site for the corresponding STAT factors. From there, various different cell-signalling pathways occur. The binding of IFN at all of the class II cytokine receptors results in the activation of the STAT3 and STAT1 signalling pathways, whereas the binding of IFN at type I IFNR also activates the STAT1 and STAT2 and the mitogen-activated protein kinases (MAPK) cell-signalling pathways [311, 313].

Types of interferons

Type I interferons

Type 1 IFNs are a large subset of interferons with a high level of homology (between 75 and 99%) [314]. IFN- α and β are the most widely studied type I IFNs but a number of others exist including IFN- ϵ , IFN- κ and IFN- ω [315]. Type I IFNs bind to the IFN α/β receptor that consists of two subunits - IFNAR1 and IFNAR2 [316]. Differences in binding affinity have been shown between type I IFNs [317, 318]. When binding occurs, it signals cells through the JAK-STAT pathway. The differences in binding affinity can result in differing downstream cell signalling through the JAK-STAT pathway resulting in the differing biological activities of the different IFNs [319].

Interferon alpha

IFN- α , a type I IFN, is produced by a number of cells including fibroblasts, T cells, macrophages, monocytes dendritic cells and natural killer cells [298]. It is a

protein that is produced both endogenously and commercially for use in the treatment of hepatitis C and in cancer therapy [320]. Endogenously, IFN- α is responsible for promoting the activation of immune response in order to mediate antimicrobial and antitumour activity [321]. IFN- α is expressed early in an infection and as a result is thought of as an important cytokine priming the innate-immune response [322]. In the innate-immune response, IFN- α stimulates natural killer cells and increases their proliferative and cytolytic activity, increases their secretion of IFN- γ and primes the cells for IL-2 and IFN- γ [322].

With regards to the adaptive immune response, IFN- α is thought to provide a link between the innate and adaptive immune response [323]. In the adaptive immune response IFN- α acts in many different ways including increasing dendritic cell and macrophage secretion of IFN- γ , it alters the Th1-Th2 balance towards Th1 response and increases major histocompatibility complex (MHC) class I and II expression [322, 324]. In addition to these effects, IFN- α directly or indirectly regulates a large number of chemokines and cytokines including IFN- γ , IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, IL-13, IL-15, TNF- α and GM-CSF [322, 325].

Numerous subtypes of IFN- α are produced endogenously and are used routinely in hepatitis C therapy [305, 326]. It has been hypothesised on numerous occasions that endogenous IFN- α may play a role in the aetiology of depression [327], but to date only studies evaluating the effects of exogenously administered IFN- α and the onset of depressive symptoms have been undertaken. When given exogenously, IFN- α has been shown to induce rapid-onset depression, usually within two weeks of initiation [53, 229,

230, 328], at an estimated prevalence of between 20-30% and is reversible on cessation of therapy [53]. This indicates a probable involvement of endogenous IFN- α in the pathophysiology of depression although further studies are required to confirm this.

Recent studies have evaluated the neurotoxic effects of IFN- α in rat cortical neuron cultures [329]. IFN- α is toxic towards these cells when exposed for 24 h. In further support of these results, the same study showed that by blocking the IFNAR and subsequent JAK/STAT signalling, you can protect against the neurotoxic effects of IFN- α [329]. Furthermore, the role of NMDA receptors in IFN- α induced neurotoxicity were evaluated and it was found that blocking the GluN2A subunit of NMDA receptors afforded protection [329], suggesting that IFN- α is responsible for producing NMDA receptor agonist activity, either directly or indirectly. IFNAR knockout mice treated with a GluN2A antagonist were fully protected against IFN- α neurotoxicity, providing further evidence for the role of IFN- α in NMDA receptor agonism. This study highlights a possible mechanism by which IFN influences depression [329]. Activation of the JAK/STAT pathway in neurons increase in the release of glutamate, calcium and pro-inflammatory cytokines all potential contributors to neurotoxicity and potential mechanisms of IFN-induced depression [330].

IFN- α has been shown to have a profound effect on the KP. Exogenously administered IFN- α can pass the BBB in concentrations high enough to act on microglial cells and macrophage receptors resulting in activation of IDO in these cells centrally [69]. This then results in the formation of KMs that then exert their neuromodulatory effects [69] and their possible role in the pathophysiology of

depression. IFN- α has been shown to have a weaker direct IDO-stimulating effect than IFN- γ and may have indirect effects on IDO [69, 229]. This is thought to occur by IFN- α stimulating the production of IFN- γ and TNF- α via monocytes and lymphocytes, which are strong inducers of IDO [69].

IFN- α has been shown to have numerous effects on the serotonergic arm of Trp catabolism [229]. In addition to the increased metabolism of 5-HT via the upregulation of IDO, IFN- α has also been shown to upregulate the serotonin transporter (5-HTT), thereby decreasing extracellular 5-HT levels and to modulate 5-HT_{1A} and 5-HT₂ receptors [229].

Interferon beta

IFN- β is a naturally occurring anti-inflammatory cytokine [331] that belongs to the type I IFN family but only shares approximately 35% homology with IFN- α [332]. It is an important pleiotropic cytokine that balances the Th1-Th2 response in the CNS in addition to limiting the migration of inflammatory cells across the BBB [333]. Endogenously, only a limited number of studies have been undertaken to assess the role of IFN- β . A recent study has shown that IFN- β exerts its endogenous anti-inflammatory effects through inhibiting numerous cytokines including IFN- γ secretion from CD4⁺ lymphocytes [334].

IFN- β is also available as a recombinant form used primarily in the treatment of multiple sclerosis (MS) [335]. Studies have shown that IFN- β exerts its anti-

inflammatory effects by increasing the production of IL-10, an anti-inflammatory IL and through inhibition of IL-17, a pro-inflammatory cytokine [335].

IFN- β therapy is not associated with an increase in depressive symptoms but with a decrease in depressive symptoms [320]. When administered as treatment for hepatitis C, IFN- β produces significantly less depressive symptoms in patients and differences in depression levels between placebo and active groups, using the Montgomery-Åsberg Depression Rating Scale (MADRS), cannot be detected [75, 320]. This is likely to be due to its regulatory effects on the pro-inflammatory-anti-inflammatory balance [333].

IFN- β has been shown to upregulate the expression of IDO, in a dose-dependent manner [336], although it has been shown to have a weaker direct IDO stimulating effect than IFN- γ [69, 229]. This results in an increase in the concentration of KMs in the CNS however to a lesser degree than after exposure to IFN- γ [289]. The stimulation of IDO is thought to occur predominantly in the periphery due to IFN- β 's limited ability to cross the BBB and is further supported by IDO being the only KP enzyme affected by IFN- β [289].

IFN- β is an interesting molecule due to its differing effects. On one hand, it is an anti-inflammatory cytokine showing possible antidepressant activity but at the same time upregulates the KP, responsible for production of neurotoxins and strongly implicated in the pathophysiology of depression [336]. One explanation for this

phenomenon may be due to IDO being the only enzyme in the KP affected, whereas IFN- γ affects downstream enzymes in the “neurotoxic arm” of the KP [81] and further shifting the balance towards the production of neurotoxic metabolites. To date, there are limited numbers of studies evaluating the effects of IFN- β on the incidence of depression.

IFN- β has recently been evaluated for its neurotoxic effects towards rat cortical neurons [329]. It was shown not to have neurotoxic effects [329] therefore strengthening the evidence for IFN- β not causing depression. Interestingly, the opposite results were observed when a commonly used cell line for neurotoxicity studies, SH-SY5Y neuroblastoma cells, was exposed to IFN- β [337]. This toxicity occurs through the activation of the JAK/STAT pathway and highlights the difference in effect between immortal and mortal cells and further highlights the relevance of appropriate models [337].

It appears from the available studies that IFN- β may provide protection against depression an effect, which may be attributed to its ability to balance the Th1-Th2 inflammatory response. This further strengthens the evidence supporting the neuroinflammatory theories of depression.

Type II interferons

IFN- γ is the sole cytokine identified to be a type II IFN [297]. Similar to type I IFNs, IFN- γ binds to a heterodimeric receptor, INFGR [297]. Again, the binding of

IFN- γ to this receptor results in the activation of the JAK-STAT cell signalling pathway [297].

Interferon gamma

IFN- γ is a pleiotropic cytokine with a primary responsibility of promoting the activation of numerous cell types, including monocytes and macrophages, involved in antimicrobial and antitumour activities [290]. The main physiological actions of IFN- γ are mediated by the induction of two enzymes – IDO and inducible nitrous oxide (iNOS) [290]. IFN- γ has been well reported to have antiviral activities [338, 339] however its pro-inflammatory properties are more potent [339, 340].

IFN- γ has been shown to be the main pro-inflammatory inducer of IDO [44] and acts in a dose-dependent manner [336]. It does this through up-regulating the transcription of the enzyme in the immune cells monocyte-derived macrophages and microglia [69]. In addition to IFN- γ 's effects on up-regulating the expression of IDO, it also upregulates enzymes further down the KP. These include KMO, responsible for the conversion of KYN to 3-HK, KYNU, responsible for the conversion of 3-HK to 3-HA and 3-HAOO, responsible for the conversion of 3-HA to QA [81], leading to the synthesis of the various neurotoxic metabolites. The effects that IFN- γ have on the enzymes in the KP results in numerous physiological effects including anti-viral and anti-tumoural properties, for which it is used clinically for [341] and the secondary effects of depression, as witnessed in patients being administered this therapy [341]. Both of these effects are thought to be attributed to IFN- γ 's ability to induce the activity

of IDO and accumulate the resultant KMs [341]. IDO is also responsible for the metabolism of 5-HT. Therefore, when stimulated with IFN- γ under inflammatory conditions, decreased levels of 5-HT are observed [128]. There is overwhelming evidence suggesting IFN- γ plays a pivotal role in the pathophysiology of depression.

Like IFN- α , IFN- γ has also been shown to be neurotoxic in mouse primary neurons [342]. Again, this is via activation of the JAK/STAT1 pathway however enhancing toxicity by glutamate toxicity at AMPA receptors, not NMDA receptors [342]. In addition, IFN- γ has been shown to induce IDO activity through the JAK/STAT pathway [343].

Type III interferons

Type III IFNs are similar to type I IFNs [344] and are sometimes referred to as the IFN- λ family [345]. This category of IFNs is comprised of 3 proteins IFN- λ 1, IFN- λ 2 and IFN- λ 3 or IL-28A, IL-28B and IL-29 respectively [346]. These proteins bind to a heterodimeric receptor comprising of IL-28R and IL-10R chains [347]. Type III IFN receptors are preferentially located on epithelial cells and in the central nervous system, and are expressed to a lesser degree than type I IFNs in response to viral infection (54). Type III and type I IFNs have very similar patterns of cell signalling, particularly through the STAT pathway [348-350].

To date, there are only limited studies undertaken on type III IFNs and no studies examining depression or the multiple parameters associated with the

neuroinflammatory hypotheses of depression. However, given the similarity between type III and type I IFNs, further studies investigating these effects are warranted. More specifically, studies evaluating the numerous subtypes of IFN- λ are also warranted.

4.1.5 Role of antidepressant medications on parameters associated with neuroinflammation

Numerous classes of antidepressant medications are currently marketed for the treatment of depression including the tricyclic antidepressants (TCAs) [351], monoamine oxidase inhibitors (MAOIs) [351], selective serotonin re-uptake inhibitors (SSRIs) [351], serotonin noradrenaline re-uptake inhibitors (SNRIs) [351], noradrenaline re-uptake inhibitors (NARIs) [352], and various miscellaneous antidepressant medications including, mirtazapine, agomelatine and bupropion [352]. Each of the classes of antidepressant medications will be explored below for their effects on each of the components of the neuroinflammatory hypotheses of depression.

A number of currently marketed antidepressant medications have been shown to alter the pro-inflammatory cytokines outlined above. However, one shortcoming of measuring cytokine levels is variability in the results obtained mainly due to the low levels of circulating cytokines and the fact that results are usually measured *ex vivo* [353]. The effects of these medications has however been evaluated in *in vitro* and *in vivo* studies in addition to *ex vivo*.

TCAAs were a common antidepressant medication in the past however other classes of antidepressants have become more popular due to their more favourable tolerability profiles [354]. TCAAs act primarily as serotonin noradrenaline reuptake inhibitors but also act as antagonists at numerous other receptors including H1 receptors, 5-HT_{2A} and α 1 adrenoceptors, the main reasons for their poor tolerability [355].

A number of TCAAs have been shown to alter inflammatory mediators in various ways. On one hand, clomipramine increases IL-1 β , a pro-inflammatory cytokine, release whereas amitriptyline, decreases the pro-inflammatory TNF- α but increases IL-6, another pro-inflammatory cytokine in treatment responders *ex vivo* [353]. Conversely, the same study showed opposite effects in patients classified as treatment non-responders [353]. This highlights a possible neuroinflammatory mechanism underlying treatment non-response.

Imipramine and clomipramine both decreased the release of numerous pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α from monocytes and IL-2 and IFN- γ release from T lymphocytes when these cells are pre-incubated in the antidepressants for 24 h *in vitro* [353]. This suggests that “pre-treatment” with antidepressant medications is an important factor in their anti-inflammatory properties and may provide an explanation to the delayed onset of antidepressant activities of these drugs. Additionally, imipramine, amitriptyline and nortriptyline decrease TNF- α and IL-1 β production in rat microglia [356-358] and imipramine decreases the production of IL-6 and NO from mouse microglia [357, 359] again supporting the anti-inflammatory

actions of these compounds. Clomipramine and imipramine attenuated NF- κ B [357, 358] and p38 MAPK activation, both responsible for regulation of the pro-inflammatory cytokines, and decreased the expression of numerous inflammatory genes including iNOS, IL-1 β and TNF- α [357].

In addition to the *ex vivo* and the *in vitro* studies, a small number of studies have evaluated the effects of TCAs *in vivo*. Clomipramine was shown to increase TNF- α levels *in vivo* [360] whereas imipramine and desimipramine decrease symptoms of TNF- α induced depression in a mouse model of depression [361]. *In vivo* studies showed that there is a degree of conflict between research groups. For example, conflicting results for the effect of TCAs on IL-6 levels have been observed with some studies showing no effect where others show a decrease in IL-6 levels (Reviewed in [353]).

SSRIs are commonly used antidepressant and as their name suggests, inhibit the reuptake of serotonin from the synapse into the presynaptic nerve terminal of the neurons [362]. This class of medications has been shown to be highly effective antidepressant agents however treatment resistant cases of depression are still experienced [363, 364].

Various SSRIs have been shown to alter parameters associated with neuroinflammation. The SSRIs sertraline and citalopram have been shown to cause a decrease in serum levels of TNF- α both *in vivo* [360] and *in vitro* [353]. *In vitro* studies

have shown that the SSRI citalopram decreases the pro-inflammatory cytokines IL-1 β , IL-6, TNF- α release from monocytes and IL-2 and IFN- γ release from T lymphocytes when these cells are pre-incubated in the antidepressants for 24 h. Other effects of the SSRIs sertraline and fluoxetine decrease the production of IFN- γ and increased anti-inflammatory IL-10 production [353] indicating anti-inflammatory activity and improvement in the key biomarkers seen in depression. Fluvoxamine were found to decrease pro-inflammatory IL-6 production [359, 365] whereas conflicting data was observed with sertraline and fluoxetine. The SSRIs fluvoxamine and fluoxetine have opposing effects on NO, a general marker of inflammation, a decrease in NO and an increase in NO respectively [359].

SNRIs, as their name suggests, inhibit the re-uptake of both serotonin and noradrenaline from the synaptic cleft via the inhibition of SERT and NET [366]. The SNRI venlafaxine has been the widest studied compound in the class and has been shown to have a number of effects on inflammatory mediators. This drug has very little effects on TNF- α levels *in vivo* [360] but decreases pro-inflammatory IL-6 production [359, 365]. Additionally, venlafaxine has been shown to decrease the production of the IFN- γ another important pro-inflammatory mediator [365].

Reversible inhibitors of monoamine oxidase (RIMAs) reversibly inhibit monoamine oxidase-A (MAO-A) in the central nervous system, resulting in increased concentrations of noradrenaline, serotonin and dopamine [367]. The most common RIMA and the focus of studies is moclobemide. A limited number of studies have been undertaken to evaluate the effects of moclobemide on inflammatory mediators.

Moclobemide decreases the production of the pro-inflammatory IFN- γ and increases production of the anti-inflammatory IL-10 production [353], thereby potentially restoring the Th1/Th2 imbalance seen in neuroinflammatory conditions.

Agomelatine, a melatonergic antidepressant, attenuated LPS-induced inflammation *in vivo* through the prevention of nuclear translocation of NF- κ B, indicating an anti-inflammatory effect [368]. Additionally, chronic treatment with agomelatine was found to completely prevent the increase in IL-1 β and IL-6 protein levels associated with LPS stimulation [368]. This evidence suggests the antidepressant agomelatine may play a significant role in reducing inflammation in addition to its antidepressant effects.

Results of *in vivo* studies are varied and conflicting in their results. With this in mind, it appears *in vitro* models used to evaluate the effects of antidepressants on cytokine production appear to give the most consistent results however this poses a limitation with regards to interpreting the results. Studies show that changes to inflammatory mediators occur across all classes of antidepressants, indicating that this effect may play an important role in their mechanisms of action. These results support the neuroinflammatory hypotheses of depression and the role of neuroinflammation in the pathophysiology of depression. However, due to the limited number of studies and the lack of consistency of the observations outlined, further studies are required to fully assess the effects of antidepressant medications on cytokine production.

Numerous current antidepressant therapies have been shown to modulate various points in the KP. The two SSRIs, citalopram and fluoxetine and two TCAs, imipramine and amitriptyline increased the concentration of the neuroprotective Trp catabolite, KA and decreased the concentration of the neurotoxic catabolite 3-HK in glial cell cultures in a time-dependent manner. The TCAs showed a higher increase in KA than the SSRIs but both the classes of antidepressants had relatively the same effect in decreasing the levels of 3-HK [369].

Additionally, citalopram, fluoxetine and amitriptyline, at a concentration of 10 μ M, were found to strongly induce the expression of both *Kat1* and *Kat2*, enzymes responsible for the biosynthesis of KA, with imipramine being only a weak inducer of those enzymes. Imipramine was shown to have greater effect on inhibiting *Kmo* than the other antidepressants in a time-dependent manner. This data indicates that these antidepressants alter the balance between the “neurotoxic and neuroprotective arms” of the KP and possibly restoring the balance. Further, this evidence strengthens the hypothesis that the KP plays a role in the pathophysiology of depression [369].

The effects of amitriptyline, mianserin and lithium on plasma KYN levels have been assessed. Patients receiving amitriptyline were placed on a drug-free regimen for 7 to 10 days before receiving amitriptyline 75 mg daily initially for 3 days and then increased to 75 mg twice a day for the duration of the study (six weeks). The patients receiving lithium and mianserin were placed on a drug-free regimen for 14 days preceding dosing of their assigned antidepressant – 20 mg mianserin three times a day or a lithium dose to maintain the patients steady-state plasma levels between 0.8-1.2

mmol/L and was continued for the duration of the study (6 weeks) [370]. No significant differences between the KYN levels of control patients and those of acutely depressed patients were observed. It was however found that mianserin and lithium significantly increased plasma KYN levels and although amitriptyline increased KYN levels, this was not a statistically significant increase [370]. A major limitation to this study is that only KYN levels were measured. This however may not give a truly accurate picture of the situation as changes in the concentration of the neuroprotective compound KA and the subsequent ratios were not assessed. As outlined earlier, antidepressant medications have different effects on the enzymes in the KP [369] and therefore differing effects on the concentration of KYN. By assessing the ratios of KM in both plasma and cerebrospinal fluid (CSF), a more accurate result may be obtained.

Agomelatine has been shown to alter KP metabolites and potentially subsequent KMs. Chronic administration of agomelatine resulted in the prevention of upregulation of KMO when exposed to LPS stimulus, a bacterial toxin routinely used in *in vitro* neuroinflammation studies. Additionally, pre-treatment with agomelatine and subsequently challenged with LPS, resulted in the upregulation of KAT II mRNA levels [368] indicating that a shift in the KM balance, towards the “neuroprotective arm” may occur in patients chronically undergoing agomelatine therapy and receiving pro-inflammatory stimulus.

The observations presented above highlight the need for further studies into the effects of antidepressant medications on the KP. Initial results indicate that the KP

plays an important role in the pathophysiology of depression and provides further support to the neuroinflammatory hypotheses of depression.

A number of studies have been undertaken to assess the antioxidant effects of antidepressants both *in vitro* and *in vivo* across the various antidepressant classes. *In vitro* studies have evaluated a wide selection of antidepressant medications in a number of cell lines. The TCA amitriptyline and the SSRI fluoxetine, protect against H₂O₂ mediated cell death in PC12 cells [371]. Other TCAs, imipramine and desimipramine, were shown to increase mRNA of a number of antioxidant defenses including superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione reductase (GR), conferring antioxidant effects in human monocytic U937 cells [372].

In addition to *in vitro* studies, the acute and chronic antioxidant effects has been evaluated in *in vivo* animal studies. The TCA imipramine and the SNRI venlafaxine were both shown to decrease CNS nitrate/nitrite levels after acute treatment [373, 374] however another TCA amitriptyline did not alter nitrate/nitrite levels in a separate acute study [54]. A number of studies have evaluated the effects of antidepressant medications on lipid peroxidation. The SSRIs, escitalopram and fluoxetine decreased lipid peroxidation in *in vivo* rat studies [375, 376] as did the TCA imipramine [246] and the SNRI venlafaxine [374].

In vivo studies have evaluated the effects of various antidepressant medications on antioxidant defenses. The SSRI fluoxetine increased SOD, an important antioxidant

defense, in hippocampal neuronal cells in an acute treatment model [247]. Similar results were observed in whole brain isolates in a chronic restraint stress model [377] however, opposite results were seen in a second chronic model [378]. Differences between the studies included different doses of fluoxetine being administered in addition to different species of rats being used; indicating that fluoxetine's effects on SOD may in fact be concentration-dependent. The SNRI venlafaxine and TCA imipramine were both also found to restore SOD activity in a chronic stress model of depression [377]. In addition to restoring SOD activity, these compounds also restored the activity of other important antioxidant defenses, catalase (CAT), GST and GR in the same model [377].

The studies above highlight the antioxidant and positive effects of current antidepressant therapies on antioxidant defense mechanisms. This provides further support to the role of oxidative stress in the pathophysiology of depression and the validity of the neuroinflammatory hypotheses of depression.

4.1.6 Summary

Numerous models of microglial- and macrophage-like and neuronal cells exist as an alternative to using human primary cell lines. A model of macrophage and microglial-like cells was identified that displays high levels of similarity to primary human microglia and macrophages. Furthermore, a model of neuronal cells has also been identified that displays high levels of phenotypic similarity to human primary neurons.

As outlined earlier, the KP is an important feature in depression and neuroinflammation which is under the control of inflammatory mediators. Furthermore, extensive studies have been undertaken evaluating the physiological effects of type I and type II IFNs however only a limited numbers of studies have evaluated the physiological effects of type III IFNs. Numerous studies have shown that type I and type II IFNs modulate various parameters associated with the neuroinflammatory theories of depression including neuroinflammation and Trp catabolism. Conversely, there have been no studies undertaken assessing the effects of type III IFNs. Given the similar effects that type III IFNs have to type I IFNs, further studies are warranted.

Currently marketed antidepressant medications have been shown to alter numerous inflammatory markers associated with depression. A number of these drugs will serve as controls in the current study.

The main aim of the current study is to develop surrogate models of both microglial and neuronal cells. Furthermore, this study aimed to further develop the microglial model into an activated phenotype using IFN treatment.

4.2 Hypothesis, aims and objectives

4.2.1 Hypothesis

The primary hypothesis of this study is that PMA differentiated THP-1 human monocytic cells stimulated with IFN will provide an adequate surrogate activated microglial model to assess the effects of compounds on the KP and markers of inflammation.

The secondary hypothesis of this study is that differentiated SH-SY5Y neuroblastoma cells will provide a better model to assess the neurotoxicity of KMs, antidepressant medications and cytokines.

4.2.2 Aims and objectives

The primary aim was to develop and optimize an *in vitro* model of IFN-induced neuroinflammation using THP-1 human monocytic cells.

Objectives

The primary aim was achieved through the following objectives:

- Assessing the effects of multiple IFNs at numerous concentrations in undifferentiated and PMA differentiated THP-1 human monocytic cells. Parameters assessed included viability, free radical production, IL-6, IL-1 β , TNF- α , IDO activity, PGE₂; and

- Assessing the effects of antidepressants on viability, free radical production, IL-6, IL-1 β , TNF- α , IDO activity, PGE₂.

The secondary aim was to develop and validate an *in vitro* model of neurotoxicity using SH-SY5Y neuroblastoma cells.

Objectives

The secondary aim was achieved through the following objectives:

- The optimization and validation of a dbcAMP differentiation method assessing viability, neurite outgrowth, the activity of KP enzymes and NA and 5-HT synthesis; and
- The evaluation of dbcAMP differentiated in comparison to undifferentiated SH-SY5Y neuroblastoma cells utilising KMs, cytokines and antidepressants using parameters of viability, free radical production, apoptosis and necrosis.

4.2.3 Expected outcomes

It was expected that this study would provide an alternative model to primary human cell cultures of microglial- or macrophage-like cells to assess the effects of compounds on the various parameters associated with depression, including, the effects on pro-inflammatory cytokine concentrations and KP enzymes and compounds. Furthermore, it was expected that a model of neuronal-like cells would be developed to assess the neurotoxic effects of compounds.

4.3 Methods

4.3.1 The effects of interferons on markers of inflammation in undifferentiated and differentiated THP-1 human monocytic cells

Routine cell culture

Human monocyte cells (THP-1) were obtained from Sigma-Aldrich (Sigma, St Louis, MO). Cells were grown and maintained at 37°C and 5% CO₂ in complete RPMI-1640 medium (Gibco, Mulgrave, Victoria, Australia) containing 4.5 g/L of glucose, 2.383 g/L of HEPES buffer, L-glutamine, 1.5 g/L of sodium bicarbonate, 110 mg/L sodium pyruvate, 10% foetal bovine serum (Bovogen, Australia), 0.05 mM of 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA) and 0.1 mg/mL of gentamicin (Gibco, Mulgrave, Victoria, Australia).

Preparation of working solutions

Stock solutions of all compounds of interest including caffeine (PCCA, Australia), CA (Sigma-Aldrich, St Louis, MO, USA), CGA (Sigma-Aldrich, St Louis, MO, USA), FA (Sigma-Aldrich, St Louis, MO, USA), PA (Sigma-Aldrich, St Louis, MO, USA), trigonelline (Sigma-Aldrich, St Louis, MO, USA) and salsolinol (Sigma-Aldrich, St Louis, MO, USA) were made up to a concentration of 10 mM in sterile PBS and were further diluted in media to produce the working solutions. IFN- γ (ORF genetics, Iceland) and IFN- α_{2a} (PeproTech, NJ, USA) were made up to a concentration of 100 μ g/mL in PBS containing 0.1% BSA (Sigma-Aldrich, St Louis, MO, USA) and were further diluted in media to produce the working solutions. PMA (Sigma-Aldrich, St Louis, MO, USA) was made up in DMSO to a concentration of 2 mM and was

further diluted in media to produce the working solutions. All stock solutions were stored at -20°C and working solutions prepared fresh on the day of the experiment.

Differentiation of THP-1 cells

THP-1 human monocytic cells were differentiated according to an unpublished method [379]. Briefly, THP-1 cells were plated at a density of 3×10^6 trypan blue excluding cells per mL in either 24 or 96 well plates and left for 24 h. PMA to a final concentration of 40 nM was added to each well and the cells left to differentiate for 5 days. After 5 days, the media was replaced and cells were ready for further treatments.

Viability

Resazurin proliferation assay

Reduction of the redox dye resazurin to resorufin was used to measure the proliferation of cell cultures [380, 381].

Briefly, human monocyte (THP-1) cells were seeded at 3×10^5 of trypan blue excluding cells/mL in 24 or 96-well microtitre plates and used either as is or after PMA differentiation. After 24 h, when used as undifferentiated cells, or after the differentiation treatment protocol as described in section 4.3.1, cells were treated with IFN stimulus for 24 h. Following the appropriate incubation, 22 μ L or 55 μ L of a 440 μ M solution of resazurin (Sigma-Aldrich, St Louis) were added to each well of a 96 or 24 well plate respectively. Cultures were then incubated for a further 4 h and

subsequently the reduction of resazurin to resorufin will be determined using fluorescence (excitation 530 nm; emission 590 nm) using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Free radical formation

2',7'-dichlorofluorescein assay

2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) is a non-fluorescent chemical used as an indicator for ROS in cells. H₂DCFDA is permeable to the cell where, upon cleavage of the acetate groups by intracellular esterases and oxidation, is then converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) [382]. DCFH-DA was used to measure global ROS production as previously described [382]. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier. Following incubation for 24 h, medium above the cells was replaced with serum-free medium containing DCFH-DA (10 μ M) for 30 min. Cells were then washed twice with PBS and fluorescence due to the oxidized dye (excitation 485 nm; emission 535 nm) was measured using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

IDO activity

IDO activity was measured using the Trp to KYN ratio in spent cell culture supernatants. This was achieved via the quantification of Trp and KYN using the HPLC methods outlined in section 2.3.8. Sample preparation was carried out as

outlined below. Method validation was undertaken according to the methods outlined in section 2.3.8.

Tryptophan

Sample preparation to isolate tryptophan from spiked plasma and cell culture medium was carried out using a method previously described by Uhe *et al* [383]. Briefly, 100 μ L of sample was mixed with 400 μ L of an acetonitrile/2-mercaptoethanol (10 mL:20 μ L) mixture, vortexed and centrifuged at 12 000 x g at 4°C for 10 minutes. The supernatant was then analysed using the HPLC method outlined above.

Kynurenine

Sample preparation to isolate KYN from spiked plasma and cell culture medium was carried out using a method previously described by Funeshima *et al* [384]. Briefly, 50 μ L of sample was diluted with 50 μ L of phosphate buffered saline. Subsequently, 20 μ L of a 10% 5-sulfosalicylic acid solution (in water) was added to each sample and the samples vortexed and chilled on ice for 30 minutes to ensure full precipitation of proteins. Samples were then centrifuged at 12000 x g for 30 minutes at room temperature and the supernatant was then analysed using the HPLC method outlined above.

Quantification of TNF- α

The TNF- α Human ELISA development kit 900-K25 (PeproTech, Rocky Hill, NJ, USA) was used in this study to quantify TNF- α . This kit contained capture antibody, detection antibody, human TNF- α standard and avidin-HRP conjugate. The ELISA buffer kit 900-K00 (PeproTech, Rocky Hill, NJ, USA) was used in conjunction with this ELISA. This kit contained PBS, wash buffer, diluent, ABTS liquid substrate and Corning ELISA microplates #3590 (Corning, NY, USA).

To determine the effects of 24 h pre-exposure of IFNs on TNF- α concentrations in undifferentiated and differentiated THP-1 monocytic cells, they were treated according to the protocols outlined in section 4.3.1 above. All steps were performed according to the manufacturer's instructions. Briefly, all buffers, samples and assay specific reagents were prepared according to the manufacturer's instructions. EIA plates were prepared by pipetting 100 μ L of the diluted capture antibody into each well of a 96 well EIA plate (Corning, NY, USA) and the plates incubated at room temperature overnight. After the appropriate incubation, the liquid was aspirated and 300 μ L of blocking buffer was added to each well and the plates incubated for a further 4 h. Liquid was then again aspirated and each well washed with 300 μ L of wash buffer three times. Subsequently, 100 μ L of either standard or sample was added to each well of the EIA plate and incubated at room temperature for 2 h on a plate shaker. After the appropriate incubation the liquid was aspirated and each well washed four times with wash buffer. Next, 100 μ L of the diluted detection antibody was added to the appropriate wells of the plate and the plates incubated at room temperature on a plate shaker for 2 h. The liquid was again aspirated and each well washed with 300 μ L of wash buffer four times. Next, 100 μ L of avidin peroxidase solution was added to each

well of the plate and the plates incubated at room temperature on a plate shaker for 45 min. The liquid was again aspirated and each well washed with 300 μ L of wash buffer four times and 100 μ L of ABTS substrate solution was added to each well and the plate was then incubated in the dark for approximately 15 min at room temperature. The absorbance of each well was then read at 405 nm using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Quantification of interleukin-6

The IL-6 Human ELISA development kit 900-K16 (PeproTech, Rocky Hill, NJ, USA) was used in this study to quantify IL-6. This kit contained capture antibody, detection antibody, human IL-6 standard and avidin-HRP conjugate. The ELISA buffer kit 900-K00 (PeproTech, Rocky Hill, NJ, USA) was used in conjunction with this ELISA. This kit contained PBS, wash buffer, diluent, ABTS liquid substrate and Corning ELISA microplates #3590 (Corning, NY, USA).

To determine the effects of 24 h pre-exposure of IFNs on IL-6 concentrations in undifferentiated and differentiated THP-1 monocytic cells, they were treated according to the protocols outlined in section 4.3.1 above. All steps were performed according to the manufacturer's instructions. Briefly, all buffers, samples and assay specific reagents were prepared according to the manufacturer's instructions. EIA plates were prepared by pipetting 100 μ L of the diluted capture antibody into each well of a 96 well EIA plate (Corning, NY, USA) and the EIA plates incubating at room temperature overnight. After the appropriate incubation, the liquid was aspirated and 300 μ L of blocking buffer was added to each well and the plates incubated for a further 4 h. Liquid was then again

aspirated and each well washed with 300 μ L of wash buffer three times. Subsequently, 100 μ L of either standard or sample was added to each well of the EIA plate and the plates incubated at room temperature for 2 h on a plate shaker. After the appropriate incubation the liquid was aspirated and each well washed four times with wash buffer. Next, 100 μ L of the diluted detection antibody was added to the appropriate wells of the plate and the plates incubated at room temperature on a plate shaker for 2 h. The liquid was again aspirated and each well washed with 300 μ L of wash buffer four times. Next, 100 μ L of avidin peroxidase solution was added to each well of the plate and incubated at room temperature on a plate shaker for 45 min. The liquid was again aspirated and each well washed with 300 μ L of wash buffer four times and 100 μ L of ABTS substrate solution was added to each well and the plate was then incubated in the dark for approximately 15 min at room temperature. The absorbance of each well was then read at 405 nm using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Quantification of interleukin 1- β

The IL-1 β Human ELISA Set ab47351 (Abcam, Melbourne, Australia) was used in this study to quantify IL-1 β . This kit contained IL-1 β capture antibody, detection biotinylated anti-IL-1 β antibody, IL-1 β standard, streptavidin-HRP and ready-to-use TMB. Other materials required and used included Costar 3590 96 well EIA/RIA plate (Corning, NY, USA), reconstitution and coating buffer (1X PBS), wash buffer (1X PBS + 0.05% Tween20), blocking buffer (1X PBS + 5% BSA), HRP diluent buffer (1X PBS + 1% BSA + 0.1% Tween20), stop reagent (1 M sulfuric acid (Ajax Finechem, Brisbane, Australia).

To determine the effects of 24 h exposure of IFNs on IL-1 β concentrations in undifferentiated and differentiated THP-1 monocytic cells, the cells were treated according to the protocols outlined in section 4.3.1 above. All steps were performed according to the manufacturer's instructions. Briefly, all buffers, samples and assay specific reagents were prepared according to the manufacturer's instructions. EIA plates were prepared by pipetting 100 μ L of the diluted capture antibody into each well of a 96 well EIA plate (Corning, NY, USA) and incubated at 4°C overnight. After the appropriate incubation, the liquid was aspirated and 250 μ L of blocking buffer was added to each well and the plates incubated for a further 4 h. Liquid was then again aspirated and each well washed with 400 μ L of wash buffer three times. Subsequently, 100 μ L of either standard or sample and 50 μ L of the diluted detection antibody was added to the appropriate wells of the plate and the plates incubated at room temperature on a plate shaker for 3 h. The liquid was again aspirated and each well washed with 400 μ L of wash buffer three times. Next, 100 μ L of Streptavidin-HRP solution was added to each well of the plate and the plates incubated at room temperature on a plate shaker for 30 min. The liquid was again aspirated and each well washed with 400 μ L of wash buffer three times and 100 μ L of ready-to-use TMB substrate solution was added to each well and the plates were then incubated in the dark for 5 min at room temperature. 100 μ L of stop reagent was then added to each well and the absorbance of each well was read at 450 nm using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Quantification of PGE₂

PGE₂ concentrations were quantified using the protocol outlined in section 2.1.3. Briefly, the PGE₂ Express ELISA kit (Cayman Chemical, Michigan, USA) was used to

quantify PGE₂ concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

4.3.2 The effect of antidepressant medications on markers of inflammation in differentiated THP-1 human monocytic cells

Routine cell culture

THP-1 human monocytic cells were grown and maintained according to the protocol outlined in section 4.3.4.

Differentiation of THP-1 monocytic cells

The differentiation of THP-1 cells was undertaken according to the procedures outlined in section 4.3.1. Briefly, THP-1 cells were seeded at 3×10^5 cells/mL of trypan blue excluding cells in 24 or 96-well microtitre plates. 50 ng/mL of PMA was then added to each well and left for 5 days for the differentiation process to occur. The THP-1 cells were then used for the assays described below.

Preparation of working solutions

The antidepressants used in this study, amitriptyline, fluoxetine and venlafaxine were all made up to a concentration of 10 mM in sterile PBS and stored at -20°C. Working solutions were made up fresh on the day of the experiment using media. Final concentrations of the antidepressants tested were 10 µM, 100 µM and 1000 µM.

Resazurin

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.1. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.1 and 22 μ L or 55 μ L of a 440 μ M of resazurin was added to each well of a 96 or 24 well plate respectively and incubated for 4 h. The reduction of resazurin to resorufin was determined by fluorescence.

2',7'-dichlorofluorescein assay

The DCFH-DA assay was performed according to the methods outlined in section 4.3.1. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.1. The spent media was then replaced serum free media containing 10 μ M of DCFH-DA and the plates incubated for 30 min and the fluorescence subsequently read.

IDO activity

IDO activity was assessed using the HPLC protocol outlined in section 2.3.8. Briefly, the concentrations of Trp and KYN were quantified in spent supernatant, using HPLC, and the ratio of KYN to Trp calculated and used as a measure of IDO activity.

Quantification of TNF- α

TNF- α concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the TNF- α human ELISA kit (PeproTech, Rocky Hill, NJ, USA) was

used to quantify TNF- α concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

Quantification of IL-6

IL-6 concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the IL-6 human ELISA kit (PeproTech, Rocky Hill, NJ, USA) was used to quantify IL-6 concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

Quantification of IL-1 β

IL-1 β concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the IL-1 β human ELISA kit (Abcam, Melbourne, Australia) was used to quantify IL-1 β concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

4.3.3 Differentiation of SH-SY5Y neuroblastoma cells

Routine cell culture

Human neuroblastoma cells (SH-SY5Y) were obtained from Sigma-Aldrich (St Louis, MO, USA). Cells were grown and maintained at 37°C and 5% CO₂ in complete Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Mulgrave, Victoria, Australia) containing 4.5 g/L of glucose, L-glutamine, 110 mg/L of sodium pyruvate, phenol red, 10% foetal bovine serum (Bovogen, Australia) and 0.1 mg/1mL of gentamicin (Gibco, Mulgrave, Victoria, Australia).

Preparation of stock solutions

Stock solutions of dibutyryl cyclic AMP (Enzo, NY, USA) were made up to a concentration of 10 mM in sterile PBS and were further diluted in media to produce the working solutions. All stock solutions were stored at -20°C and working solutions prepared fresh on the day of the experiment. The final concentrations of dbcAMP used were 10 µM, 100 µM and 1000 µM.

Differentiation protocol

A previously described method [284] was optimized for this study. Briefly, SH-SY5Y cells were plated at a density of 10^5 cells/mL in T₁₇₅ flasks, 24 or 96 well plates and incubated for 24 h. A range of concentrations (0.1 to 1 mM) of dbcAMP were used to treat cells for 2 days and a complete media change containing the required concentration was undertaken and the cells incubated for a further 24 h. Cells were grown and maintained at 37°C and 5% CO₂ in complete Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Mulgrave, Victoria, Australia) containing 4.5 g/L of glucose, L-glutamine, 110 mg/L of sodium pyruvate, phenol red, 10% foetal bovine serum (Bovogen, Australia) and 0.1 mg/1mL of gentamicin (Gibco, Mulgrave, Victoria, Australia) for the duration of the differentiation process.

Crystal violet staining

Cells were differentiated according to the protocol outlined above and crystal violet staining undertaken. Briefly, cells were placed on ice and washed 2X with cold PBS. Cells were then fixed on ice for 10 minutes with ice-cold methanol. Methanol was then aspirated and the cells washed 3 times with water. The cells were allowed to

dry at room temperature overnight and visualised using Leica DM2000 microscope (Leica Microsystems, Victoria, Australia).

Neurite outgrowth quantification

The Molecular Probes Neurite Outgrowth Kit (Life Technologies, Scoresby, Australia) was used to quantify the relative neurite projections after exposure to varying concentrations of dbcAMP. This kit does this via a dual-colour fluorescent stain that allows the relative quantification of neurite projections.

This kit contained Cell Membrane Stain (1000X), Cell Viability Indicator (1000X) and Background Suppression dye (100X). Additional materials required but not supplied included PBS (Sigma-Aldrich, St Louis, MO, USA).

Quantification of neurite outgrowths was undertaken using the Molecular Probes Neurite Outgrowth Kit (Life Technologies, Scoresby, Australia) and was performed according to the manufacturer's instructions. Briefly, all buffers and solutions were prepared prior to the experiment according to the manufacturer's instructions. Subsequently, the spent media was aspirated from each well and each well washed three times with warm PBS. Next, 400 μ L of the prepared working Stain solution was added to each well and the plates were incubated at 37°C for 20 minutes. The solution was then aspirated and each well washed three times with warm PBS. 400 μ L of working background suppression solution was then added to each well and visualized on an Olympus IX53 fluorescence microscope using DAPI and Cy1 filters.

Resazurin viability assay

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.1 with minor modifications. Briefly, cells were plated and treated with dbcAMP and the plates incubated for 24 h. The media was then replaced with fresh media containing 44 μ M of resazurin was added to each well and the plated incubated for 3 h. The reduction of resazurin to resorufin was determined by fluorescence.

Quantification of noradrenaline

Quantification of NA was undertaken using a Norepinephrine ELISA kit (Abnova, Taipei, Taiwan) and was performed according to the manufacturer's instructions.

This assay is an enzyme immunoassay used to quantify NA. This assay extracts NA from the biological matrix using a cis-diol-specific affinity gel. NA is subsequently acylated and enzymatically derivatized. The competitive ELISA kit uses the microtitre plate format, whereby the antigen is bound to the solid phase. Subsequently, the derivatized standards, controls and samples and the solid phase bound analytes compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit immunoglobulin G (IgG)-peroxidase conjugate using tetramethylbenzidine (TMB) as a substrate. The reaction is monitored at 450 nm. Quantification of unknown samples is achieved by comparing their absorbance with a standard curve.

The Norepinephrine ELISA kit (Abnova, Taipei, Taiwan) was used to quantify the production of NA pre- and post-differentiation. This kit contained wash buffer concentrate, enzyme conjugate (anti-rabbit IgG conjugated with peroxidase), substrate (tetramethylbenzidine (TMB)), stop solution (0.2 M sulfuric acid (H₂SO₄)), NA-normetanephrine microtitre strips, NA antiserum (from rabbit), adjustment buffer, standards A to F, acylation buffer, acylation reagent, assay buffer, coenzyme (S-adenosyl-L-methionine), enzyme (catechol-O-methyl transferase (COMT)), extraction buffer, extraction plate, hydrochloric acid and controls 1 and 2. The assay was performed as per manufacturer's instructions.

Briefly, sample preparation, extraction and acylation was performed according to manufacturer's instructions. 25 µL of the enzyme solution was pipetted into all wells of the NA microtitre strips followed by 20 µL of the extracted standards, controls and samples into the appropriate wells and the plate incubated for 30 minutes at room temperature on a shaker. Next 50 µL of the NA antiserum was pipetted into all wells and the plate incubated for 2 hours at room temperature on a shaker. The contents of the wells were then be aspirated and washed before 100 µL of the substrate was then added to all the wells and the plate incubated for 25 minutes at room temperature on a shaker. 100 µL of the stop solution was added to each well and the absorbance read at 450 nm with a reference wavelength of between 620 nm and 650 nm within 10 minutes. Results were then calculated from the calibration curve and are represented as nanograms per millilitre.

Quantification of serotonin

5-HT was quantified using the HPLC method below utilising the native fluorescence of this analyte.

Apparatus

The HPLC system used was the system outlined in section 2.3.8. The column used was a GraceSmart C18 (5 μ m, 250 x 4.6 mm) reverse phase column (Grace Discovery Science, Columbia, MD, USA) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Briefly, 50 μ L of cell culture supernatant was mixed with 10 μ L of 10% 5-sulfosalicylic acid (Sigma-Aldrich, St Louis, MO, USA) solution and the proteins allowed to precipitate on ice for 30 minutes [383]. Subsequently, samples were then centrifuged at 12000 x g for 30 minutes at 4°C. Samples were then ready for analysis using the method outlined below.

Chromatographic conditions

The HPLC method used to quantify 5-HT is as follows. Mobile phase A consisted of a 0.1% GAC solution in water and mobile phase B consisted of 100% ACN. A gradient method over 20 minutes at 30°C, with a flow rate of 1 mL/min was employed for the elution of serotonin. The optimal gradient conditions were found to

be 0.01 to 10 minutes from 1% solvent B to 90% solvent B, 10.01 to 17 minutes 90% solvent B to 1% solvent B and then 17.01 to 20 minutes at 1% to re-equilibrate the system. The fluorescence of serotonin was monitored at an excitation of 280 nm and an emission of 320 nm.

Kynurenine pathway enzyme activities

The activities of enzymes in the KP were assessed by quantifying the various KMs and subsequently comparing the ratio between the various treatments.

Tryptophan

Apparatus

The HPLC system used was that outlined in section 2.3.8. The column used was a Phenomenex Gemini-NX C18 (3 μ m, 150 x 4.6 mm) reverse phase column fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Sample preparation to isolate Trp from spent cell culture supernatant was carried out using a method previously described by Uhe *et al* [383]. Briefly, 100 μ L of sample was mixed with 400 μ L of an acetonitrile/2-mercaptoethanol (10 mL:20 μ L) mixture, vortexed and centrifuged at 12 000 x g at 4°C for 10 minutes. The supernatant was then analysed using the HPLC method outlined below.

Chromatographic conditions

Mobile phase A consisted of a 0.1% glacial acetic acid (GAC) solution in water and mobile phase B consisted of 100% acetonitrile. An isocratic method over 15 minutes at 30°C, with a flow rate of 1 mL/min was employed for the elution of Trp. The optimal conditions were found to be 10% solvent B. The fluorescence of Trp was monitored at excitation 285 nm and emission 360 nm.

Kynurenine and 3-hydroxykynurenine

Apparatus

The system outlined above was employed for the analysis of KYN and 3-HK with the exception of the column. The column used was a Phenomenex Gemini-NX C18 (5 μ m, 250 x 4.6 mm) reverse phase column fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Sample preparation was undertaken according to a previously described method [385]. Briefly, 50 μ L of cell culture supernatant was mixed with 10 μ L of 10% 5-sulfosalicylic acid (Sigma-Aldrich, St Louis, MO, USA) solution and the proteins allowed to precipitate on ice for 30 minutes. Subsequently, samples were then centrifuged at 12000 x g for 10 minutes at 4°C. Samples were then ready for analysis using the method outlined below.

Chromatographic conditions

Mobile phase A consisted of a 0.1% GAC solution in water and mobile phase B consisted of 100% acetonitrile. An isocratic method over 15 minutes at 30°C, with a flow rate of 1 mL/min was employed for the elution of KYN. The optimal conditions were found to be 10% solvent B. The UV absorbance of KYN was monitored at 365 nm.

Kynurenic acid

Apparatus

The system outlined above was employed for the analysis of KA with the exception of the column. The column to be used will be a GraceSmart RP 18 (5 µm, 250 x 4.6 mm) reverse phase column (Grace Davison Discovery Sciences, Melbourne, Australia) fitted with a Phenomenex SecurityGuard guard cartridge (Phenomenex, Lane Cove, Australia).

Sample preparation

Briefly, 50 µL of cell culture supernatant was mixed with 10 µL of 10% 5-SSA (Sigma-Aldrich, St Louis, MO, USA) solution and the proteins allowed to precipitate on ice for 30 min. Subsequently, samples were then centrifuged at 12000 x g for 10 min at 4°C. Samples were then ready for analysis using the method outlined below.

Chromatographic conditions

The HPLC method used to analyse KA employed an isocratic method run over 10 min using 0.1% GAC (solvent A) and acetonitrile (solvent B) (90:10 % v/v) and 10 µL injections of sample was employed to quantify KA. The fluorescence of KA was monitored at excitation 344 nm and emission 398 nm and quantification was made after comparison to a 10 point standard curve.

Method validation

The HPLC methods were validated according to the methods outlined in section 2.3.8 above.

4.3.4 Toxicity of kynurenine metabolites on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Routine cell culture

Cells were maintained according to the protocol outlined in section 4.3.3 above.

Differentiation of SH-SY5Y neuroblastoma cells

Differentiation of SH-SY5Y neuroblastoma cells was undertaken as previously described in section 4.3.3 above. Briefly, cells were plated at a density of 2×10^5 trypan blue excluding cells/mL in 24 well plates and left to attach. After 24 h, the media was replaced with fresh media containing 100 µM of dbcAMP. A subsequent media change,

with media containing 100 μM of dbcAMP, was done 48 h later and the cells left to differentiate for a further 24 h, with a total differentiation time of 72 h.

Preparation of stock solutions

All of the KMs, Trp, KYN, 3-HK, KA, 3-HA and QA and the positive control salsolinol (Sigma-Aldrich, St Louis, MO, USA) were all prepared at 10 mM solutions in PBS and further diluted in media to prepare the working solutions. All stock solutions were stored at -20°C and working solutions made up fresh on the day of the experiment in media. The final concentrations of the compounds used in the experiments was 1 μM , 10 μM , 100 μM and 1000 μM .

Viability

Resazurin

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.1 with minor modifications. Briefly, cells were plated and treated with the appropriate KM according to the methods described in section 4.3.4 and the plates incubated for 24 h or 72 h. The media was then replaced with fresh media containing 44 μM of resazurin was added to each well and incubated for 3 h. The reduction of resazurin to resorufin was determined by fluorescence.

LDH

Lactate dehydrogenase (LDH) activity was used as an indicator of cell membrane integrity and a measure of cytotoxicity [386, 387]. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier. Following incubation for a further 24 h, LDH activity was determined using a LDH cytotoxicity kit (Cayman Chemical, Michigan, USA). All steps were performed according to the manufacturer's instructions. Absorbance was read using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland) at absorbance 490 nm.

Free radical production

DCFH-DA assay

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a non-fluorescent chemical used as an indicator for reactive oxygen species (ROS) in cells. H_2DCFDA is permeable to the cell where, upon cleavage of the acetate groups by intracellular esterases and oxidation, is then converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). DCFH-DA was used to measure global ROS production as previously described [382]. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier. Following incubation for 24 h, medium above the cells was replaced with serum-free medium containing DCFH-DA (10 μ M) for 30 min. Cells were then washed twice with PBS and fluorescence due to the oxidized dye (excitation 485 nm; emission 535 nm) was measured using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

DHR 123 assay

Dihydrorhodamine 123 (DHR 123) is a non-fluorescent probe that is freely permeable cells and when exposed to free radicals, is oxidised to the fluorescent rhodamine 123 which was used to measure mitochondrial ROS/RNS formation. The DHR 123 assay was carried out according to previously described methods [382]. Briefly, cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier. Following incubation for 24 h, medium above the cells was replaced with serum-free medium containing DHR 123 (25 μ M) for 30 minutes. Cells were then washed twice with PBS and fluorescence due to the oxidized dye (excitation 485 nm; emission 535 nm) was measured using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Apoptosis

Annexin V activation

An early marker of apoptosis is redistribution of phospholipids, such as phosphatidylserine and phosphatidylethanolamine, from the inner to the outer layer of the membrane bilayer, resulting in them being exposed to the cell surface [388, 389]. This process allows detection of the exposed phospholipids due to their high affinity for annexin V, a phospholipid binding protein. This assay uses a FITC-conjugated annexin V (a fluorochrome labelled annexin V) as a probe for phosphatidylserine on the outer membrane of the cell. In addition, propidium iodide solution was used as a marker of cell death.

The Annexin V FITC Assay Kit purchased from Sapphire Bioscience (Cayman Chemical, Michigan, USA) consisting of cell-based Annexin V FITC, cell-based assay Annexin V binding buffer (10X) and cell-based propidium iodide solution was used as a marker of apoptosis in this study.

Annexin V activity was determined using an Annexin V FITC assay kit (Cayman Chemicals, Michigan, USA). All steps were performed according to the manufacturer's instructions. Briefly, cells were cultured in a black 96-well plate according to the experimental conditions outlined in section 4.3.4. Plates were then subsequently centrifuged at 400 x g using an Eppendorf 5810 R centrifuge and the resultant supernatant discarded. 100 µL of diluted assay buffer was added to the remaining cells in each well and the plate centrifuged at 400 x g using an Eppendorf 5810 R centrifuge and the supernatant aspirated. Cells were then incubated in 50 µL of Annexin V FITC/Propidium iodide staining solution at room temperature for 10 minutes and subsequently centrifuged at 400 x g for 5 min using an Eppendorf 5810 R centrifuge and the supernatant aspirated. Finally, 100 µL of diluted assay binding buffer was added to each well and the plate read using a Tecan Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland) at excitation and emission 485 nm and 535 nm respectively.

Necrosis

Propidium iodide fluorimetry

Propidium iodide solution was used as a marker of necrosis. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier in section 4.3.4. Following incubation for a further 24 h, necrosis was determined using an Annexin V FITC assay kit (Cayman Chemicals, Michigan, USA) performed according to the protocol outlined in section 4.3.4. Fluorescence was read using an Infinite M200 Pro microplate fluorometer (Tecan, Mannedorf, Switzerland) (excitation 560 nm; emission 595 nm).

4.3.5 Toxicity of cytokines on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Routine cell culture

SH-SY5Y human neuroblastoma cells were cultured as per the protocol described in section 4.3.3.

Preparation of stock solutions

All of the cytokine (IFN- γ , IFN- α_{2A} , PGE₂, IL-1 β , IL-6 and TNF- α) were all prepared at 100 μ g/mL solutions in PBS containing 0.1% BSA and further diluted in media to prepare the working solutions. All stock solutions were stored at -20°C and working solutions made up fresh on the day of the experiment in media. The final concentrations of IFNs used was 12.5 ng/mL, 25 ng/mL and 50 ng/mL and the final concentrations of the remaining compounds was 2 pM, 20 pM, 200 pM and 2000 pM.

Viability

Resazurin viability assay

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.5. The media was then replaced with fresh media containing 44 μM of resazurin and the plates incubated for 3 h. The reduction of resazurin to resorufin was determined by fluorescence.

LDH cytotoxicity assay

The LDH assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The LDH cytotoxicity kit (Cayman Chemical, Michigan, USA) was used to determine LDH leakage and was performed according to the manufacturer's instructions.

Free radical formation

DCFH-DA assay

The DCFH-DA assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.5. The spent media was then replaced with serum free media containing 10 μM of DCFH-DA, and the plates incubated for 30 min and the fluorescence subsequently read.

DHR 123 assay

The DHR 123 assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The spent media was then replaced serum free media containing 25 μ M of DHR 123, and the plates incubated for 30 min and the fluorescence subsequently read.

Apoptosis

Annexin

The Annexin V-FITC assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure annexin V activation and was performed according to the manufacturer's instructions.

Necrosis

Propidium iodide fluorimetry

The propidium iodide assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure propidium iodide fluorescence and was performed according to the manufacturer's instructions.

4.3.6 Toxicity of antidepressant medications on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Routine cell culture

SH-SY5Y human neuroblastoma cells were cultured as per the protocol described in section 4.3.3.

Viability

Resazurin

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.3. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The media was then replaced with fresh media containing 44 μ M of resazurin and the plates incubated for 3 h. The reduction of resazurin to resorufin was determined by fluorescence.

LDH

The LDH assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The LDH cytotoxicity kit (Cayman Chemical, Michigan, USA) was used to determine LDH leakage and was performed according to the manufacturer's instructions.

Free radical formation

DCFH-DA assay

The DCFH-DA assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The spent media was then replaced serum free media containing 10 μ M of DCFH-DA and the plates incubated for 30 min and the fluorescence subsequently read.

DHR 123 assay

The DHR 123 assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The spent media was then replaced serum free media containing 25 μ M of DHR 123, incubated for 30 min and the fluorescence subsequently read.

Apoptosis

Annexin

The Annexin V-FITC assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure annexin V activation and was performed according to the manufacturer's instructions.

Necrosis

Propidium iodide fluorimetry

The propidium iodide assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 4.3.4. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure propidium iodide fluorescence and was performed according to the manufacturer's instructions.

4.3.7 Statistical analysis

One-way ANOVA with Tukey's post hoc test (where significance was observed) were used in this study and were performed using GraphPad InStat version 3.06 (2003) with $P < 0.05$ (*,#), $P < 0.01$ (**,##), and $P < 0.001$ (***,###). All graphs were drawn using Graphpad Prism v6 (San Diego, USA).

4.4 Results

4.4.1 Development of a model of interferon-induced inflammation

Undifferentiated THP-1 human monocytic cells

Resazurin proliferation assay

Statistically significant increases in proliferation were seen in undifferentiated THP-1 human monocytic cells was observed after the treatment with various IFNs, that is, human IFN- α_{2A} , human IFN- β , human IFN- γ , murine IFN- γ and human IFN- $\lambda 3$. Human IFN- α_{2A} (12.5 ng/mL and 50 ng/mL), human IFN- β (12.5 ng/mL), human IFN- γ

(25 ng/mL and 50 ng/mL), murine IFN- γ (100 units/mL) and human IFN- λ 3 (50 ng/mL). These increases in viability were only modest in nature with a maximum increase of approximately 20% observed.

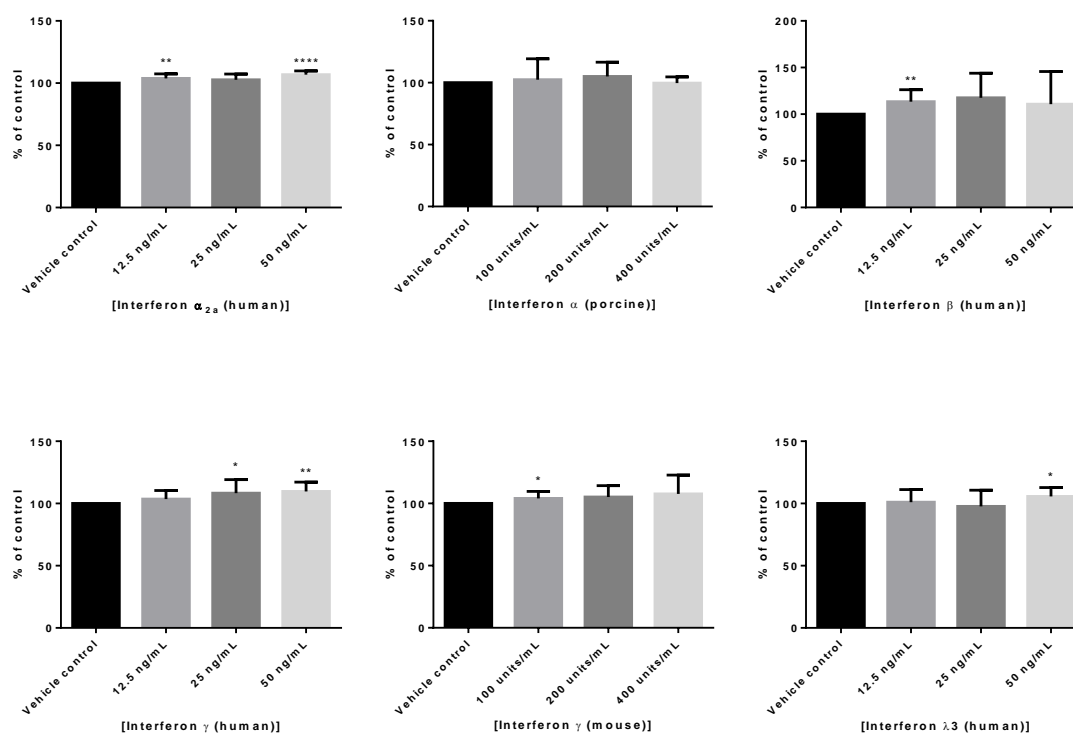


Figure 46 – The effects of the various interferons on the proliferation of undifferentiated THP-1 monocytic cells using the resazurin reduction viability assay (n = 9)

Quantification of IL-6 production

Statistically significant increases in IL-6 concentrations were seen after undifferentiated THP-1 cells were treated with IFN- λ 3 (50 ng/mL). Conversely, treatment with mouse IFN- γ (25 ng/mL) significantly decreased IL-6 release. Although

not statistically significant, treatment with human IFN- α_{2A} and human IFN- γ trend towards an increase in IL-6 concentrations and are approaching significance.

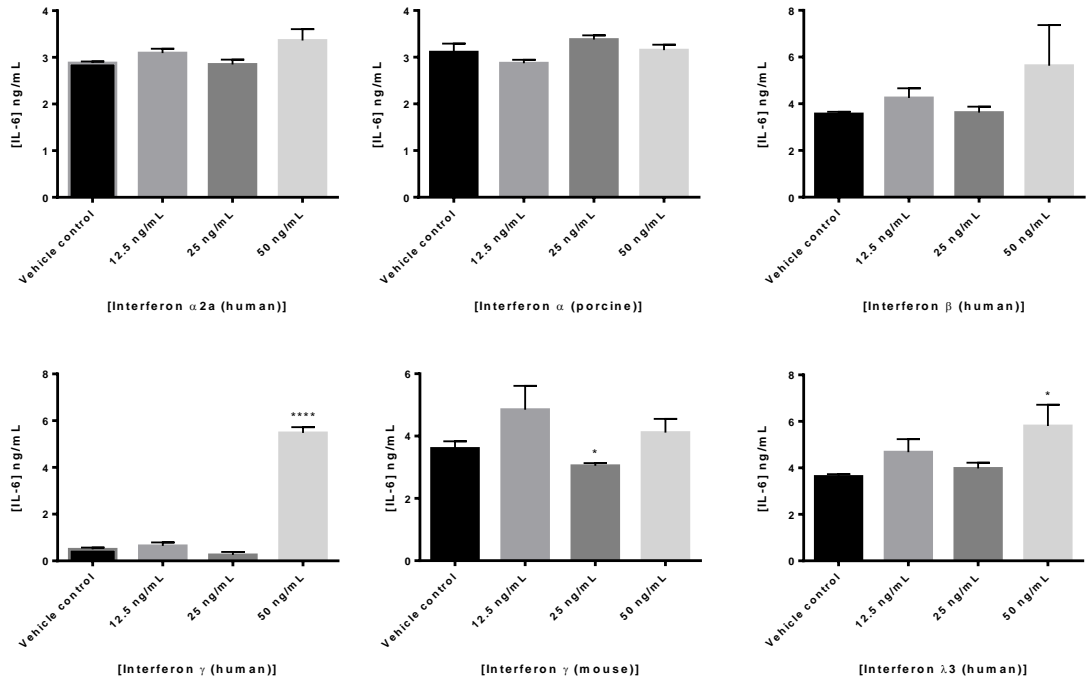


Figure 47 – IL-6 concentrations after undifferentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of TNF- α concentrations

A statistically significant increase in TNF- α concentrations was observed after undifferentiated THP-1 cells were treated with mouse IFN- γ (12.5 ng/mL and 50 ng/mL). Trending increases were also observed after treatment with human IFN- α_{2A} (50 ng/mL). Conversely, a decrease in TNF- α concentrations was observed after exposure to human IFN- γ (25 ng/mL). Once again, these changes in TNF- α production in the undifferentiated THP-1 cells were modest in nature.

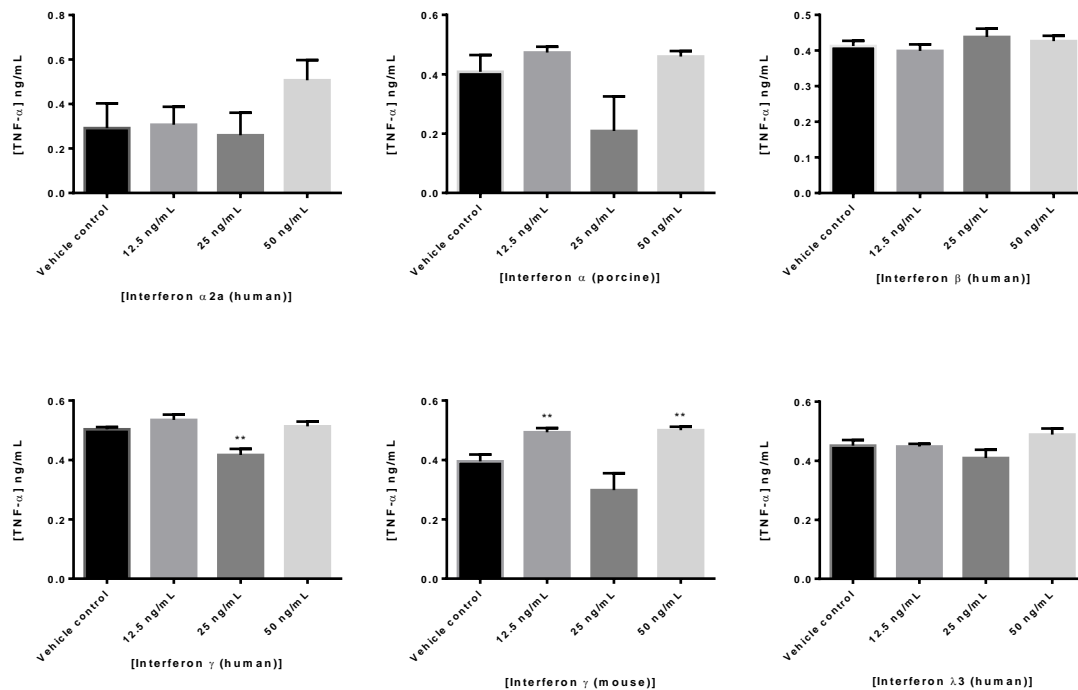


Figure 48 – TNF- α concentrations after undifferentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of IL-1 β concentrations

Increased IL-1 β concentrations were observed after undifferentiated THP-1 cells were treated with mouse IFN- γ (12.5 ng/mL) and porcine IFN- α (25 ng/mL). Decreased IL-1 β concentrations were observed after exposure to human IFN- λ 3 (25 ng/mL).

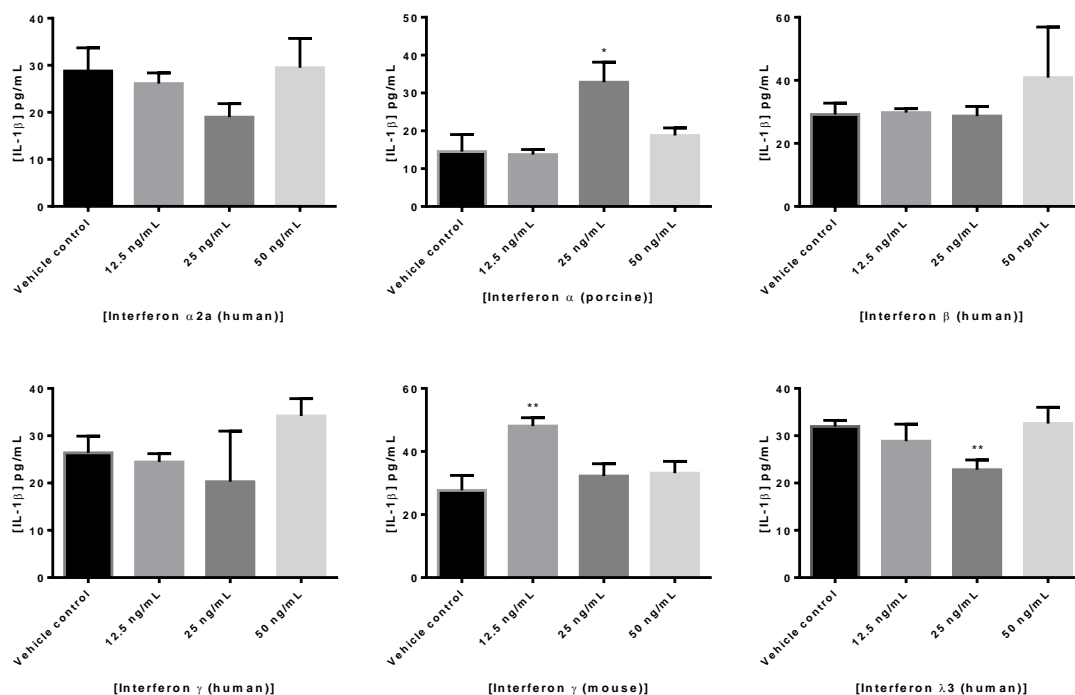


Figure 49 – IL-1β concentrations after undifferentiated THP-1 cells were treated with the various interferons (n = 6)

Quantification of PGE₂ concentrations

No statistically significant changes in PGE₂ concentrations were observed after IFN treatment in undifferentiated THP-1 cells. There was, however, a trending increase in PGE₂ concentrations after treatment with IFN-γ as seen in figure 50 below.

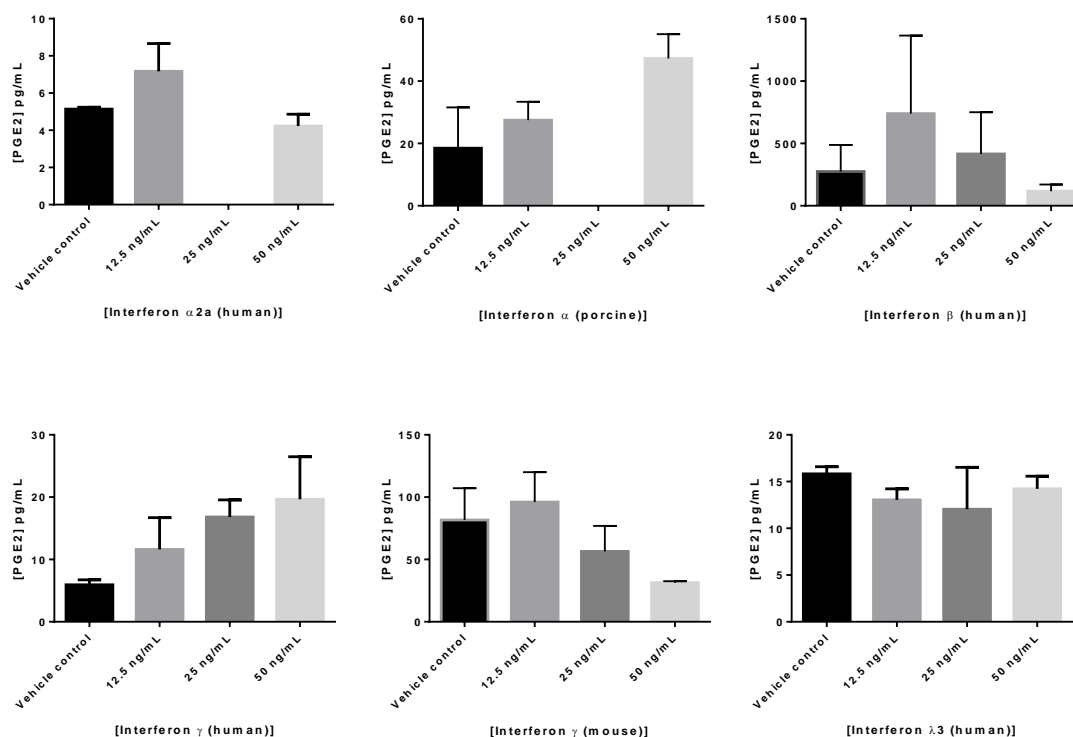


Figure 50 – PGE₂ concentrations after undifferentiated THP-1 cells were treated with the various interferons (n = 6)

Validation of the HPLC methods for the isolation and quantification of tryptophan and kynurenine

The HPLC method for the quantification of Trp and KYN in spent cell culture supernatant described in section 4.3.1 was shown to be accurate, precise, repeatable and specific. An example HPLC chromatogram utilising the sample preparation and analytical methods in section 4.3.1 is shown below in figure 53. The retention time of Trp and KYN using the abovementioned method was found to be 5.840 ± 0.079 min and 4.404 ± 0.044 (n = 30) respectively.

Linearity

The standard curve was prepared as described in section 4.3.1. The results represented in figure 51 are an average of the area of triplicate injections from 3 separate runs ($n = 9$) \pm 1 standard deviation of each of the 10 concentrations tested and resulted in a linear curve across the concentration range tested.

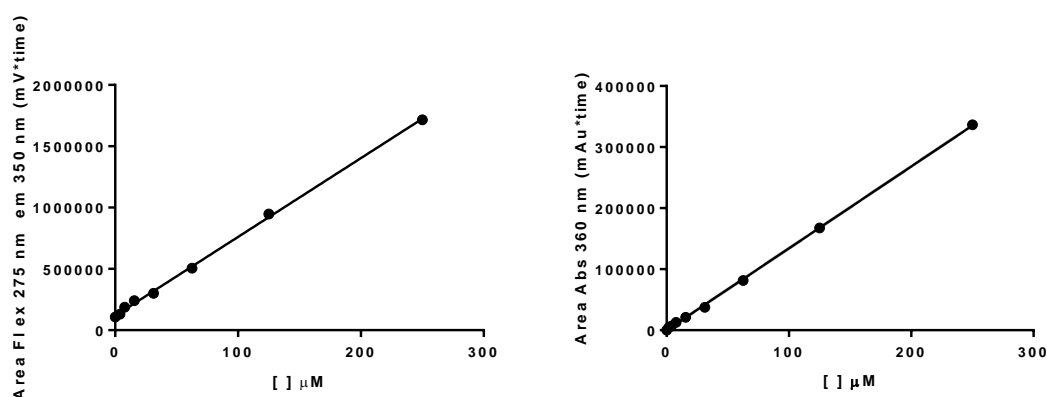


Figure 51 – Calibration curve for the quantification of tryptophan at 275 nm and kynurenine at 360 nm (area) ($n = 3$; $Y = 6425 \cdot X + 118455$ and $Y = 1341 \cdot X + 101.1$; $R^2 = 0.9989$ and 0.9996 , under the chromatographic conditions described in section 4.3.1), which produced a linear curve across the concentration range tested (% RSD < 5%).

Accuracy and precision

Figure 52 below demonstrates the accuracy and precision of both the sample preparation method and the HPLC method for the isolation and quantification of Trp and KYN. All three of the concentrations tested, 6.25 μ M, 12.5 μ M and 25 μ M, were

within the acceptable range of 85 to 115% of expected. The HPLC method used to quantify Trp and KYN in spent cell culture supernatant samples was found to be precise. Figure 52 below shows the precision of the method with regards to the area, height and retention time represented as the coefficient of variance. All results fell within the acceptable range of less than 10%.

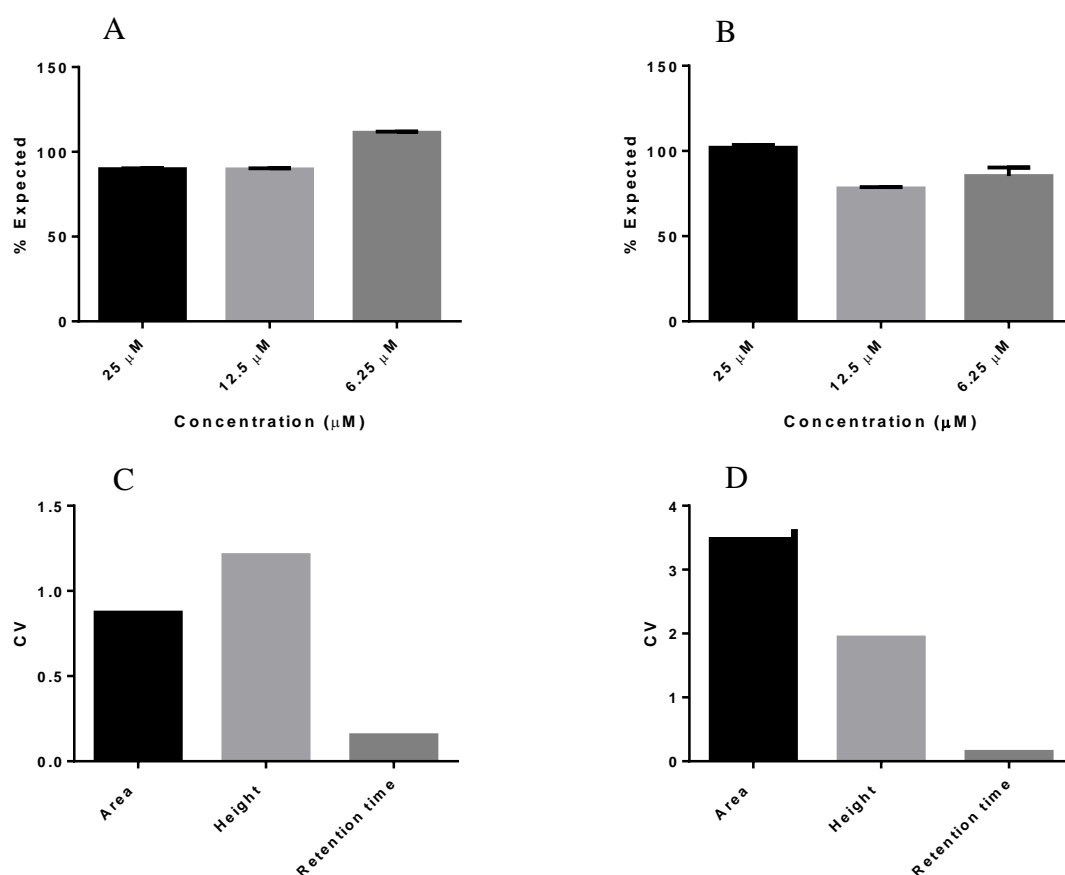


Figure 52 – The a) accuracy of the HPLC method used to quantify tryptophan; b) accuracy of the HPLC method used to quantify kynurenine; c) precision of the HPLC method used to quantify tryptophan; d) precision of the HPLC method to quantify kynurenine (n = 30).

Selectivity

The HPLC method used to quantify Trp and KYN was found to be selective for both analytes of interest. This can be seen in figure 53 below.

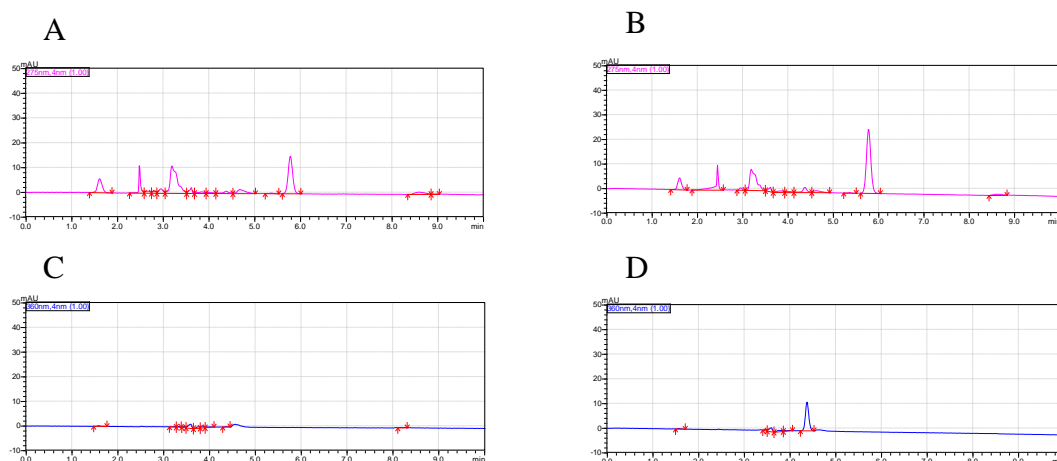


Figure 53 – HPLC chromatograms highlighting the selectivity of the method used to quantify Trp and KYN at fluorescence ex 275 nm and em 350 nm and absorbance 360 nm, respectively a) blank media at ex 275 nm em 350 nm; b) Trp spiked media sample; c) blank media sample at 360 nm; d) KYN spiked media sample.

Lower limits of detection and quantification

The limit of detection (signal to noise ratio 3-to-1) was found to be 0.0003 mM and 0.012 mM and the limit of quantification (signal to noise ratio 10-to-1) was found to be 0.006 mM and 0.019 mM for Trp and KYN, respectively.

Quantification of IDO activity

No significant changes in Trp or KYN concentrations were observed in undifferentiated THP-1 cells treated with human IFN- α_{2A} . This resulted in no change to the KYN to Trp ratio in undifferentiated THP-1 cells as seen in figure 54 below.

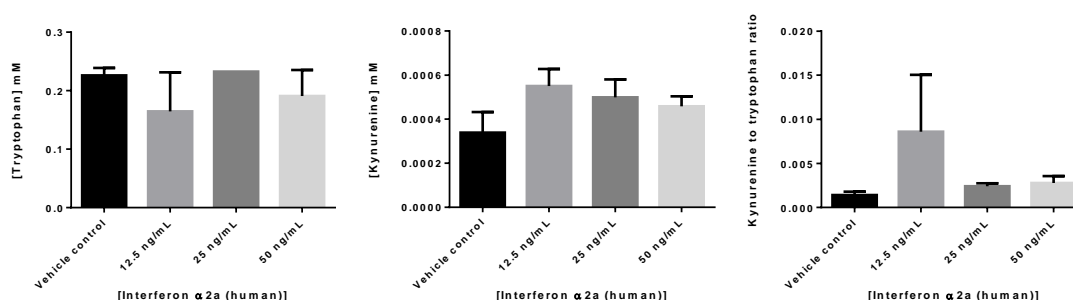


Figure 54 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with human interferon- α_{2A} (n = 9)

Exposure of undifferentiated THP-1 cells to human IFN- β (12.5 ng/mL and 25 ng/mL), reduced Trp concentration significantly, however, KYN concentrations were too low to quantify in 6 of the samples. Therefore, only trends towards an increased KYN to Trp ratio can be presented, as seen in figure 55 below.

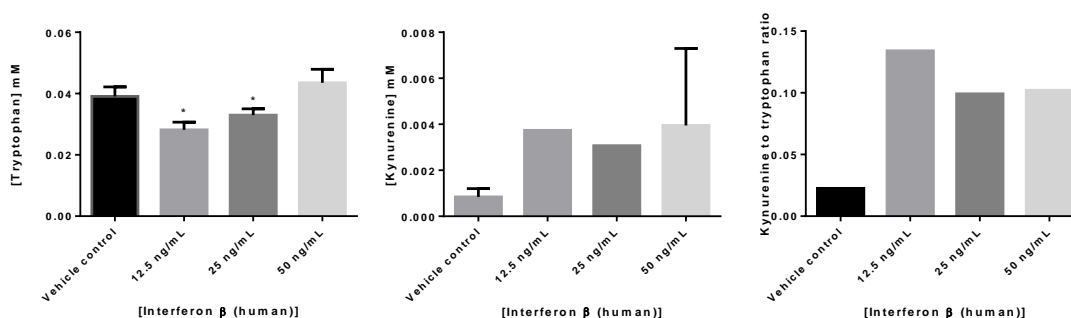


Figure 55 - Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with human interferon-β (n = 9)

Human IFN-γ exposure to undifferentiated THP-1 cells resulted in a decrease in Trp concentrations in comparison to the vehicle control however this was not statistically significant. Statistically significant increases in KYN were, however, observed at all concentrations tested. This resulted in the significant increase in the KYN to Trp ratio at all concentrations tested indicating an increase in the activity of IDO by twice as much as vehicle control as seen in figure 56.

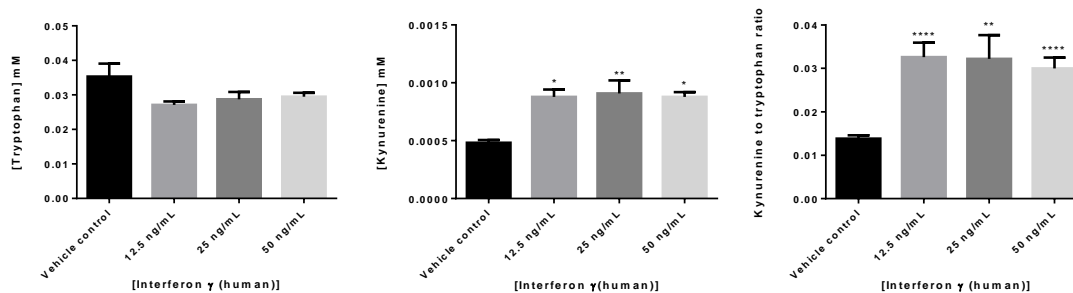


Figure 56 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with human interferon- γ (n = 9)

Exposure of undifferentiated THP-1 cells to human IFN- λ 3 resulted in no statistically significant change to Trp or KYN concentrations. Trends towards increased Trp and decreased KYN were observed. No statistically significant change in IDO was observed however a trend towards decreased IDO can be seen below in figure 57.

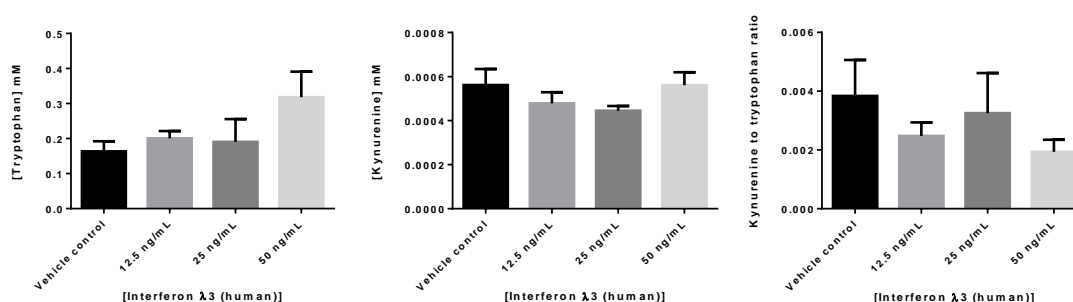


Figure 57 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with human interferon- λ 3 (n = 9)

Exposure of undifferentiated THP-1 cells to porcine IFN- α resulted in no statistically significant change to Trp or KYN concentrations. Trends towards increased Trp and decreased KYN were observed. No statistically significant change in IDO was observed however a trend towards decreased IDO can be seen below in figure 58.

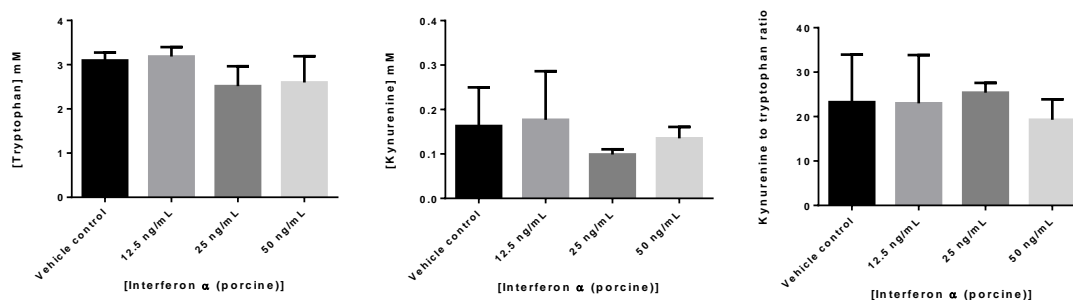


Figure 58– Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with porcine interferon- α (n = 9)

Exposure of undifferentiated THP-1 cells to mouse IFN- γ resulted in no statistically significant change to Trp or KYN concentrations. Trends towards increased Trp and decreased KYN were observed. No statistically significant change in IDO was observed however a trend towards decreased IDO can be seen below in figure 59.

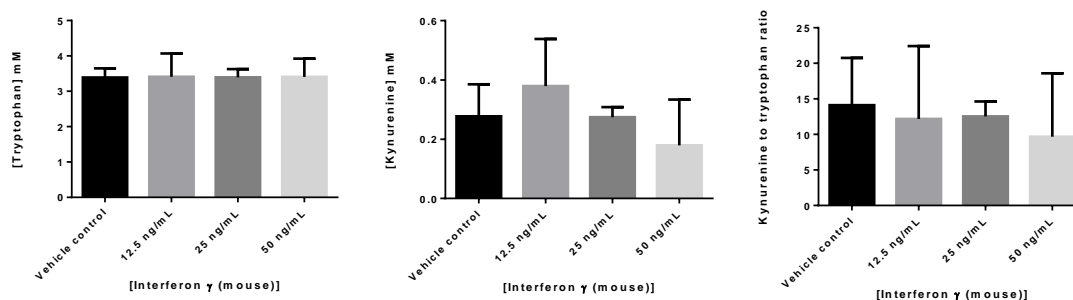


Figure 59 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in undifferentiated THP-1 cells treated with mouse interferon- γ (n = 9)

Differentiated THP-1 human monocytic cells

Resazurin

A statistically significant increase in proliferation after differentiated THP-1 cells were exposed to porcine IFN- α (400 units/mL), human IFN- β (12.5 ng/mL, 25 ng/mL and 50 ng/mL), mouse IFN- γ (200 units/mL and 400 units/mL) and human IFN- λ 3 (50 ng/mL) as seen in figure 60. IFN- β had the greatest influence of on the viability of differentiated THP-1 cells, with 7-fold increases in comparison to the vehicle control.

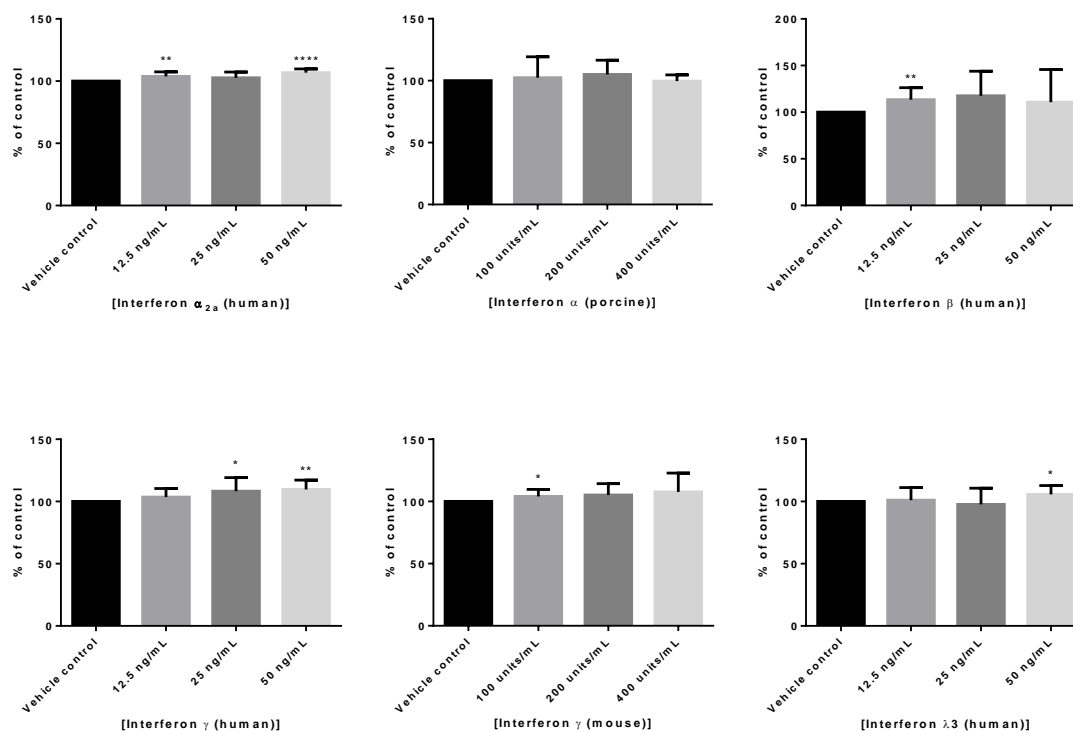


Figure 60 – The effects of the various interferons on the proliferation of differentiated THP-1 monocytic cells using the resazurin reduction viability assay (n = 9)

Quantification of IL-6 concentrations

Statistically significant increases in IL-6 concentrations were observed after differentiated THP-1 cells were exposed to human IFN- γ (50 ng/mL) human IFN- $\lambda 3$ (50 ng/mL). Trends of increased IL-6 production was observed after these cells were exposed to human IFN- α and human IFN- β in comparison to the vehicle control as seen in figure 61. The increases in IL-6 observed were only modest in nature.

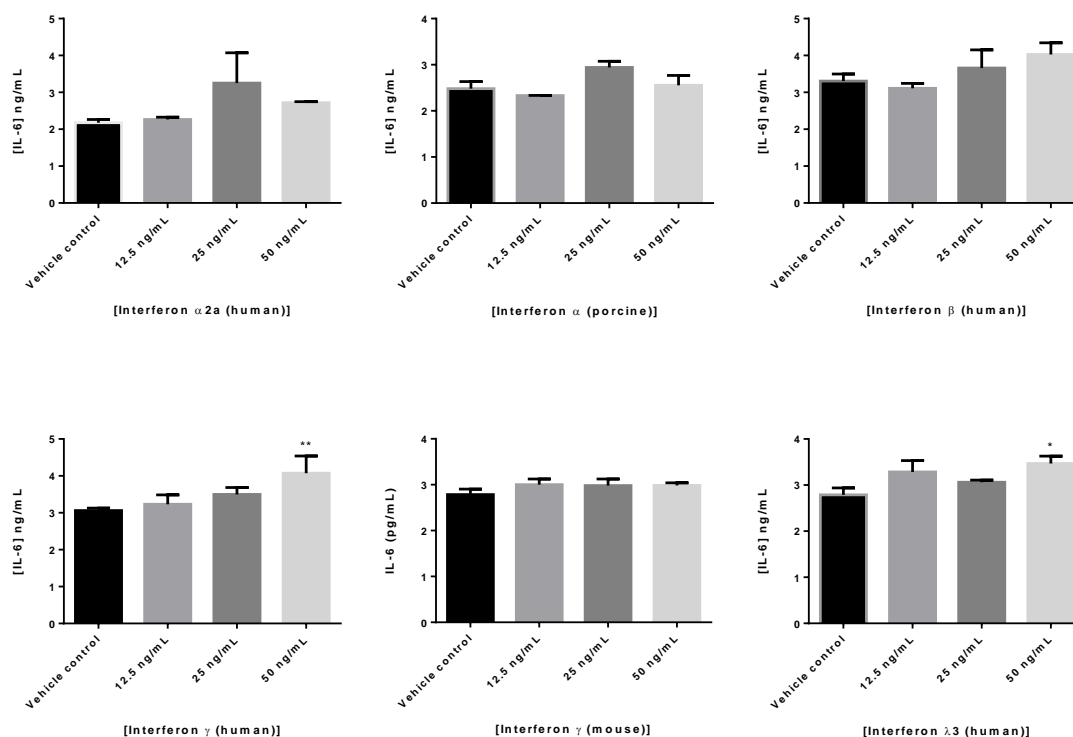


Figure 61 – IL-6 concentrations after differentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of TNF-α concentrations

Statistically significant increases in TNF-α concentrations was observed after differentiated THP-1 cells were exposed to human IFN-β (25 ng/mL) and mouse IFN-γ (25 ng/mL). Although not statistically significant, trending increases in TNF-α concentrations after exposure to human IFN-α, human IFN-γ and human IFN-λ₃ were observed as seen in figure 62.

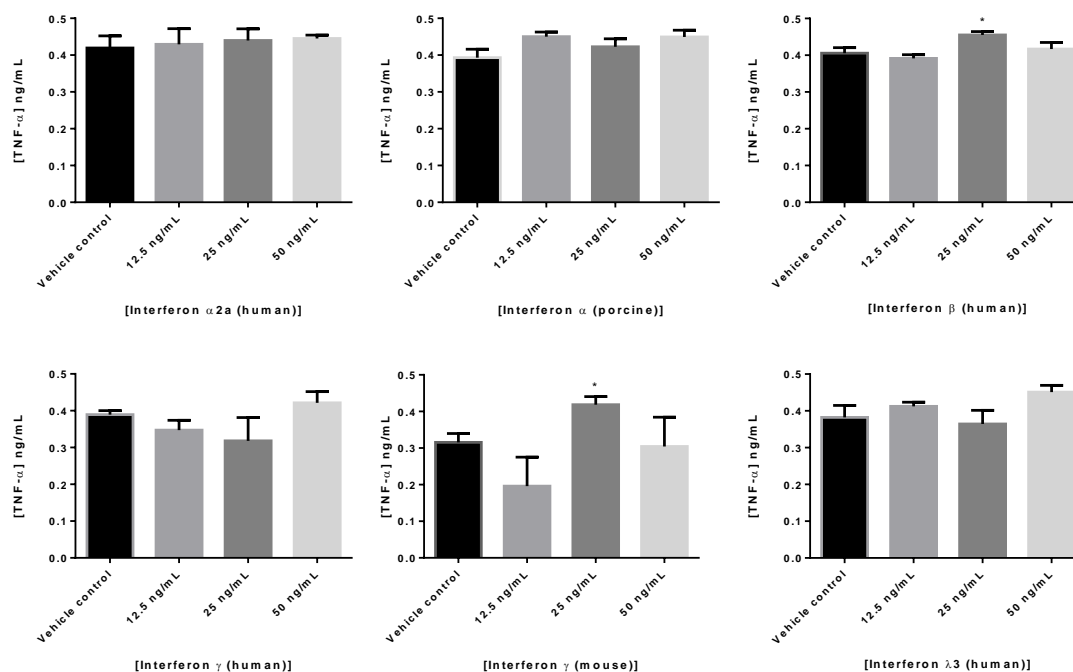


Figure 62 – TNF- α concentrations after differentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of IL-1 β concentrations

Statistically significant increases in IL-1 β concentrations were observed after exposure of differentiated THP-1 cells to human IFN- β (25 ng/mL and 50 ng/mL) and human IFN- λ 3 (25 ng/mL) as seen in figure 63 below. Although not statistically significant, IL-1 β concentrations were nearing significance after exposure to human IFN- α _{2A} (50 ng/mL) and human IFN- γ (50 ng/mL).

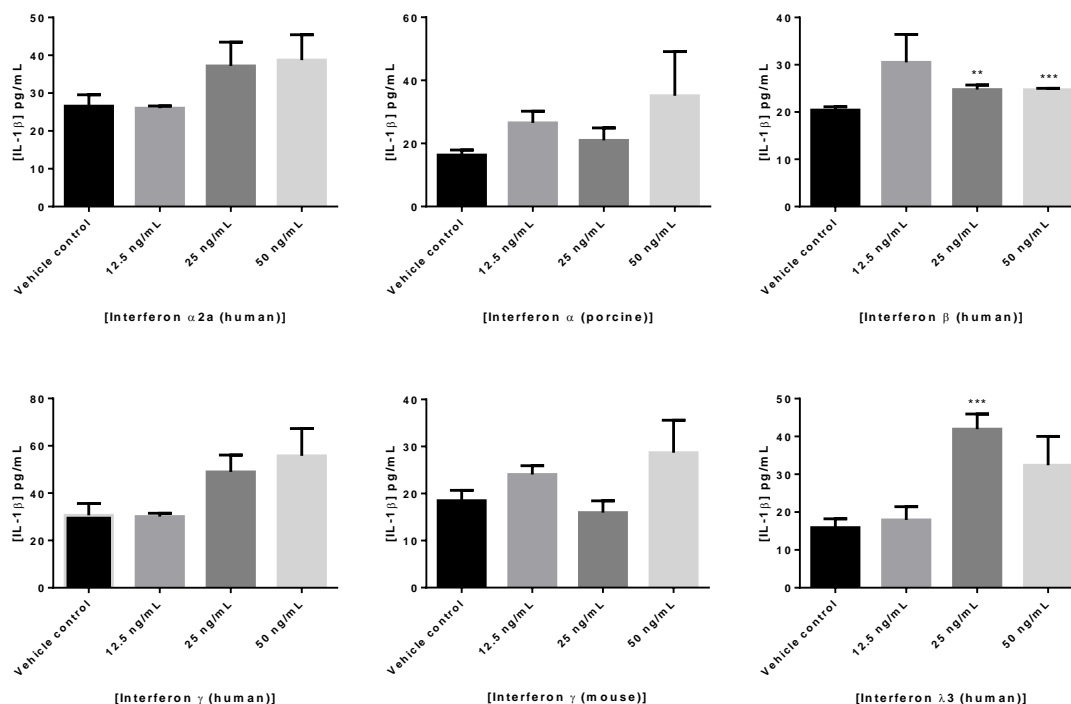


Figure 63 – IL-1 β concentrations after differentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of PGE₂ concentrations

Statistical significant increases in PGE₂ concentrations were observed after differentiated THP-1 cells were exposed to human IFN- α _{2A} (12.5 ng/mL, 25 ng/mL and 50 ng/mL), human IFN- γ (12.5 ng/mL, 25 ng/mL and 50 ng/mL) and human IFN- λ 3 (25 ng/mL) as seen in figure 64 below. Five-fold and one hundred-fold increases in PGE₂ concentrations were observed after exposure to IFN- α _{2A} and IFN- γ , respectively, in comparison to the vehicle control.

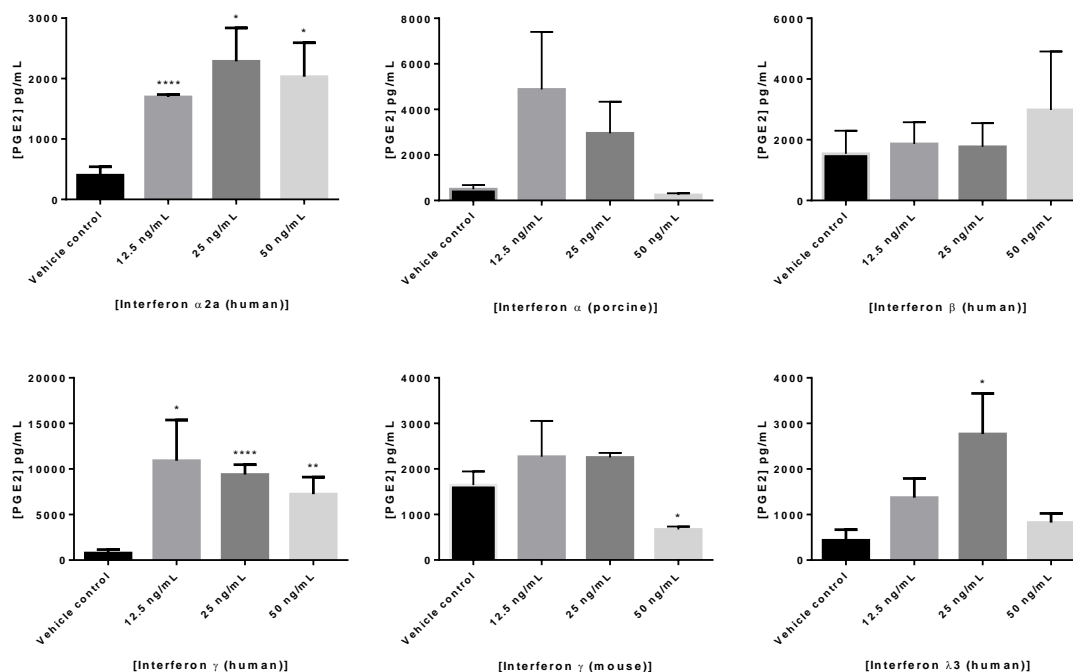


Figure 64 – PGE₂ concentrations after differentiated THP-1 cells were treated with the various interferons (n =6)

Quantification of IDO activity

No significant decrease in Trp concentrations were observed after exposure of differentiated THP-1 cells to human IFN-α_{2A} however a trending decrease was observed. Significant increases in KYN concentrations were however observed at all concentrations tested. A resultant statistically significant increase, an increase to twice as high as vehicle control, in the KYN to Trp ratio was observed at all concentrations tested as seen in figure 65 below.

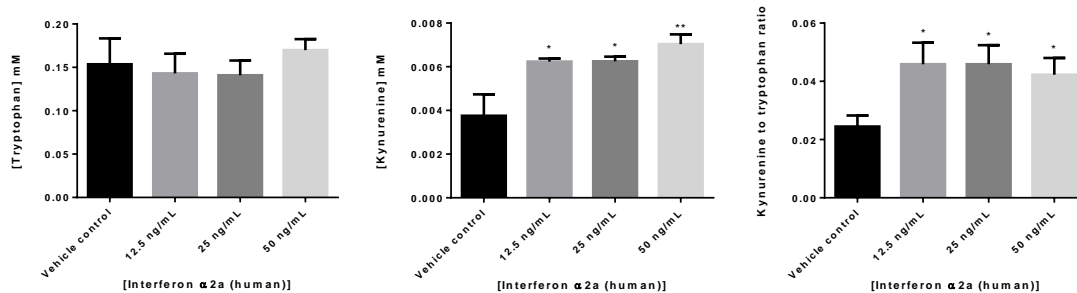


Figure 65 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with human interferon- α_{2A} (n = 9)

Exposure of differentiated THP-1 cells to human IFN- β resulted in no significant change in Trp and KYN concentrations or the KYN to Trp ratios. Trends towards decreased KYN concentrations and KYN to Trp ratio are however observed as seen in figure 66 below.

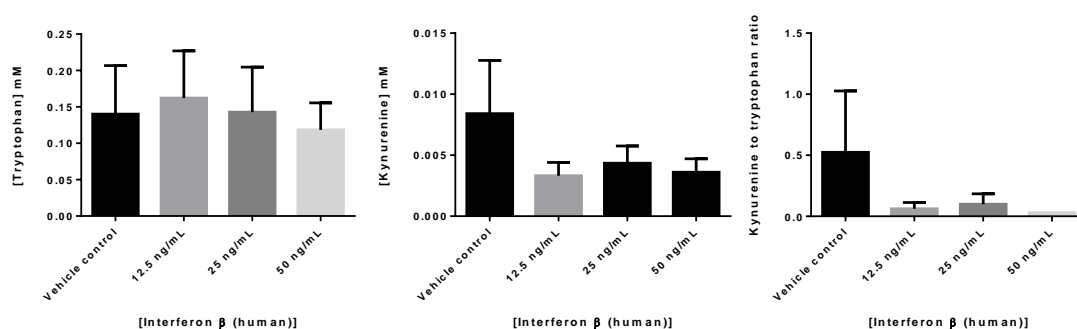


Figure 66 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with human interferon- β (n = 9)

Exposure of differentiated THP-1 cells exposed to human IFN- γ resulted in a concentration-dependent decrease in Trp concentrations and a concentration-dependent increase in KYN concentrations. This resulted in the statistically significant increase in KYN to Trp ratio at all three concentrations tested as seen in figure 67 below. Exposure of these cells to 50 ng/mL of IFN- γ resulted in a Trp to KYN ratio approximately eight-fold higher than seen in vehicle control.

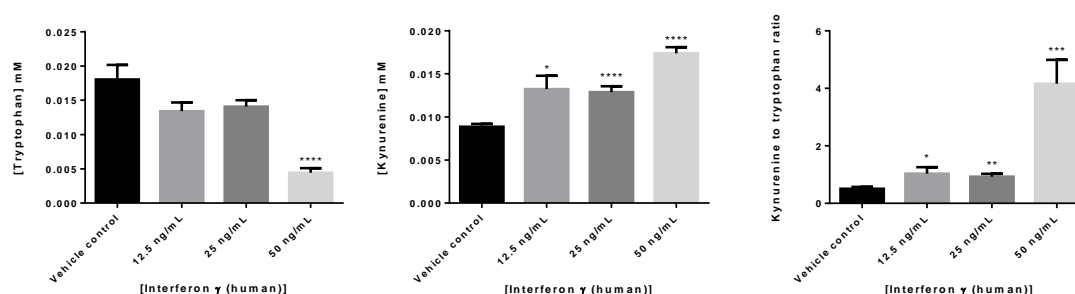


Figure 67 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with human interferon- γ (n = 9)

Exposure of differentiated THP-1 cells exposed to human IFN- λ 3 showed no statistically significant change in any of the parameters tested. Trending increases in KYN to Trp ratio was however observed at 50 ng/mL as seen in figure 68 below.

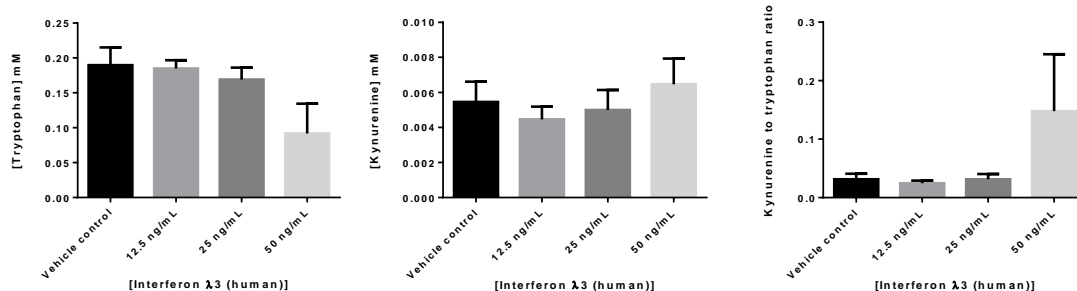


Figure 68 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with human interferon-λ3 (n = 9)

Exposure of differentiated THP-1 cells exposed to porcine IFN-α showed no statistically significant change in any of the parameters tested as seen in figure 69 below.

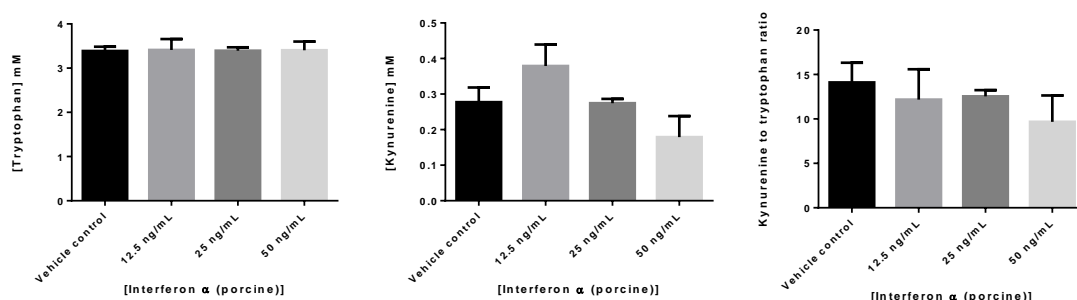


Figure 69 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with porcine IFN-α (n = 9)

Exposure of differentiated THP-1 cells exposed to murine IFN- γ showed no statistically significant change in tryptophan concentrations however KYN concentrations were increased modestly after exposure to 50 ng/mL. This resulted in no change in the KYN to Trp ratio as seen in figure 70 below.

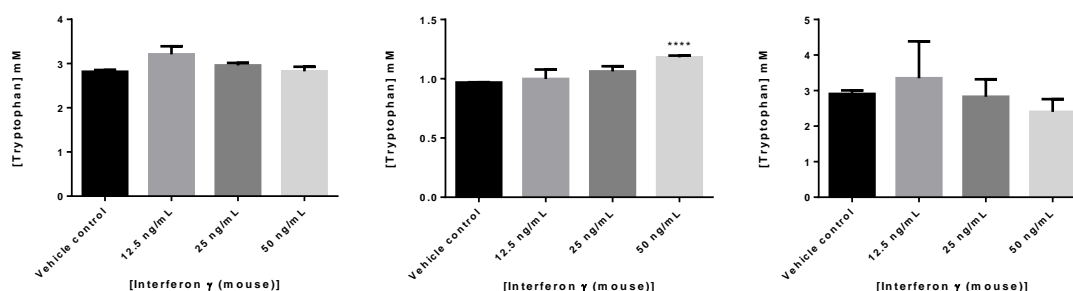


Figure 70 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells treated with mouse interferon- γ (n = 9)

4.4.2 Effect of antidepressant medications on markers of inflammation in differentiated THP-1 human monocytic cells

Resazurin

Statistically significant concentration-dependent decreases in the viability of differentiated THP-1 cells were observed after these cells were exposed to amitriptyline, fluoxetine and venlafaxine as seen in figure 71 below. Amitriptyline and fluoxetine both decreased the viability of differentiated THP-1 cells by approximately 50% after 24 h treatment with 1000 μ M. Furthermore, amitriptyline and fluoxetine, 100 μ M, decreased viability by approximately 20% and 50%, respectively. Venlafaxine had the

least effect on the viability of THP-1 cells, with a 20% and 10% reductions in viability after exposure to 1000 μM and 100 μM , respectively as seen in figure 71 below.

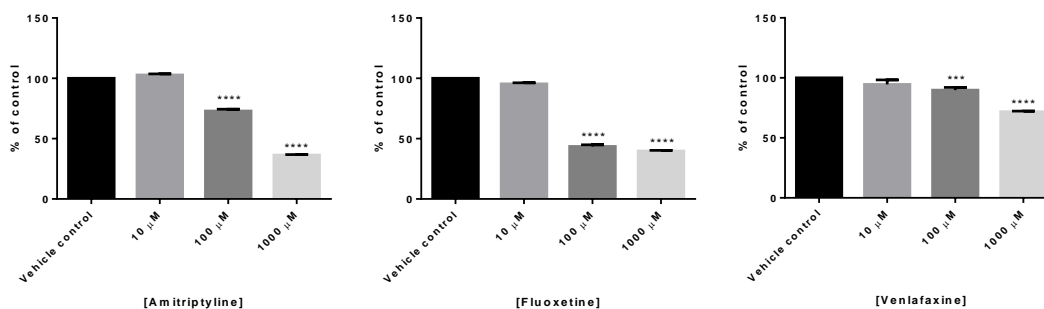


Figure 71 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine on the proliferation of differentiated THP-1 monocytic cells using the resazurin reduction viability assay (n = 9)

Statistically significant concentration-dependent decreases in the viability of differentiated THP-1 cells were observed after these cells were exposed to amitriptyline, fluoxetine and venlafaxine in combination with IFN- α_{2A} as seen in figure 72 below. The greatest decreases in viability were observed in cells pre-treated with amitriptyline and fluoxetine (1000 μM), where viability decreased by approximately half. Furthermore, amitriptyline and fluoxetine, 100 μM , decreased viability by approximately 20% and 50%, respectively. Venlafaxine had the least effect on the viability of THP-1 cells, with 20% reduction in viability after exposure to 1000 μM .

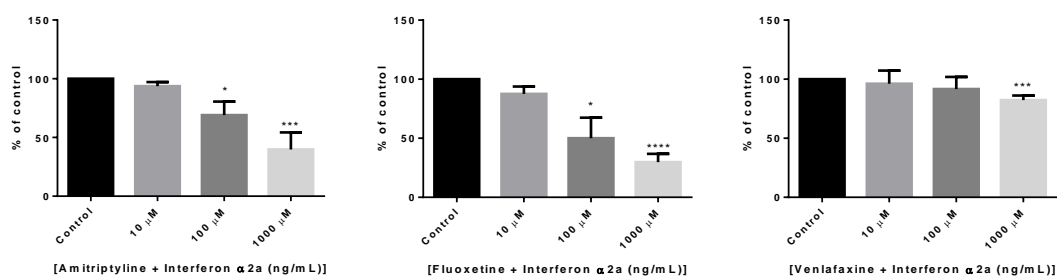


Figure 72 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine in combination with human interferon- α_{2A} on the proliferation of differentiated THP-1 monocytic cells using the resazurin reduction viability assay (n = 9)

Statistically significant concentration-dependent decreases in the viability of differentiated THP-1 cells were observed after these cells were exposed to amitriptyline and fluoxetine in addition to human IFN- γ as seen in figure 73 below. The greatest decreases in viability were observed in cells pre-treated with amitriptyline and fluoxetine (1000 μ M), where viability decreased by approximately half. Furthermore, amitriptyline and fluoxetine, 100 μ M, decreased viability by approximately 20% and 50%, respectively.

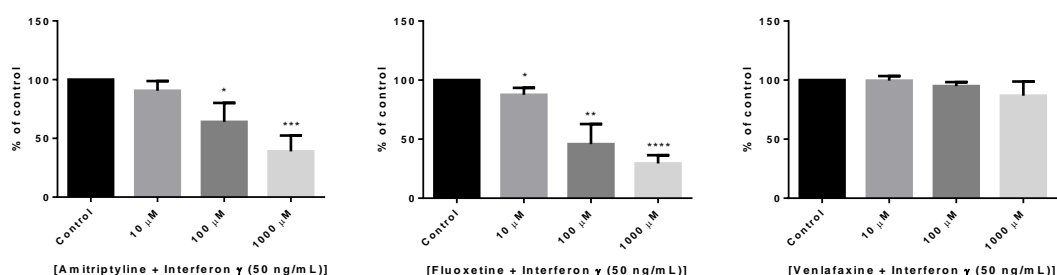


Figure 73 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine in combination with human interferon- γ on the proliferation of differentiated THP-1 monocytic cells using the resazurin reduction viability assay (n = 9)

Quantification of free radical production using the DCFH-DA assay

No statistically significant increase in free radical production was observed after differentiated THP-1 cells were exposed to amitriptyline, fluoxetine and venlafaxine. A trending increase in free radical production in a concentration-dependent manner after exposure to venlafaxine was observed as seen in figure 74 below.

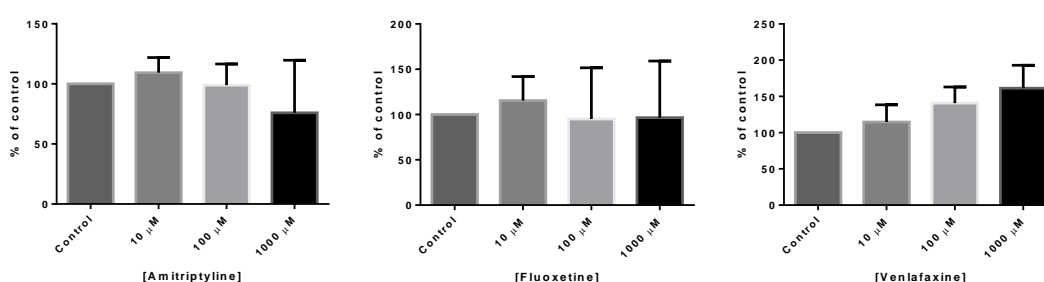


Figure 74 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine on the production of free radicals by differentiated THP-1 monocytic cells using the DCFH-DA assay (n = 9)

A statistically significant, concentration-dependent decrease in free radical production was observed in differentiated THP-1 cells after exposure to amitriptyline, fluoxetine and venlafaxine in combination with IFN- α_{2A} as seen below. The greatest decreases in free radical production were observed in cells pre-treated with amitriptyline and fluoxetine (1000 μ M), where viability decreased by approximately half. Furthermore, amitriptyline and fluoxetine, 100 μ M, decreased viability by approximately 20% and 50%, respectively as seen in figure 75 below.

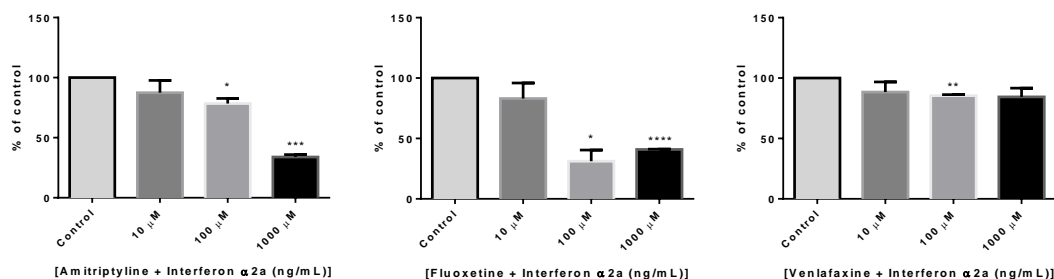


Figure 75 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine in combination with interferon- α_{2A} on the production of free radicals by differentiated THP-1 monocytic cells using the DCFH-DA assay (n = 9)

Statistically significant decreases in free radical production were observed in differentiated THP-1 cells treated with fluoxetine in combination with human IFN- γ (100 μ M and 1000 μ M) as seen in figure 76 below. The greatest decreases in viability were observed in cells pre-treated with fluoxetine (100 μ M and 1000 μ M), where viability decreased by approximately half.

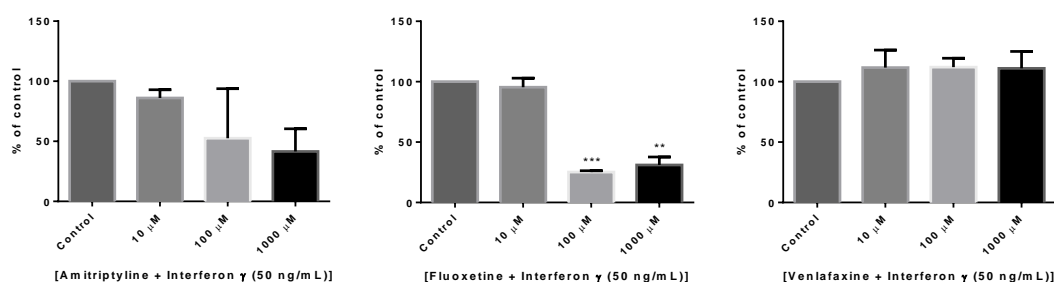


Figure 76 – The effects of a) amitriptyline; b) fluoxetine and c) venlafaxine in combination with interferon- γ on the production of free radicals by differentiated THP-1 monocytic cells using the DCFH-DA assay (n = 9)

Quantification of IDO activity

Interferon gamma

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to amitriptyline in combination with human IFN- γ (100 μ M and 1000 μ M). Conversely, significant decreases were observed in KYN concentrations after exposure to amitriptyline in combination with human IFN- γ (10 μ M, 100 μ M and 1000 μ M). This resulted in an approximately 75% decrease in the KYN to Trp ratio after exposure to amitriptyline in combination with human IFN- γ (100 μ M and 1000 μ M) as seen in figure 77 below.

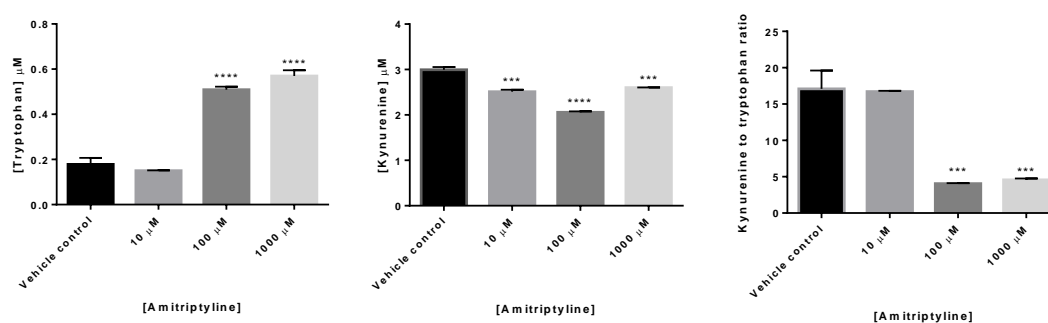


Figure 77 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with amitriptyline and human interferon- γ (n = 9)

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to fluoxetine in combination with human IFN- γ (10 μ M, 100 μ M and 1000 μ M). Conversely, significant decreases were observed in KYN concentrations after exposure to fluoxetine in combination with

human IFN- γ (10 μ M, 100 μ M and 1000 μ M). This resulted in an approximate 60% decrease in KYN to Trp ratio after exposure to fluoxetine in combination with human IFN- γ (10 μ M, 100 μ M and 1000 μ M) as seen in figure 78 below.

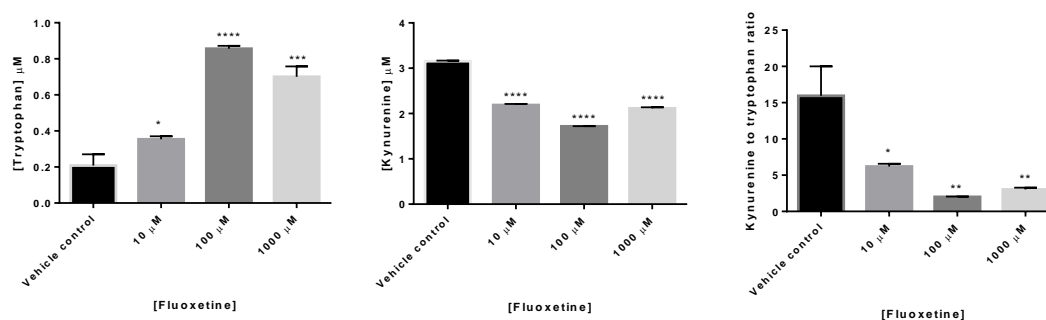


Figure 78 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with fluoxetine and human interferon- γ (n = 9)

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to venlafaxine in combination with human IFN- γ (10 μ M, 100 μ M and 1000 μ M). Conversely, significant increases were observed in KYN concentrations after exposure to venlafaxine in combination with human IFN- γ (10 μ M and 1000 μ M). This resulted in an approximate 40% decrease in KYN to Trp ratio after exposure to venlafaxine in combination with human IFN- γ (10 μ M, 100 μ M and 1000 μ M) as seen in figure 79 below.

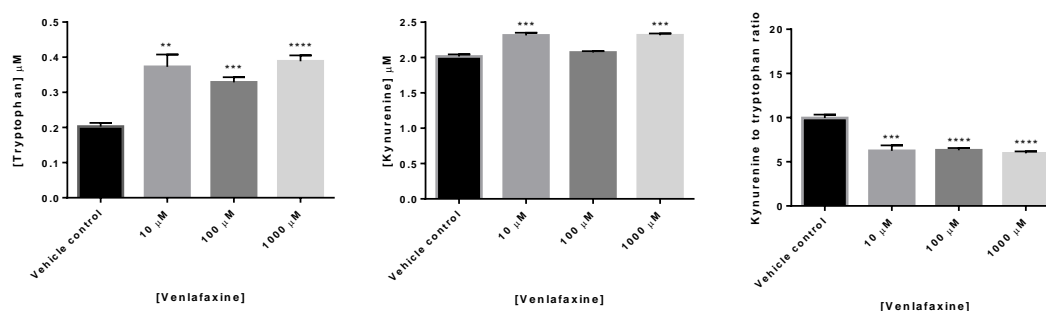


Figure 79 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with venlafaxine and human interferon- γ (n = 9)

Interferon alpha

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to amitriptyline in combination with human IFN- α_{2A} (10 μM , 100 μM and 1000 μM). Conversely, significant decreases were observed in KYN concentrations after exposure to amitriptyline in combination with human IFN- α_{2A} (10 μM , 100 μM and 1000 μM). This resulted in an approximate 50% decrease in KYN to Trp ratio after exposure to amitriptyline in combination with human IFN- α_{2A} (10 μM , 100 μM and 1000 μM) as seen in figure 80 below.

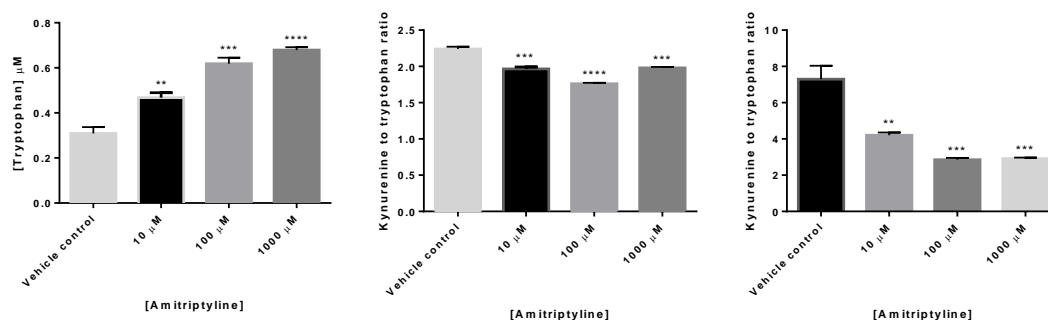


Figure 80 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with amitriptyline and human interferon- α_{2A} (n = 9)

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to fluoxetine in combination with human IFN- α_{2A} (10 μ M, 100 μ M and 1000 μ M). Conversely, significant decreases were observed in KYN concentrations after exposure to fluoxetine in combination with human IFN- α_{2A} (10 μ M, 100 μ M and 1000 μ M). This resulted in an approximate 50% decrease in KYN to Trp ratio after exposure to fluoxetine in combination with human IFN- α_{2A} (10 μ M, 100 μ M and 1000 μ M) as seen in figure 81 below.

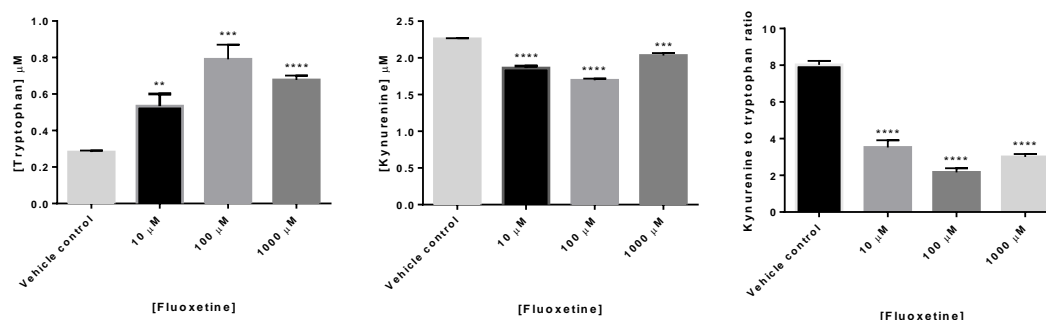


Figure 81 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with fluoxetine and human interferon- α_{2A} (n = 9)

Significant, concentration-dependent increase in Trp concentrations were observed after differentiated THP-1 cells were exposed to venlafaxine in combination with human IFN- α_{2A} (10 μM , 100 μM and 1000 μM). Conversely, significant decreases were observed in KYN concentrations after exposure to venlafaxine in combination with human IFN- α_{2A} (10 μM , 100 μM and 1000 μM). This resulted in an approximate 80% decrease in KYN to Trp ratio after exposure to venlafaxine 100 μM in combination with human IFN- α_{2A} . Furthermore, venlafaxine 10 μM and 1000 μM decreased IDO activity by approximately 20% as seen in figure 82 below.

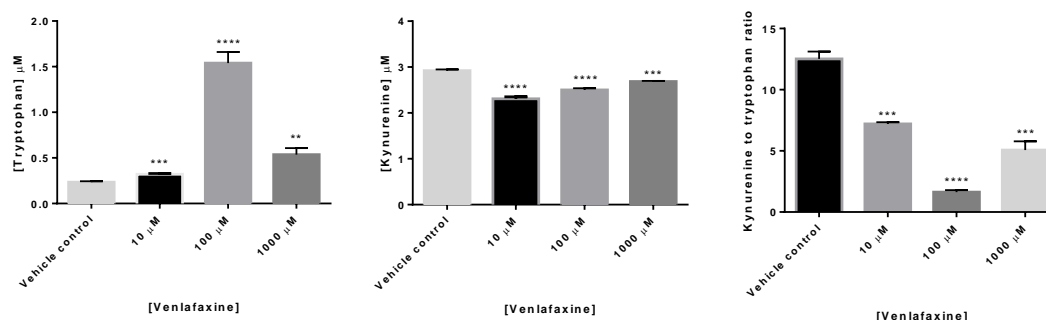


Figure 82 – Changes in a) tryptophan concentration; b) kynurenine concentration; c) kynurenine to tryptophan ratio in differentiated THP-1 cells pre-treated with venlafaxine and human interferon- α_{2A} (n = 9)

Quantification of pro-inflammatory cytokines

Interferon gamma

Amitriptyline was shown to significantly decrease the production of IL-6 at all three concentrations tested by approximately 90% in IFN- γ stimulated differentiated THP-1 cells. Conversely, IL-1 β concentrations were significantly increased, by approximately 7000-fold when pre-treated with amitriptyline and TNF- α concentrations were unchanged in comparison to the control as seen in figure 83 below.

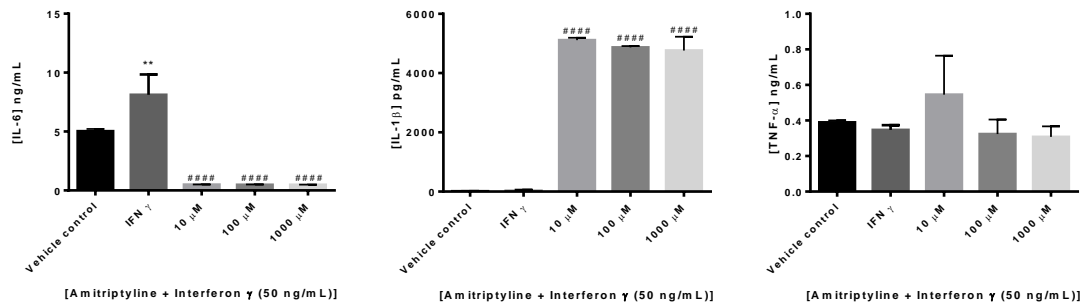


Figure 83 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with amitriptyline and stimulated with human interferon- γ (n = 6)

Fluoxetine was shown to significantly decrease the production of IL-6 at all three concentrations tested by approximately 80% in IFN- γ stimulated differentiated THP-1 cells. Conversely, TNF- α concentrations were significantly increased five-fold when pre-treated with fluoxetine and IL-1 β concentrations were unchanged in comparison to the control as seen in figure 84 below.

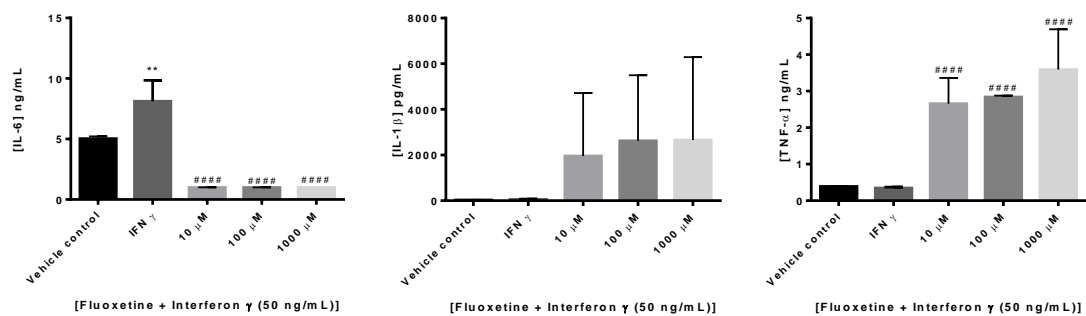


Figure 84 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with fluoxetine and stimulated with human interferon- γ (n = 6)

Venlafaxine was shown to significantly decrease the production of IL-6 at all three concentrations tested by approximately 80% in IFN- γ stimulated differentiated THP-1 cells. Conversely, IL-1 β concentrations were significantly increased 8-fold when pre-treated with 10 μ M venlafaxine and TNF- α concentrations were increased 3-fold after treatment with 100 μ M and decreased after treatment with 1000 μ M in comparison to the vehicle control as seen in figure 85 below.

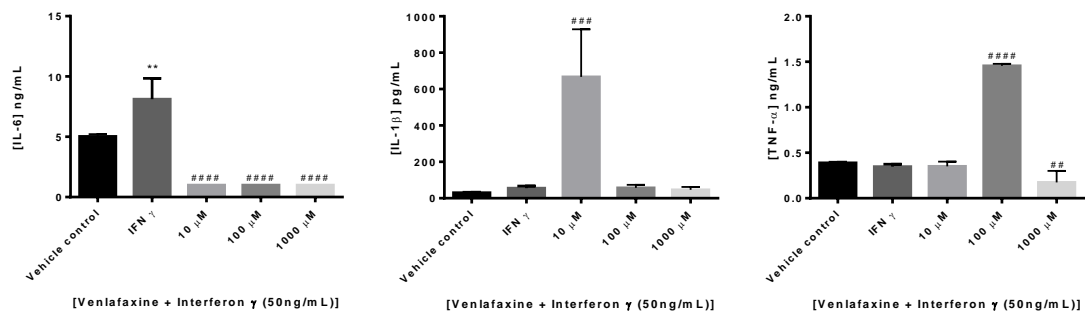


Figure 85 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with venlafaxine and stimulated with human interferon- γ (n = 6)

Interferon alpha

Amitriptyline was shown to significantly decrease the production of IL-6 at all three concentrations tested 4-fold in IFN- α_{2A} stimulated differentiated THP-1 cells. Conversely, IL-1 β concentrations were significantly increased, twice as high as IFN- α alone, when pre-treated with amitriptyline at 100 μ M and 1000 μ M and TNF- α concentrations were increased at 100 μ M and decreased at 1000 μ M in comparison to the control as seen in figure 86 below.

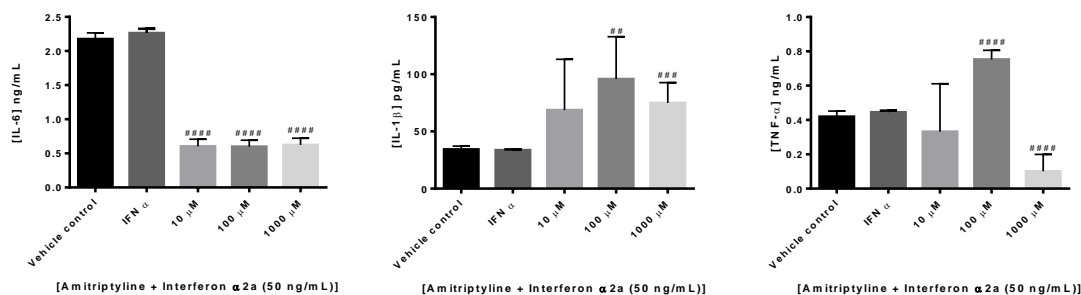


Figure 86 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with amitriptyline and stimulated with human interferon- α_{2A} (n = 6)

Fluoxetine was shown to significantly decrease the production of IL-6 at all three concentrations tested, by approximately 60% in IFN- α_{2A} stimulated differentiated THP-1 cells. Conversely, IL-1 β concentrations were significantly increased, approximately doubled, when pre-treated with Fluoxetine at all three concentrations and TNF- α concentrations were significantly decreased at 100 μ M and 1000 μ M in comparison to the control as seen in figure 87 below.

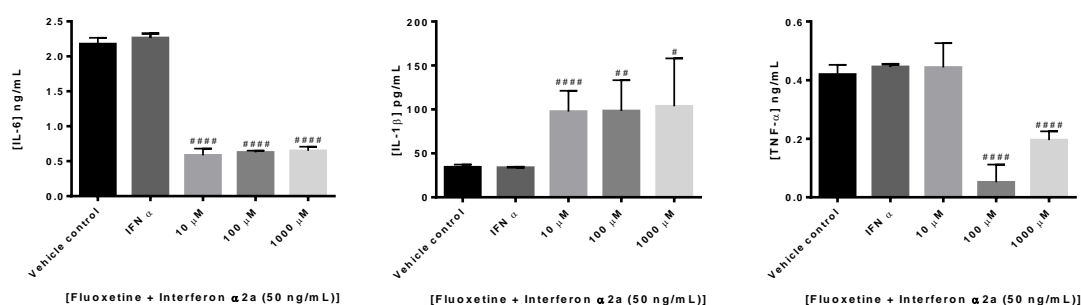


Figure 87 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with fluoxetine and stimulated with human interferon- α_{2A} (n = 6)

Fluoxetine was shown to significantly decrease the production of IL-6 at all three concentrations tested, by approximately 75%, in IFN- α_{2A} stimulated differentiated THP-1 cells. Conversely, IL-1 β concentrations were significantly increased when pre-treated with Fluoxetine at all three concentrations and TNF- α concentrations were significantly decreased at all three concentrations, by approximately 50% in comparison to the control as seen in figure 88 below.

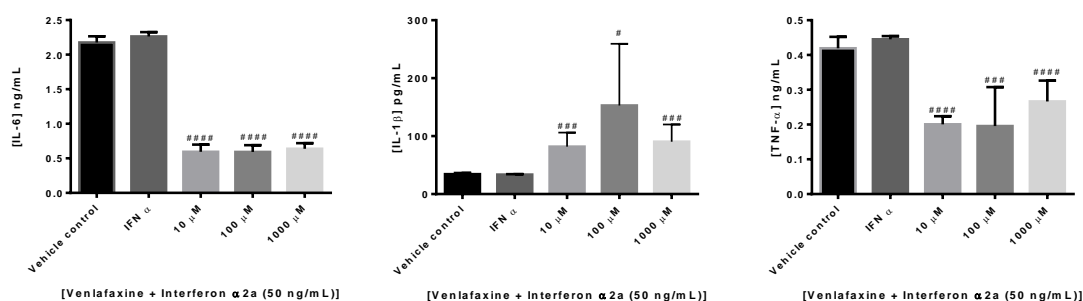


Figure 88 – IL-6, IL-1 β and TNF- α production in differentiated THP-1 cells pre-treated with venlafaxine and stimulated with human interferon- α_{2A} (n = 6)

4.4.3 Development of a model of differentiation of SH-SY5Y neuroblastoma cells

The effects of the differentiating agent on viability

Resazurin

The differentiation agent used in this study, dbcAMP did not have an effect on the viability of SH-SY5Y neuroblastoma cells across the concentration range tested in comparison to the vehicle control as seen in figure 89 below.

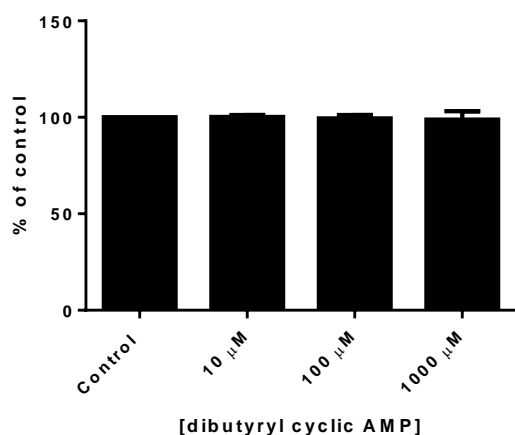
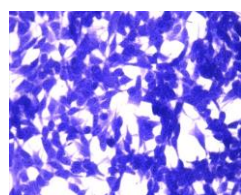


Figure 89 - The effects of dibutyryl cyclic AMP at various concentrations on the viability of SH-SY5Y neuroblastoma cells.

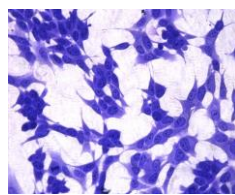
The effects of differentiation on morphology

Crystal violet stain

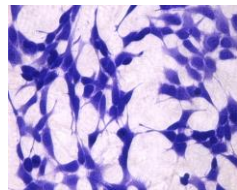
The crystal violet staining of the SH-SY5Y neuroblastoma cells shows a change in the morphology of the cells when they are exposed to 100 and 1000 μ M concentrations of dbcAMP as seen in figure 90 below. Visible changes to the appearance of the cell body was observed along with the increased length of neurite projections.



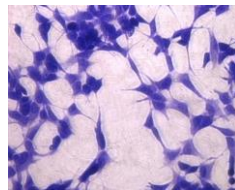
40x Control



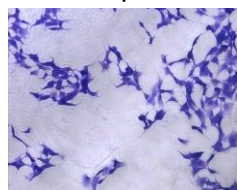
40x 10 μ M dbcAMP



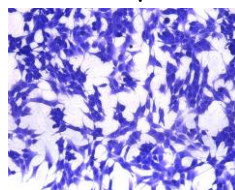
40x 100 μ M dbcAMP



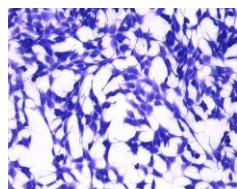
40x 1000 μ M dbcAMP



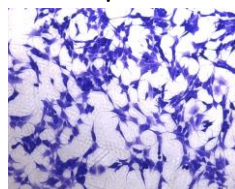
20x Control



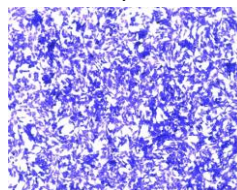
20x 10 μ M dbcAMP



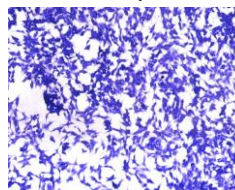
20x 100 μ M dbcAMP



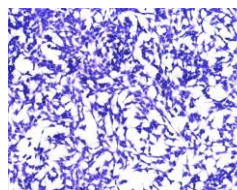
20x 1000 μ M dbcAMP



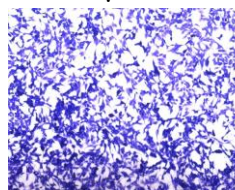
10x Control



10x 10 μ M dbcAMP



10x 100 μ M dbcAMP

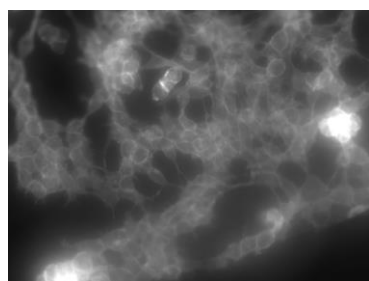


10x 1000 μ M dbcAMP

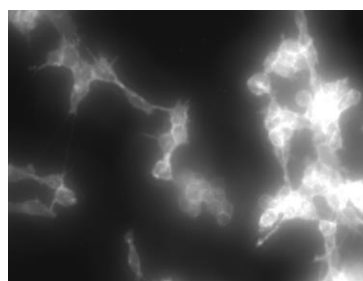
Figure 90 – Crystal violet stained SH-SY5Y neuroblastoma cells exposed to varying concentrations of dibutyryl cyclic AMP.

Neurite projection

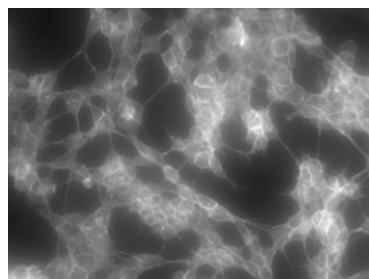
Consistent to what is observed in the crystal violet staining in section 4.5.1, an increase in neurite projections was observed after SH-SY5Y neuroblastoma cells were exposed to dbcAMP. The images (figure 91) show that the optimized method at 100 μM exposure to dbcAMP to have the greatest effect on neurite projections. After exposure to this concentration of dbcAMP, a greater number of projections along with longer projections are observed.



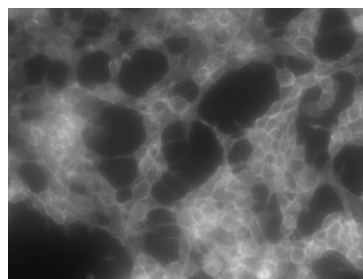
Control



10 μM



100 μM



1000 μM

Figure 91 – The effects of exposure to various concentrations of dbcAMP on the formation of neurite projections

Quantification of noradrenaline production

SH-SY5Y neuroblastoma cells exposed to 100 μ M of dbcAMP showed a statistically significant two-fold increase in NA production ($p < 0.001$) relative to sample protein as determined by the Bradford assay (data not shown) in comparison to the vehicle control. No statistically significant increases in noradrenaline were observed after treatments with the other concentrations across the range tested as seen in figure 92.

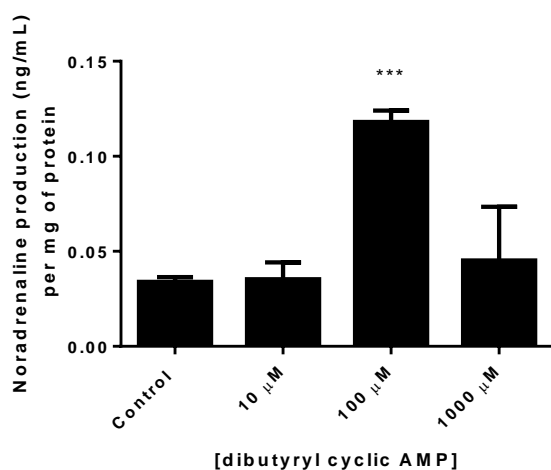


Figure 92 – Noradrenaline production relative to protein content after exposure to dbcAMP (n = 6)

Quantification of serotonin production

There was no significant change in serotonin concentrations produced after exposure to dbcAMP, in comparison to the control as seen in figure 93.

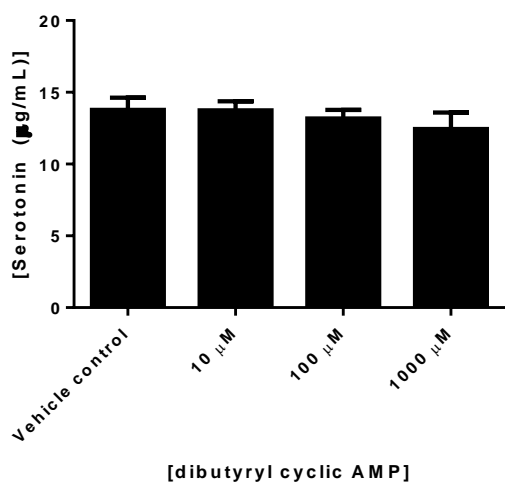


Figure 93 – Serotonin concentrations produced by SH-SY5Y neuroblastoma cells exposed to dbcAMP (n = 9)

Kynurenine Pathway enzyme activities

Significant changes to enzymes in the KP were observed after the differentiation of SH-SY5Y neuroblastoma cells using dbcAMP. The ratio of KYN to Trp, signifying IDO activity, doubled whereas the activities of KAT, signified by the KA to KYN ratio, and KMO, signified by the 3-HK to KYN decreased in differentiated SH-SY5Y neuroblastoma cells in comparison to their undifferentiated counterpart as seen in figure 94 below.

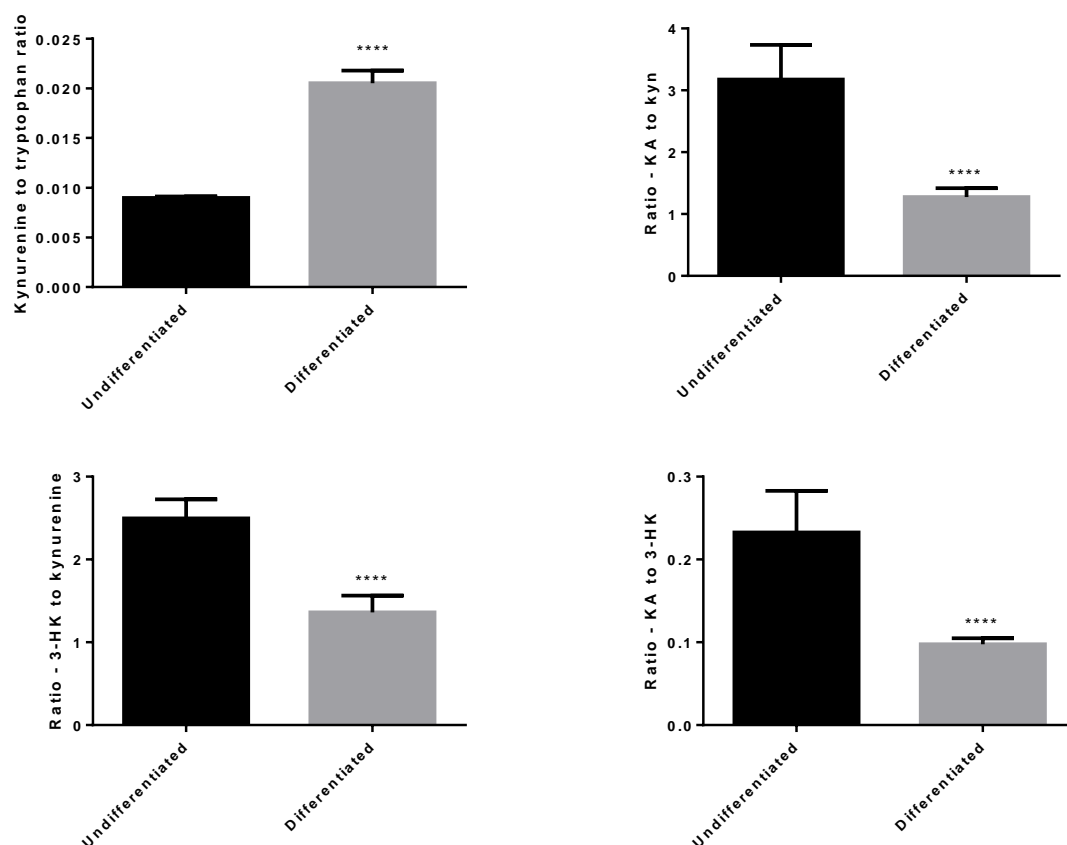


Figure 94 – The changes in the ratios of KMs in undifferentiated and differentiated SH-SY5Y neuroblastoma cells (n = 9)

4.4.4 Toxicity of kynurenine metabolites on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Undifferentiated SH-SY5Y neuroblastoma cells

Resazurin

Statistically significant decreases in the viability of SH-SY5Y neuroblastoma cells were shown after the cells were exposed to 1 μM ($p < 0.05$) and 10 μM ($p < 0.01$) of Trp and 1 μM ($p < 0.01$) and 10 μM ($p < 0.001$) of KYN as seen in figure 95. Although these results were statistically significant, the decrease in viability was modest in nature.

Significant decreases in SH-SY5Y neuroblastoma viability were observed after the exposure of these cells to 1000 μ M concentrations of 3-HK ($p < 0.001$) and 3-HA ($p < 0.001$) as seen in figure 95 and resulted in an approximate decrease in viability of approximately 80% and 70%, respectively.

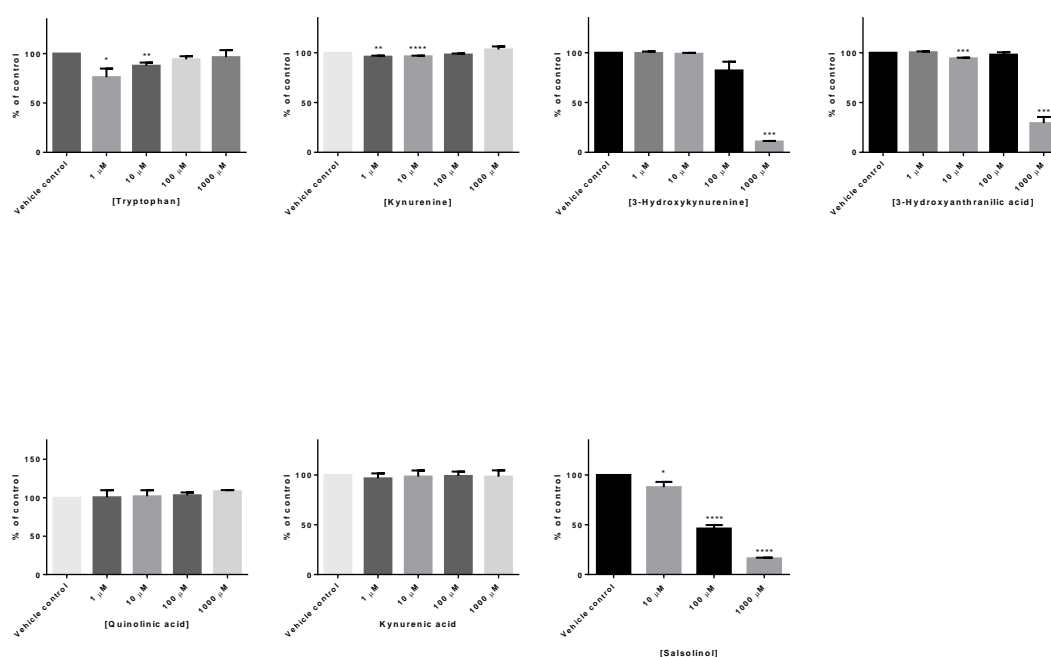


Figure 95 – The effects of kynurenine metabolites on the viability of undifferentiated SH-SY5Y neuroblastoma cells using the resazurin reduction viability assay (n = 9)

Combinations of known neurotoxic KMs provided a modest decrease in the viability of SH-SY5Y neuroblastoma cells. 100 nM, 1 μ M and 10 μ M of each of 3-HK + QA and 3-HA + QA and 10 μ M of 3-HK + 3-HA + QA (all $p < 0.05$) as seen in figure 96. The positive control of the study, salsolinol, afforded a decrease in viability of SH-

SY5Y neuroblastoma cells at 10 ($p < 0.05$), 100 ($p < 0.001$) and 1000 μM ($p < 0.001$) by up to 80%.

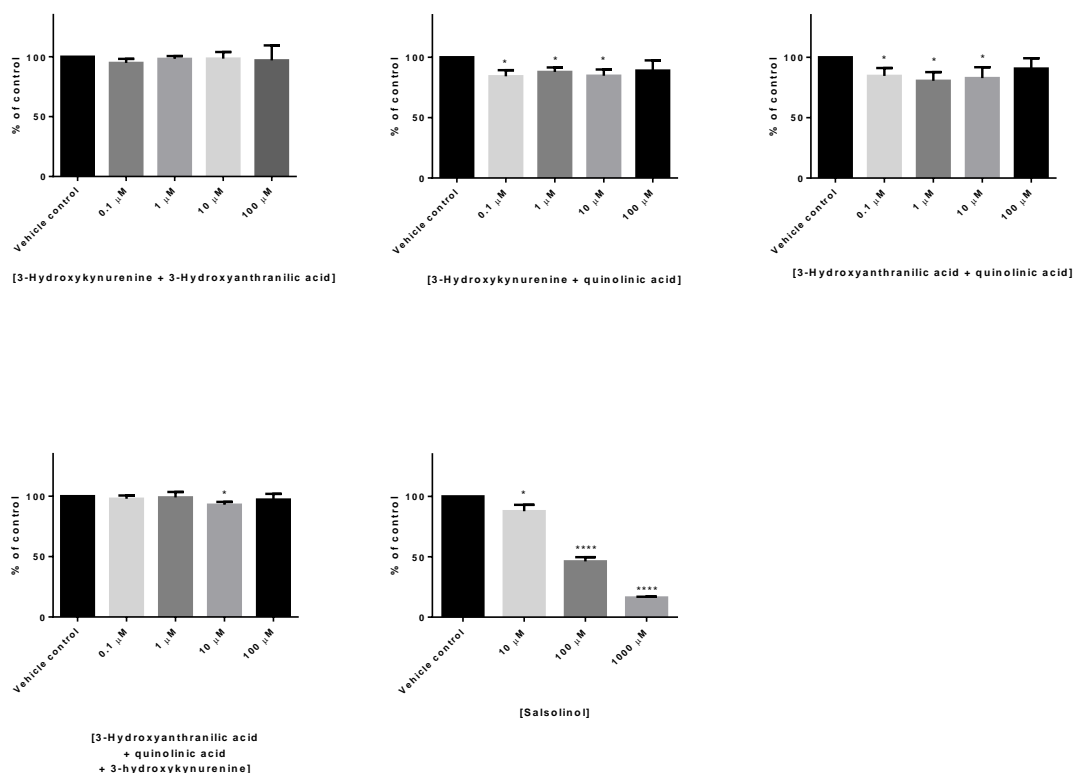


Figure 96 – The effects of combinations of kynurenine metabolites on the viability of undifferentiated SH-SY5Y neuroblastoma cells (n = 9)

72 hour treatment

Seventy two hour exposure of SH-SY5Y neuroblastoma cells to 1000 μM concentrations of 3-HK and 3-HA afforded higher percentages of death ($p < 0.001$) at approximately 90% as seen in figure 97 below. Interestingly, 72 h exposure of 1 and 10 μM concentrations of 3-HA and 1, 10 and 100 μM concentrations of QA afforded an increase in the viability of SH-SY5Y neuroblastoma cells.

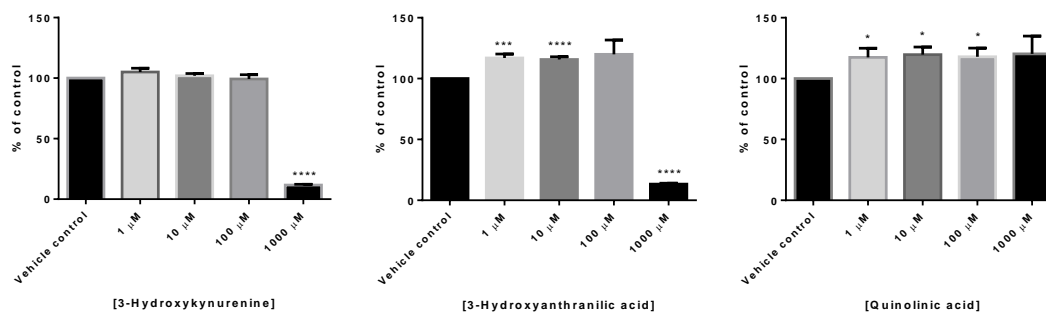


Figure 97 – The effects of 72 h exposure of undifferentiated SH-SY5Y neuroblastoma cells to neurotoxic kynurenine metabolites

Higher percentages of death of undifferentiated SH-SY5Y neuroblastoma cells were observed after 72 h exposure to combinations of KMs in comparison to 24 h exposures. Significant decrease in viability were observed after exposure to 100 μM 3-HA + 3-HK and 3-HA + 3-HK + QA ($p < 0.0001$) by approximately 60% and 40%, respectively, as seen in figure 98.

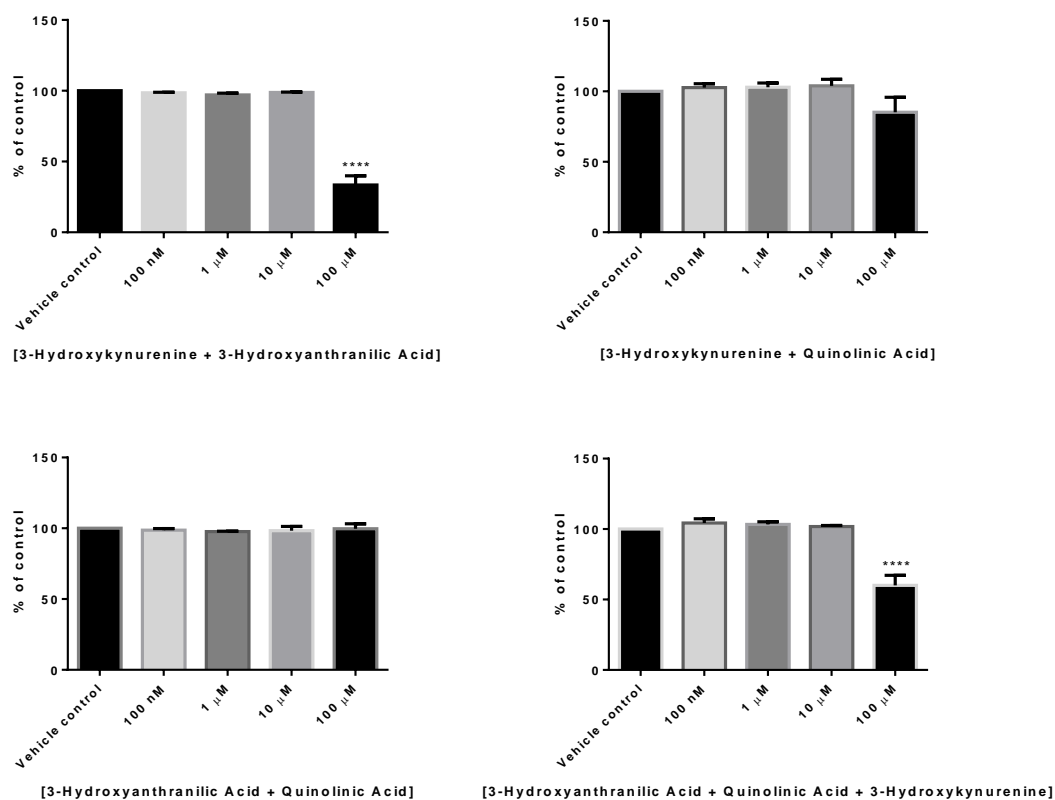


Figure 98 – The effects of 72 h exposure of undifferentiated SH-SY5Y neuroblastoma cells to combinations of neurotoxic kynurenine metabolites (n = 9)

LDH

There was no statistically significant change in LDH release with any treatment group in comparison to the vehicle control. However, consistent with the results obtained in the resazurin reduction viability assay, 1000 μM exposure of undifferentiated SH-SY5Y neuroblastoma cells to 3-HK and 3-HA resulted in trending increases in LDH release as seen in figure 99 below.

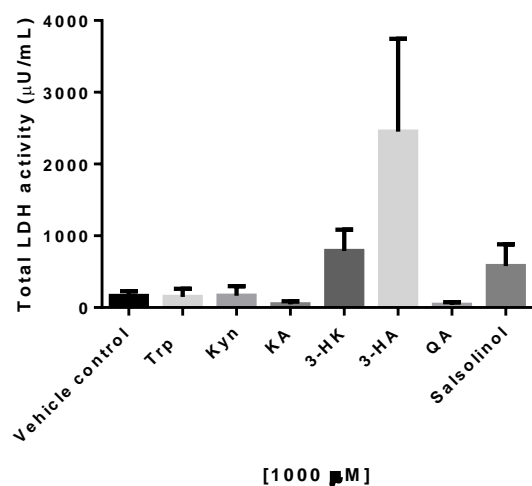


Figure 99 – The effects of 24 h exposure to kynurenine metabolites (1000 µM) on LDH activity in undifferentiated SH-SY5Y neuroblastoma cells (n = 3)

No statistically significant changes in LDH activity were observed after undifferentiated SH-SY5Y cells were exposed to combinations of neurotoxic KYN for 24 h. No detectable LDH activity was observed after undifferentiated SH-SY5Y cells were exposed to 3-HK + QA as seen in figure 100 below.

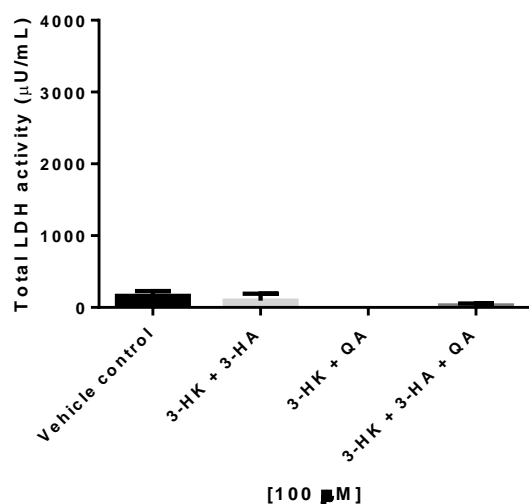


Figure 100 – The effects of 24 h exposure to combinations of kynurenine metabolites (100 μM) on LDH activity in undifferentiated SH-SY5Y neuroblastoma cells (n = 3)

The effects of KMs on apoptosis

Annexin V activation

Annexin V activation, an early marker of apoptosis, was observed in SH-SY5Y neuroblastoma cells exposed to 1000 μM of 3-HK and 3-HA, with 5-fold and 2-fold increases observed respectively as seen in figure 101 below.

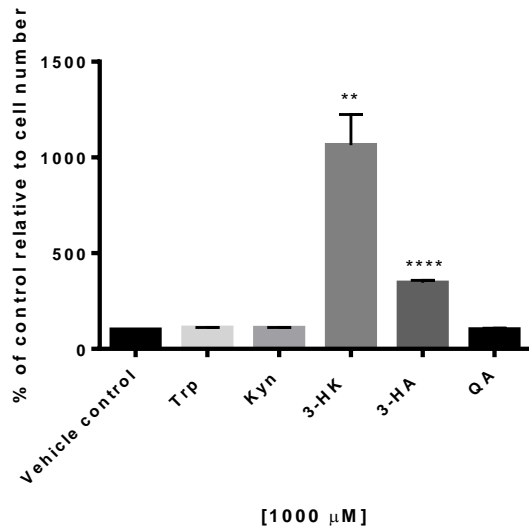


Figure 101 – The effects of kynurenine metabolites on annexin V activation in undifferentiated SH-SY5Y neuroblastoma cells (n = 3)

Annexin V activation was observed in undifferentiated SH-SY5Y neuroblastoma cells exposed to 100 μM 3-HK in combination with 100 μM QA ($p < 0.01$), however this was only a modest increase as seen in figure 102 below.

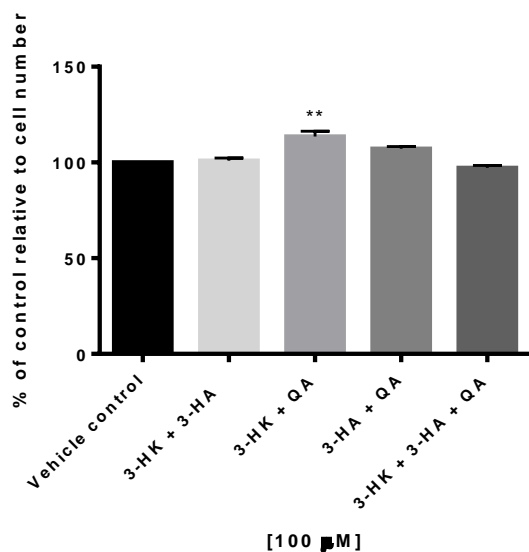


Figure 102 – The effects of combinations of neurotoxic kynurenine metabolites on annexin V activation in undifferentiated SH-SY5Y neuroblastoma cells (n = 3)

Propidium Iodide

Propidium iodide dye, a marker of necrosis, was used in this study. Undifferentiated SH-SY5Y neuroblastoma cells exposed to 1000 μ M of 3-HK and 3-HA showed 5-fold and 2-fold higher fluorescence intensity, respectively, in comparison to the vehicle control as seen in figure 103 below.

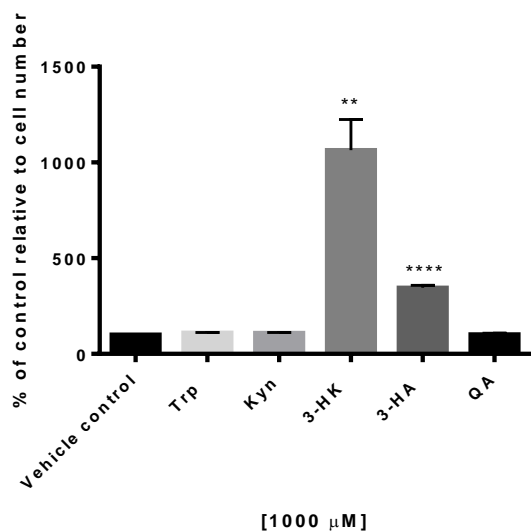


Figure 103 – The effects of exposure of kynurenine metabolites (24 h) to SH-SY5Y neuroblastoma cells on necrosis (n = 3)

Undifferentiated SH-SY5Y neuroblastoma cells exposed to 100 μ M of 3-HK in combination with QA ($p < 0.05$) and 100 μ M 3-HA in combination with QA ($p < 0.05$) showed higher fluorescence intensity in comparison to the vehicle control, however, this was modest in nature as seen in figure 104 below.

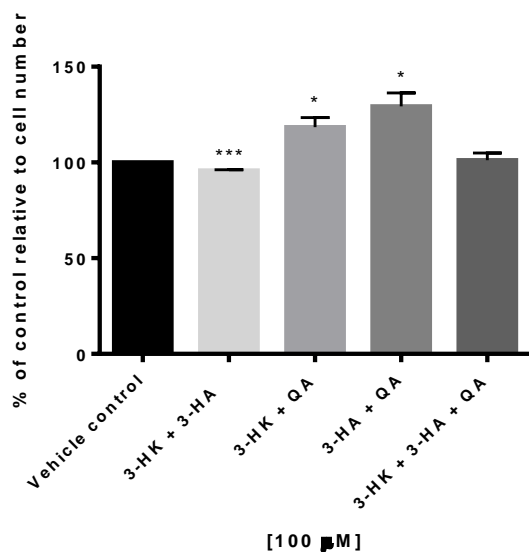


Figure 104 - The effects of exposure of combinations of neurotoxic kynurenine metabolites (24 h) to SH-SY5Y neuroblastoma cells on necrosis (n = 3)

The effects of KMs on free radical production

DCFH-DA assay

Free radical production, measured using the DCFH-DA assay, was shown to be altered after the exposure of undifferentiated SH-SY5Y neuroblastoma cells to a number of the KMs. Increases were observed in these cells after 24 h exposure to KYN (100 μM), KA (10 μM, 100 μM and 1000 μM), 3-HK (1000 μM), 3-HA (1000 μM) and QA (1000 μM), however, all increases were modest in nature. Likewise, the positive control salsolinol, showed a concentration-dependent increase in free radical production as expected. These results can be seen in figure 105 below.

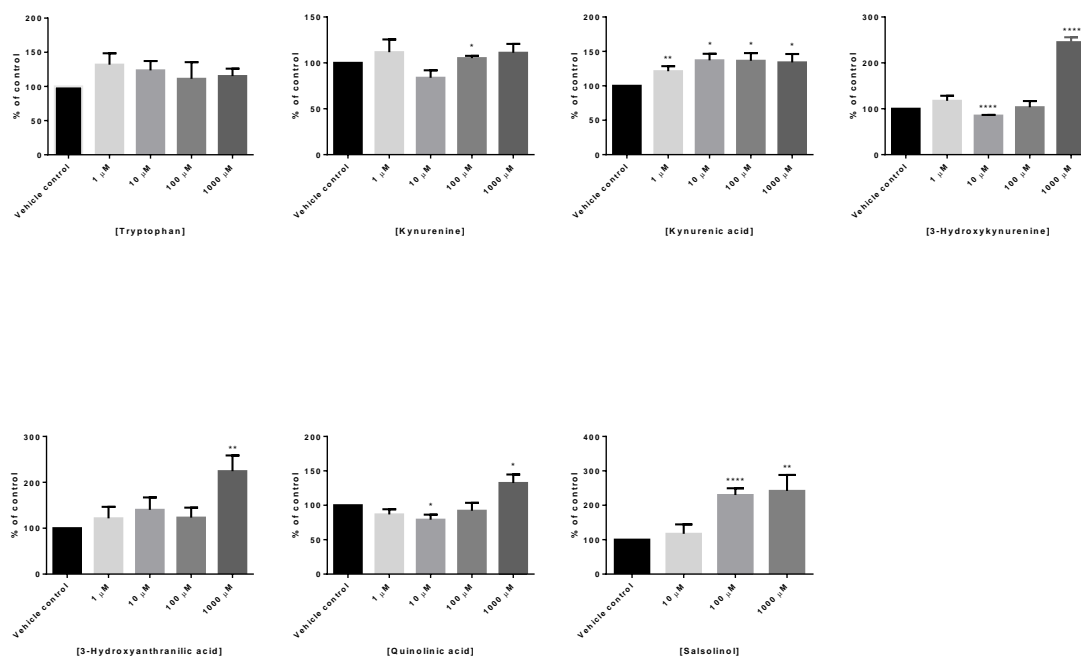


Figure 105 – The effects of kynurenine metabolites on free radical production in undifferentiated SH-SY5Y neuroblastoma cells using the DCFH-DA assay (n = 9)

Combinations of KMs were also shown to increase free radical production. Combinations of 3-HK + 3-HA (100 nM, 1 μ M, 10 μ M and 100 μ M), 3-HA + QA (1 μ M, 10 μ M and 100 μ M) and 3-HK + 3-HA + QA (100 nM, 1 μ M, 10 μ M and 100 μ M) were all shown to significantly increase free radical formation, up to 50%, in undifferentiated SH-SY5Y neuroblastoma cells after 24 h exposure as seen in figure 106 below.

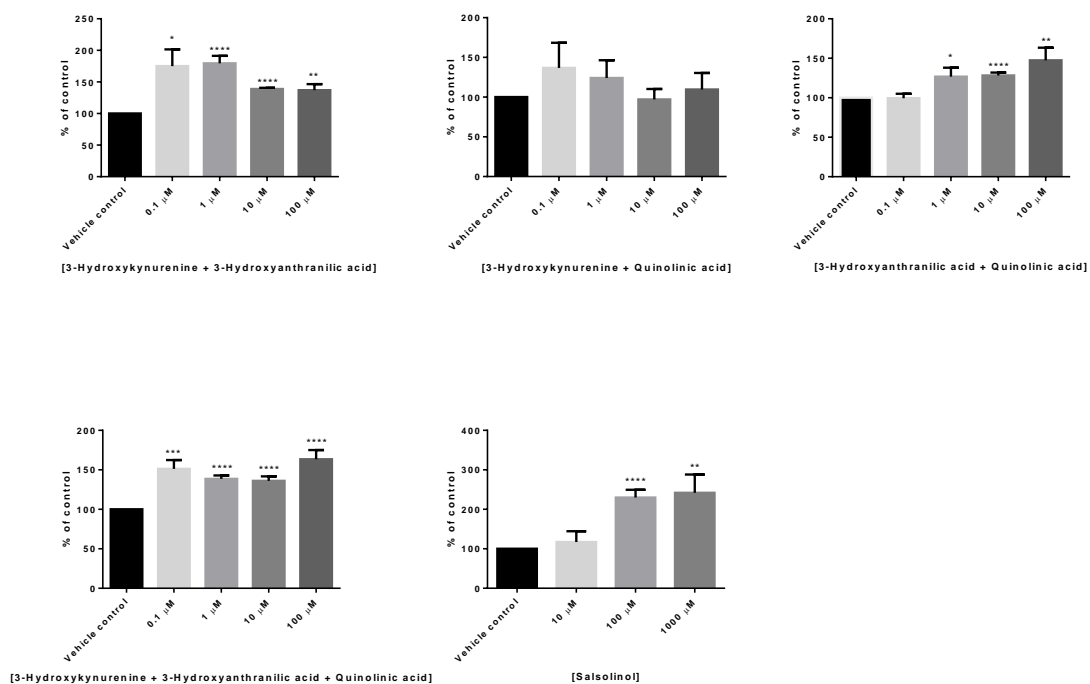


Figure 106 – The effects of combinations of kynurenine metabolites on free radical production using the DCFH-DA assay in undifferentiated SH-SY5Y neuroblastoma cells (n = 9)

DHR 123 assay

DHR 123 was used to evaluate the effects of KMs on the production of intracellular free radicals, in particular superoxide. This study showed that both 3-HK (1000 μM) ($p < 0.05$) and 3-HA (1000 μM) ($p < 0.001$) increased intracellular free radical production, 8-fold and 2-fold, respectively, as seen in figure 107 below.

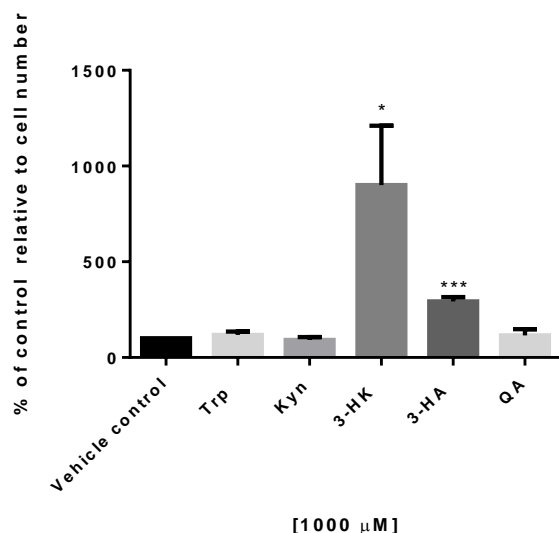


Figure 107 – The effects of kynurenine metabolites on the production on mitochondrial free radicals using DHR 123 in undifferentiated SH-SY5Y neuroblastoma cells (n = 9)

Furthermore, figure 108 below shows the effects of combinations of neurotoxic KMs on the production of mitochondrial free radicals using DHR 123. No statistically significant increases in mitochondrial free radical production were observed however, the combinations of 3-HK and 3-HA ($p < 0.05$) and 3-HK, 3-HA and QA ($p < 0.01$) both decreased the production of mitochondrial free radicals, however, these decreases are modest in nature.

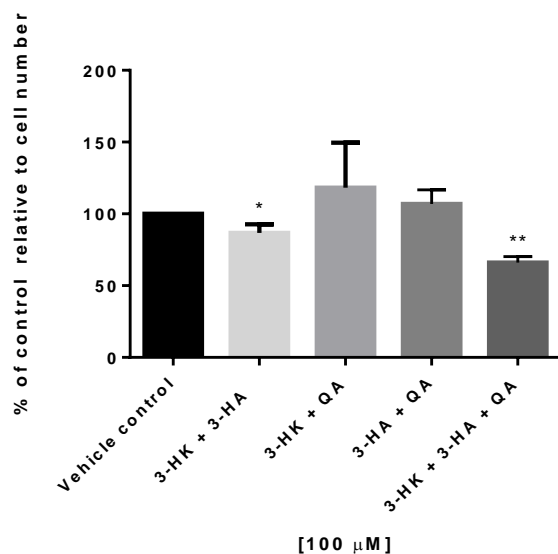


Figure 108 – The effects of combinations of neurotoxic kynurenine metabolites on the production of mitochondrial free radicals using DHR 123 in undifferentiated SH-SY5Y neuroblastoma cells (n = 9)

Differentiated SH-SY5Y neuroblastoma cells

The effects of KMs on the viability of differentiated SH-SY5Y neuroblastoma cells

Resazurin

Similar trends of cytotoxicity were observed with differentiated SH-SY5Y neuroblastoma cells as those observed in the undifferentiated phenotype. Significant decreases in viability were observed in differentiated SH-SY5Y neuroblastoma cells treated with 3-HK (1000 μ M) and 3-HA (1000 μ M), both by approximately 50% as seen in figure 109. Again, exposure to the positive control, salsolinol, resulted in the concentration-dependent decrease in viability, as observed in undifferentiated SH-SY5Y neuroblastoma cells.

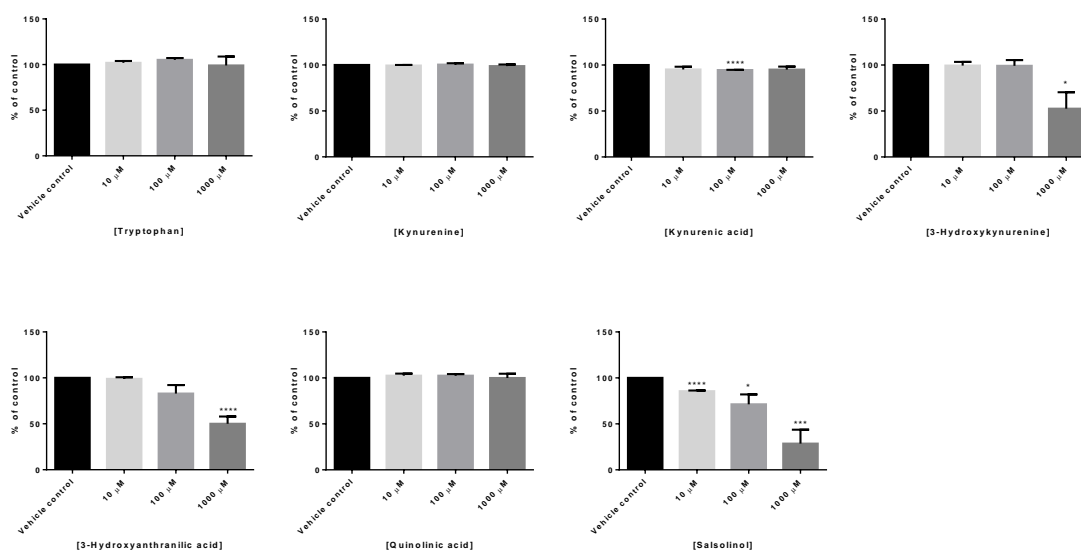


Figure 109 – The effects of kynurenine metabolites on the viability of differentiated SH-SY5Y neuroblastoma cells using resazurin (n = 9)

Differentiated SH-SY5Y neuroblastoma cells were exposed to combinations of KMs resulted in decreased viability of SH-SY5Y neuroblastoma cells. 3-HK + QA (10 μ M) and 3-HK + 3-HA + QA (10 μ M and 100 μ M) decreased the viability of these cells however this is modest in nature as seen in figure 110 below.

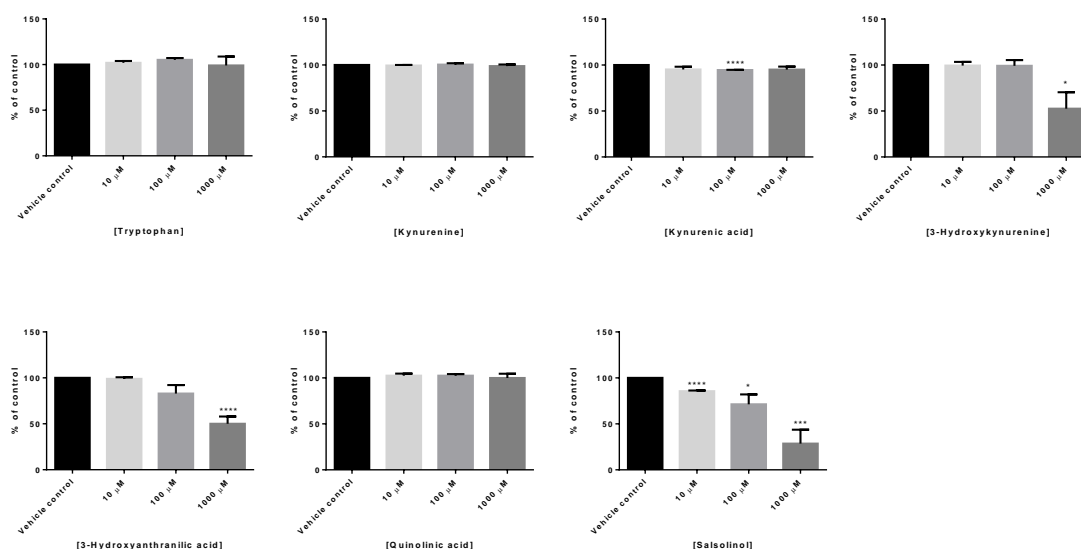


Figure 110 – The effects of combinations of neurotoxic kynurenine metabolites on the viability of differentiated SH-SY5Y neuroblastoma cells (n = 9)

72 hours

Seventy two hour exposure of differentiated SH-SY5Y neuroblastoma cells to KMs again resulted in modest decreases in proliferation using the resazurin reduction proliferation assay. KYN (10 μ M), KA (1 μ M, 10 μ M and 1000 μ M) and 3-HA (1 μ M and 10 μ M) significantly but modestly decreased the viability of these cells after 72 h exposure. Exposure to 3-HK and 3-HA (100 μ M and 1000 μ M) showed a concentration-dependent decrease in viability however this was not statistically significant as seen in figure 111 below.

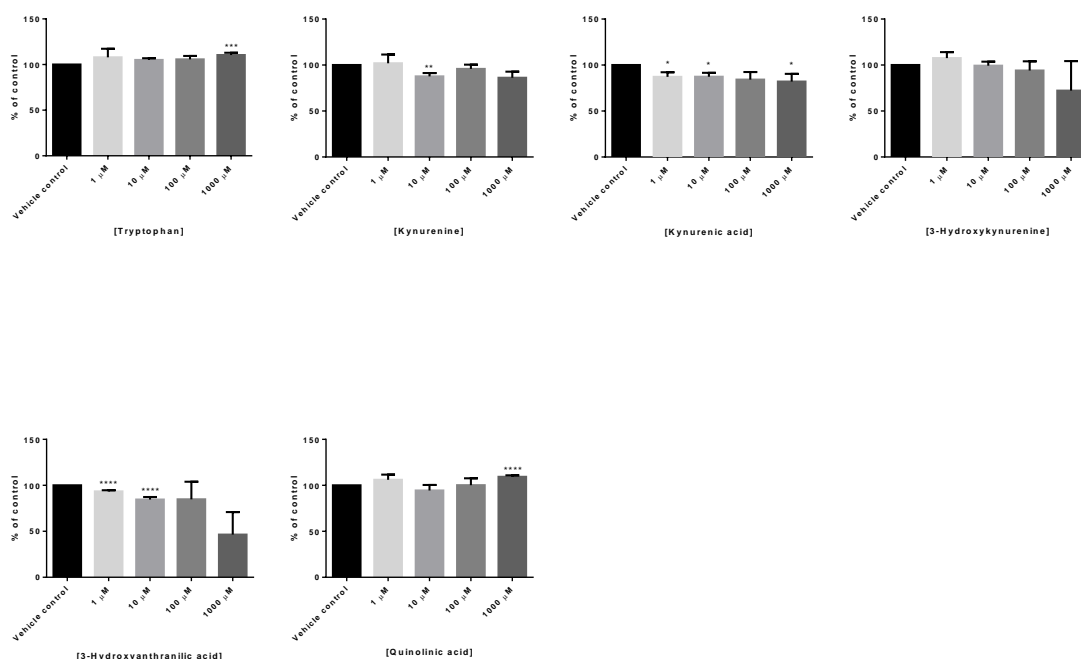


Figure 111 – The effects of 72 h exposure of kynurenine metabolites to differentiated SH-SY5Y neuroblastoma cells using the resazurin reduction assay (n = 9)

Seventy two hour exposure of SH-SY5Y neuroblastoma cells to combinations of KMs resulted in the decrease viability. Concentration-dependent decreases in viability were observed in cells exposed to 3-HA + 3-HK, 3-HA + QA and 3-HK + 3-HA + QA, with decreases of approximately 30% observed as seen in figure 112 below.

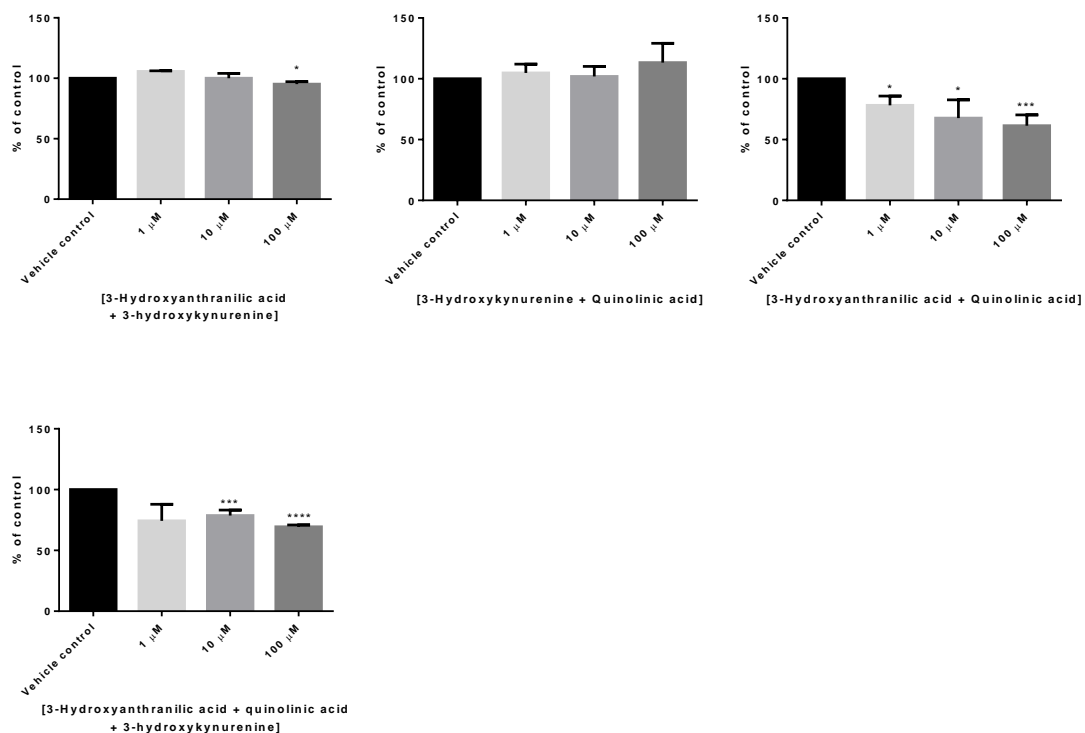


Figure 112 – The effects of 72 h exposure of combinations of neurotoxic kynurenine metabolites to the viability of differentiated SH-SY5Y neuroblastoma cells (n = 9)

LDH assay

No significant differences in LDH activity was shown after dbcAMP differentiated SH-SY5Y neuroblastoma cells exposed to KMs as seen in figure 113 below.

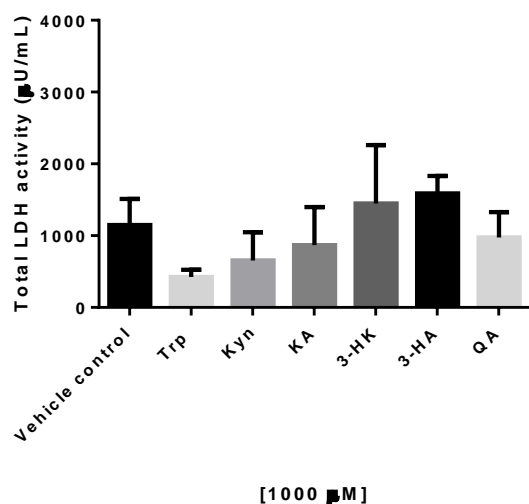


Figure 113 – Total LDH activity relative to cell number in differentiated SH-SY5Y neuroblastoma cells after 24 h exposure to kynurenine metabolites (n = 3)

No significant differences in LDH activity was shown after dbcAMP differentiated SH-SY5Y neuroblastoma cells exposed to combinations of neurotoxic KMs as seen in figure 114 below.

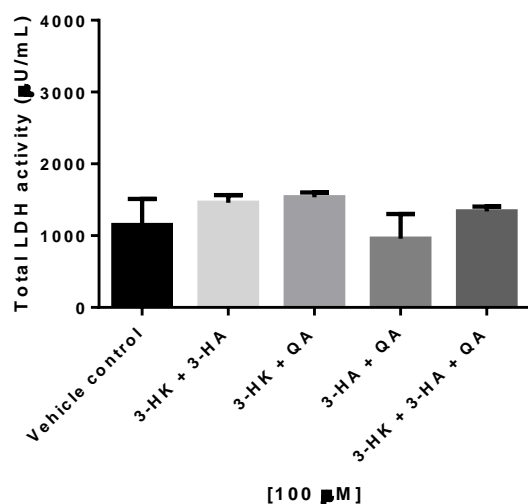


Figure 114 – Total LDH activity relative to cell number after differentiated SH-SY5Y neuroblastoma cells were exposed to 72 h combinations of kynurenine metabolites (n = 3)

The effects of KMs on apoptosis in differentiated SH-SY5Y neuroblastoma cells

Annexin V activation

Annexin V activation was observed after exposure to a number of KMs. Increased apoptosis was observed after exposure to 3-HK (1000 μ M) and 3-HA (1000 μ M) by approximately double to that observed in the vehicle control exposed cells as seen in figure 115 below.

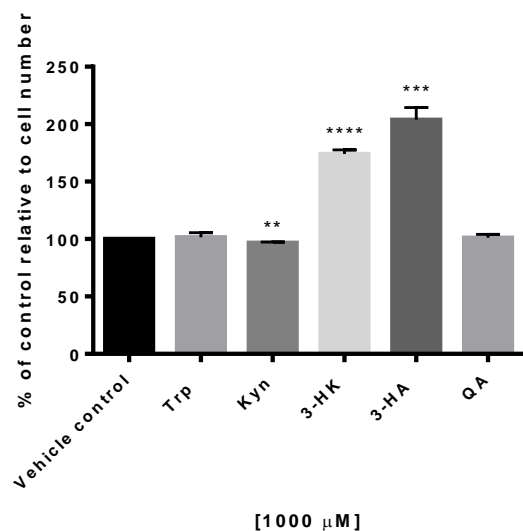


Figure 115 – Annexin V activation relative to cell number after differentiated SH-SY5Y neuroblastoma cells exposed to kynurenine metabolites (n = 3)

Annexin V activation was observed after differentiated SH-SY5Y neuroblastoma cells were exposed to a combination of 100 μ M of the two neurotoxic KMs 3-HK and QA ($p < 0.0001$), approximately 50% higher than that observed in cells exposed to the vehicle control as seen in figure 116 below.

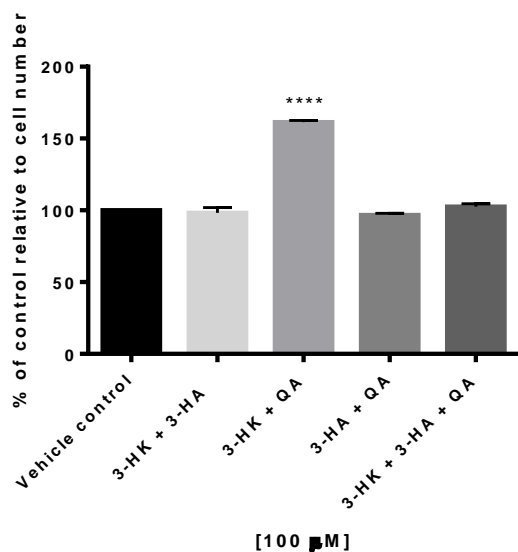


Figure 116 – Annexin V activation relative to cell number after differentiated SH-SY5Y neuroblastoma cells were exposed to combinations of neurotoxic kynurenine metabolites (n = 3)

Propidium Iodide

Necrosis in differentiated SH-SY5Y neuroblastoma cells was significantly decreased after 24 h exposure to 1000 μ M Trp ($p < 0.01$) and KYN ($p < 0.05$), by approximately 40% in comparison to the control. Although not statistically significant, trending increases in necrosis was observed after 24 h exposure to 3-HK and 3-HA as seen in figure 117 below.

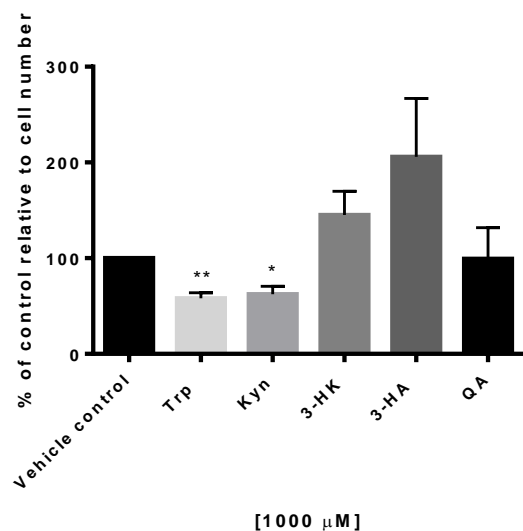


Figure 117 – The effects of kynurenine metabolites on necrosis in differentiated SH-SY5Y neuroblastoma cells (n = 3)

Necrosis in differentiated SH-SY5Y neuroblastoma cells exposed to a combination of 3-HK and QA ($p < 0.001$) and 3-HA and QA ($p < 0.001$), by approximately 100% and 20%, respectively were observed as seen in figure 118 below.

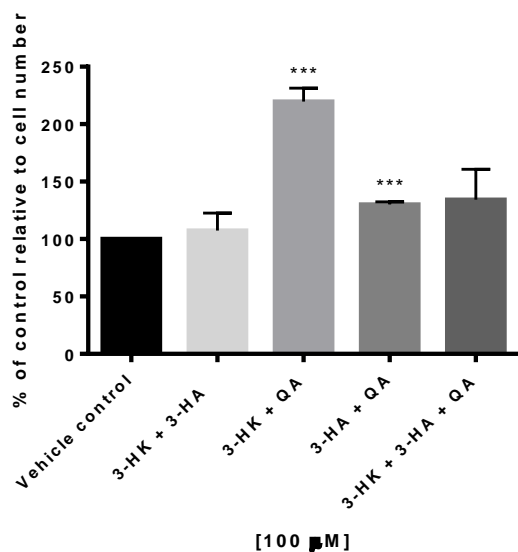


Figure 118 - The effects of combinations of neurotoxic kynurenine metabolites on necrosis in differentiated SH-SY5Y neuroblastoma cells (n = 3)

Free radical formation after differentiated SH-SY5Y neuroblastoma cells are exposed to KMs

DCFH-DA assay

Increased free radical production was observed in differentiated SH-SY5Y neuroblastoma cells 10 μ M KYN ($p < 0.01$) and in a dose-dependent manner in cells treated with the positive control salsolinol. Conversely, 100 μ M and 1000 μ M of KA and 1000 μ M KYN significantly decreased free radical production as seen in figure 119 below. All changes in free radical production were modest in nature.

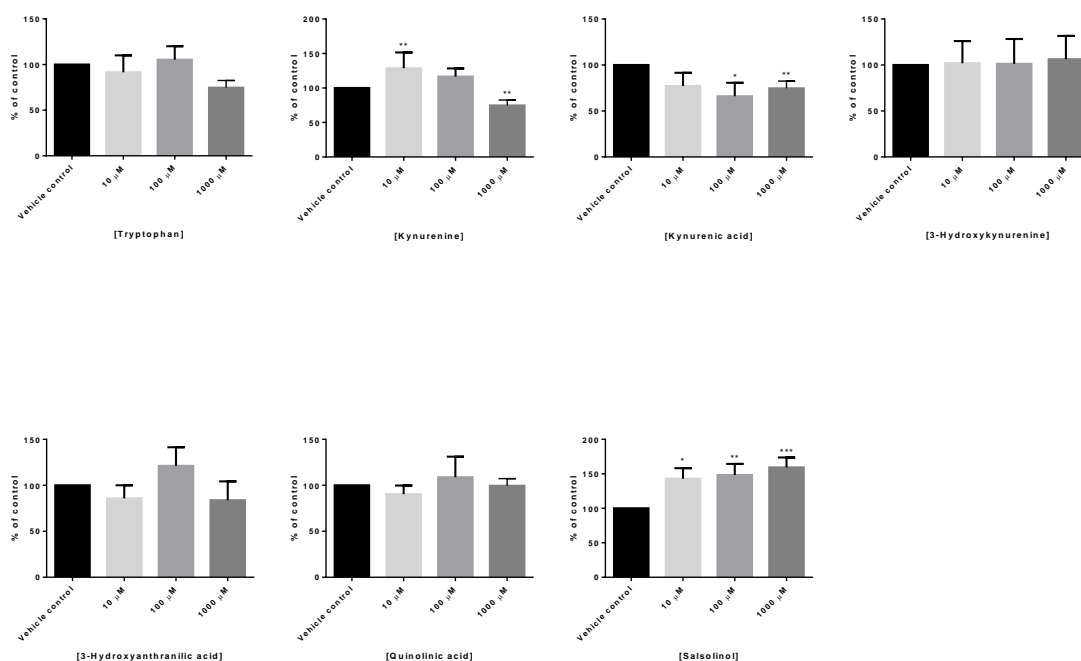


Figure 119 – Free radical production after differentiated SH-SY5Y neuroblastoma cells were exposed to kynurenine metabolites using the DCFH-DA assay (n = 9)

No significant increase in free radical production was observed in differentiated SH-SY5Y neuroblastoma cells exposed to combinations of KMs. Concentration-dependent trends in free radical production were observed however. These trends were observed after 24 h exposure to 3-HA + QA and 3-HK + 3-HA + QA as seen in figure 120 below.

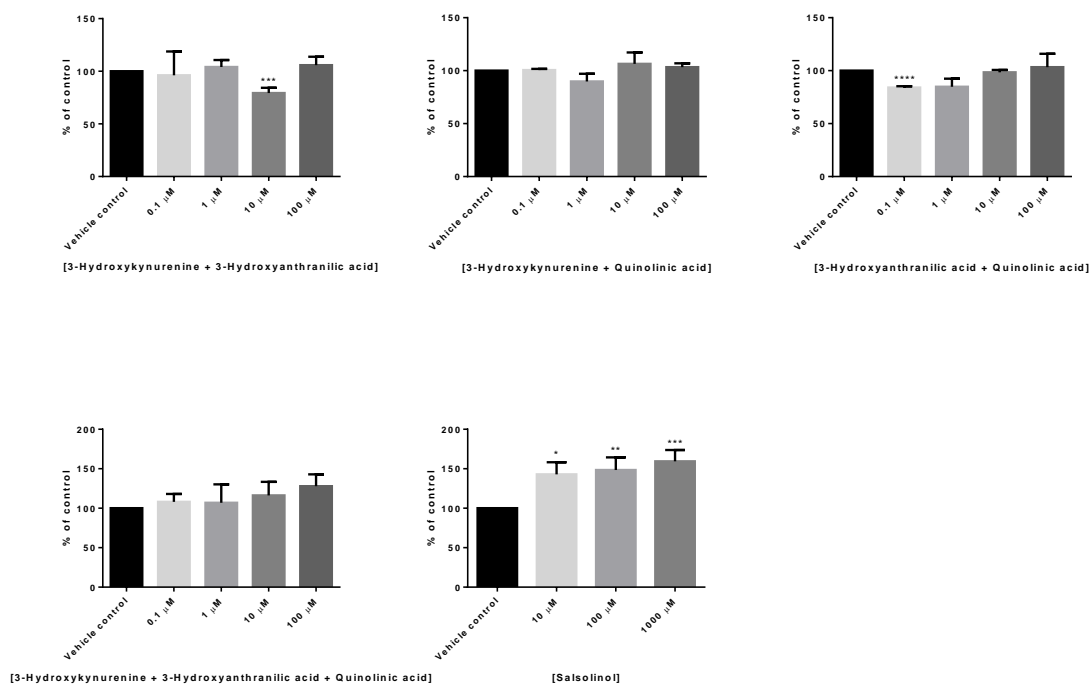


Figure 120 – Free radical production after differentiated SH-SY5Y neuroblastoma cells were exposed to combinations of neurotoxic kynurenine metabolites using the DCFH-DA assay (n = 9)

DHR 123

Statistically significant increases in mitochondrial free radical production, as observed using the DHR 123 assay, were seen in differentiated SH-SY5Y neuroblastoma cells exposed to 3-HK (1000 μ M) ($p < 0.05$) and 3-HA (1000 μ M) ($p < 0.01$), 3-fold and 1-fold increases, respectively, were observed as observed in figure 121.

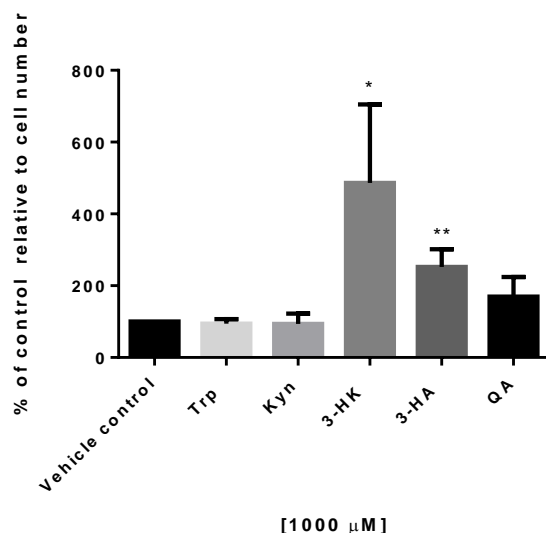


Figure 121 – Mitochondrial free radical production after differentiated SH-SY5Y neuroblastoma cells exposed to 1000 μM kynurenine metabolites using DHR 123 (n = 9)

4.4.5 Toxicity of cytokines on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Undifferentiated SH-SY5Y neuroblastoma cells

The effects of cytokines on the viability of undifferentiated SH-SY5Y neuroblastoma cells

Resazurin

Modest, statistically significant decreases in viability were observed after undifferentiated SH-SY5Y neuroblastoma cells were treated with human IFN- α_{2A} (6.25 ng/mL, 12.5 ng/mL, 25 ng/mL and 50 ng/mL) and human IFN- γ (6.25 ng/mL, 12.5 ng/mL and 25 ng/mL). A modest increase in proliferation was observed after

undifferentiated SH-SY5Y cells were exposed to 2000 pM of TNF- α as seen in figure 122 below.

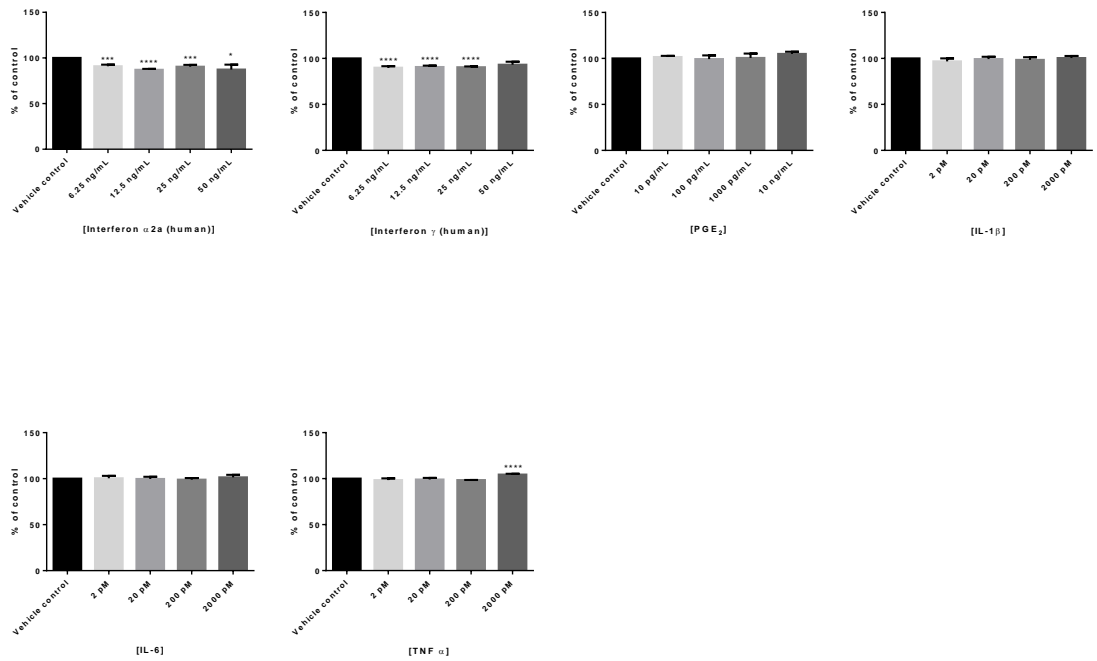


Figure 122 – Changes to the viability of undifferentiated SH-SY5Y neuroblastoma cells after 24 h exposure to pro-inflammatory cytokines (n = 9)

LDH assay

Significant increases in LDH activity were observed after undifferentiated SH-SY5Y neuroblastoma cells were exposed to IL-6, IL-1 β and TNF- α approximately 10-fold as seen in figure 123 below. No changes in LDH were observed after undifferentiated SH-SY5Y neuroblastoma cells were exposed to IFN- α , IFN- γ and PGE₂.

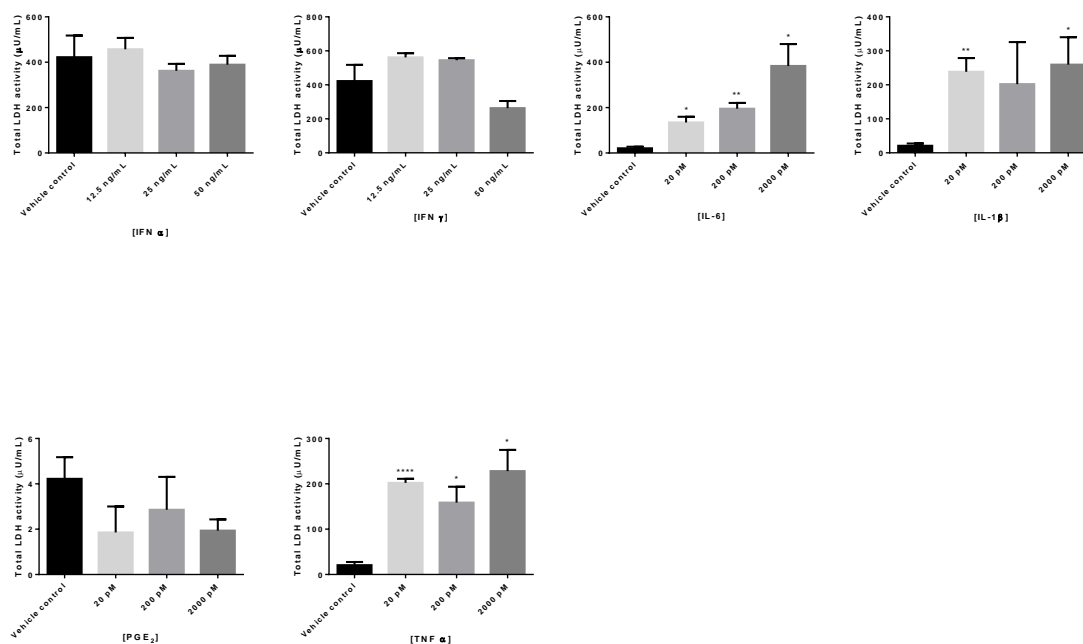


Figure 123 – Total LDH activity relative to cell number in undifferentiated SH-SY5Y exposed to pro-inflammatory cytokines (n = 3)

Free radical formation after undifferentiated SH-SY5Y neuroblastoma cells are exposed to cytokines

DCFH-DA assay

A significant decrease in oxidative stress was observed after undifferentiated SH-SY5Y cells were exposed to IL-1 β (200 pM and 2000 pM), IL-6 (2 pM) and TNF- α (2 pM, 20 pM, 200 pM and 2000 pM). The decreases in IL-1 β and IL-6 were modest in nature. Conversely, TNF- α decreases free radical production by approximately 40% as seen in figure 124 below.

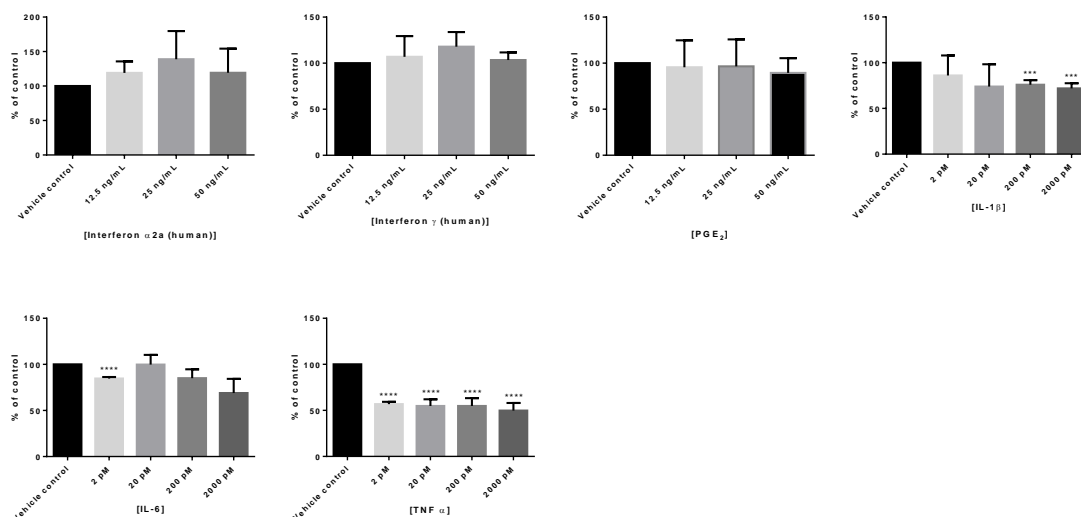


Figure 124 –Free radical production in undifferentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory cytokines (n = 9)

Markers of apoptosis after undifferentiated SH-SY5Y neuroblastoma cells are exposed to cytokines

Annexin V activation

A decrease in annexin V activation was observed with a number of cytokine treatments in comparison to the vehicle control. These included IFN-α_{2A} (12.5 ng/mL and 25 ng/mL), IFN-γ (50 ng/mL), IL-1β (20 pM and 2000 pM), IL-6 (20 pM and 200 pM) and TNF-α (20 pM and 200 pM) as seen in figure 125.

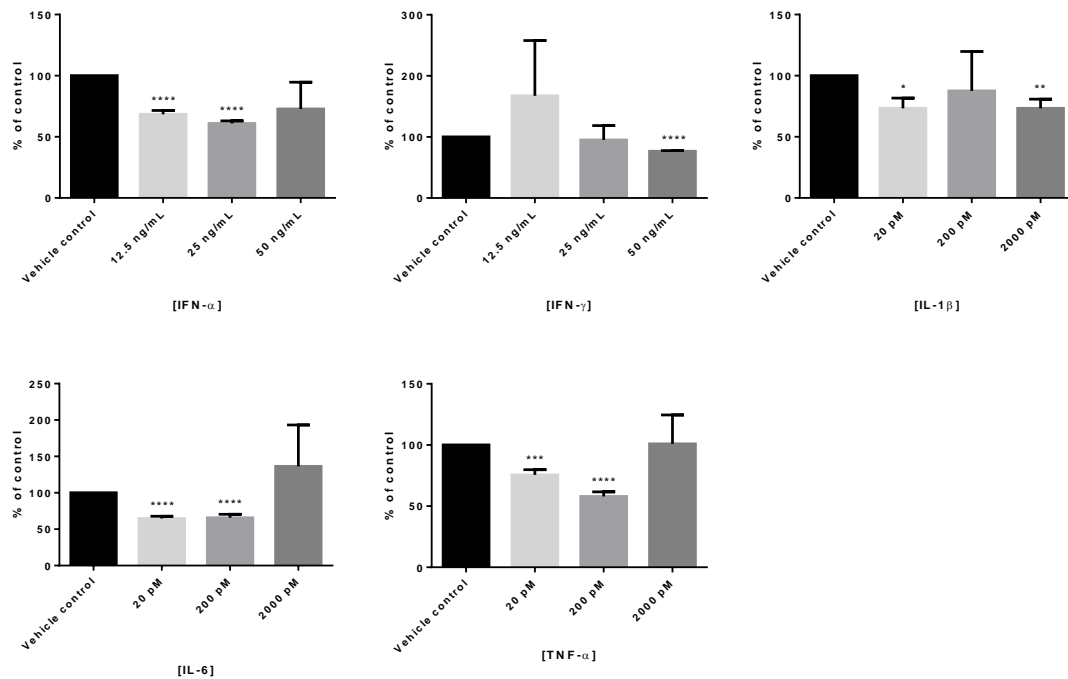


Figure 125 – Annexin V activation in undifferentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory cytokines (n = 9)

The effects of cytokines on necrosis measured using propidium iodide

In addition to a decrease in markers of apoptosis, a decrease in necrosis was also observed after the treatment of undifferentiated SH-SY5Y neuroblastoma cells with various cytokines. These include IFN- α_{2A} (50 ng/mL), IFN- γ (12.5 ng/mL), IL-1 β (20 pM and 200 pM), IL-6 (20 pM, 200 pM and 2000 pM) and TNF- α (200 pM and 2000 pM) as seen in figure 126.

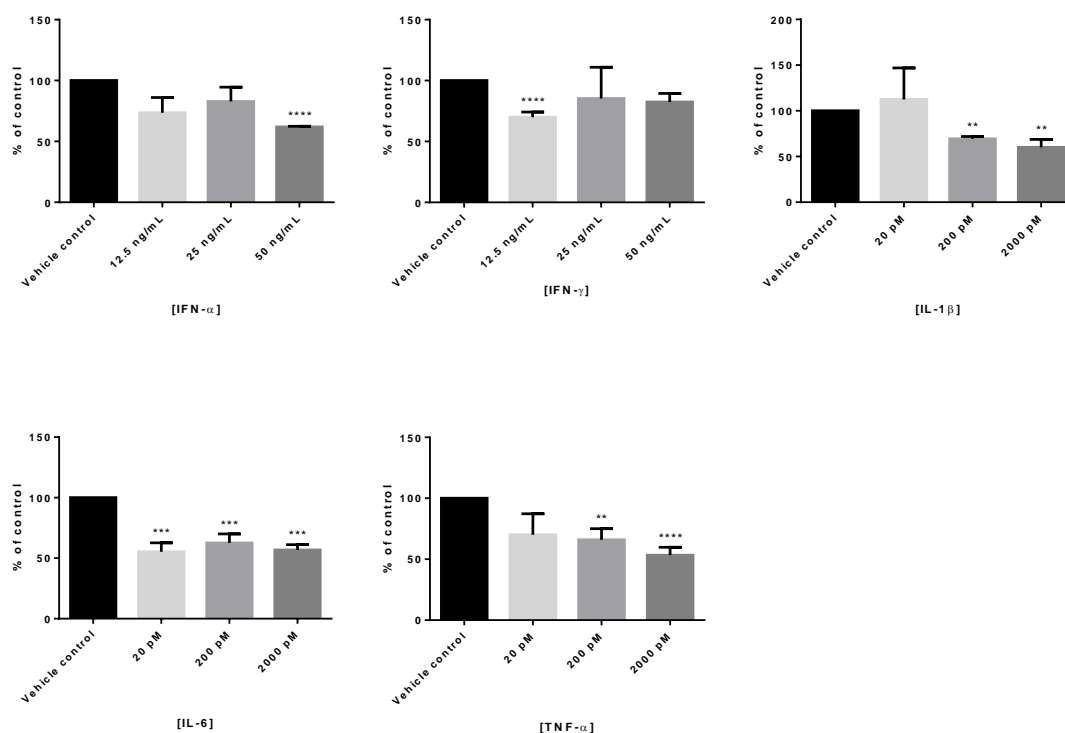


Figure 126 – Necrosis, measured using propidium iodide, in undifferentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory cytokines (n = 3)

Differentiated SH-SY5Y neuroblastoma cells

The effects of cytokines on the viability of differentiated SH-SY5Y neuroblastoma cells

Resazurin

A modest decrease in the proliferation of differentiated SH-SY5Y neuroblastoma cells was observed after treatment with IFN- α_{2A} (6.25 ng/mL, 12.5 ng/mL, 25 ng/mL and 50 ng/mL). A modest increase in proliferation was observed after the treatment of these cells with IL-6 (2000 pM) as seen in figure 127.

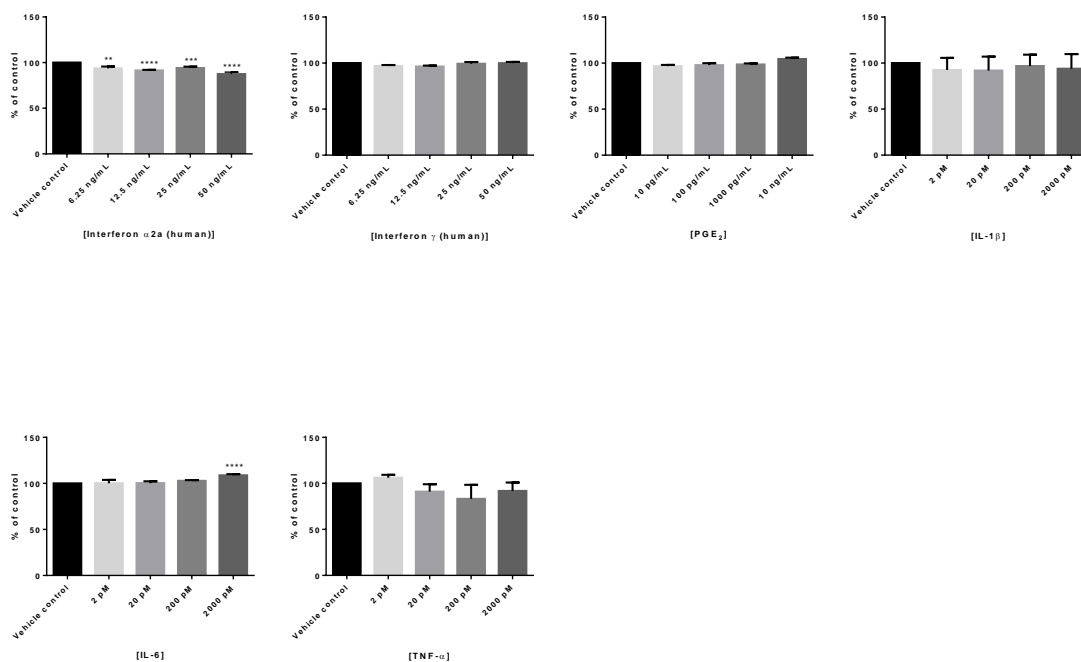


Figure 127 – Changes to the viability of differentiated SH-SY5Y neuroblastoma cells after 24 h exposure to pro-inflammatory cytokines (n = 9)

LDH assay

Statistically significant increases in LDH activity was observed after differentiated SH-SY5Y neuroblastoma cells were exposed to PGE₂. These increases were approximately double that of cells treated with the vehicle control and can be seen in figure 128 below.

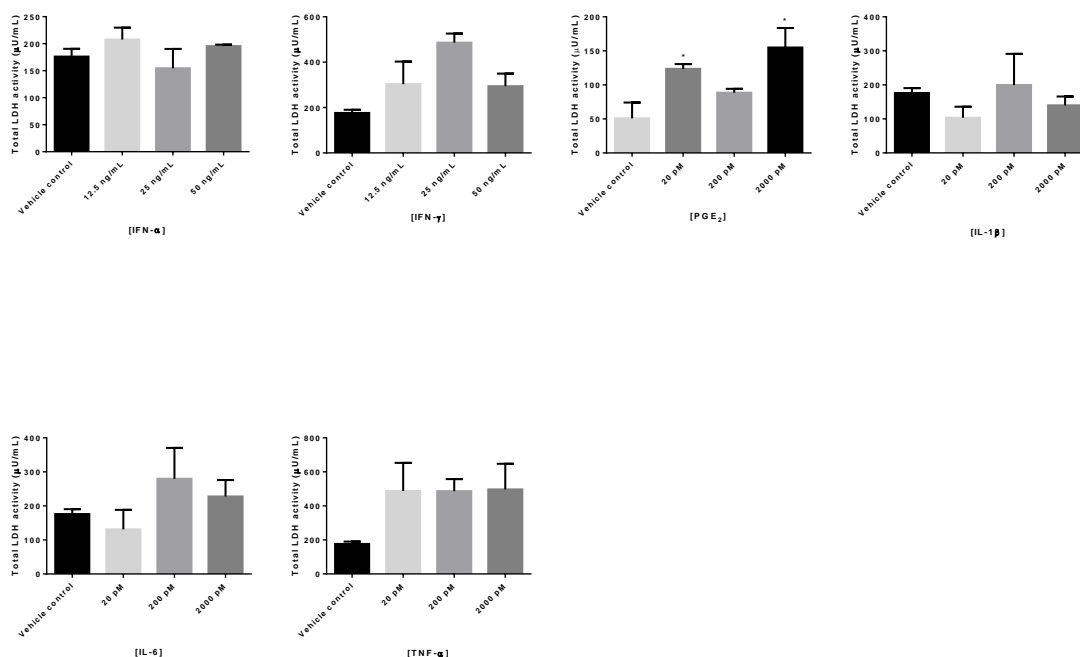


Figure 128 – Changes to the viability of undifferentiated SH-SY5Y neuroblastoma cells after 24 h exposure to pro-inflammatory cytokines (n = 9)

Free radical formation in differentiated SH-SY5Y neuroblastoma cells exposed to cytokines

DCFH-DA assay

Increased free radical production was observed after differentiated SH-SY5Y neuroblastoma cells were exposed to TNF-α (20 pM and 200 pM). Conversely, IL-6 (2000 pM) exposure decreased the production of free radicals. All changes were modest in nature as seen in figure 129 below.

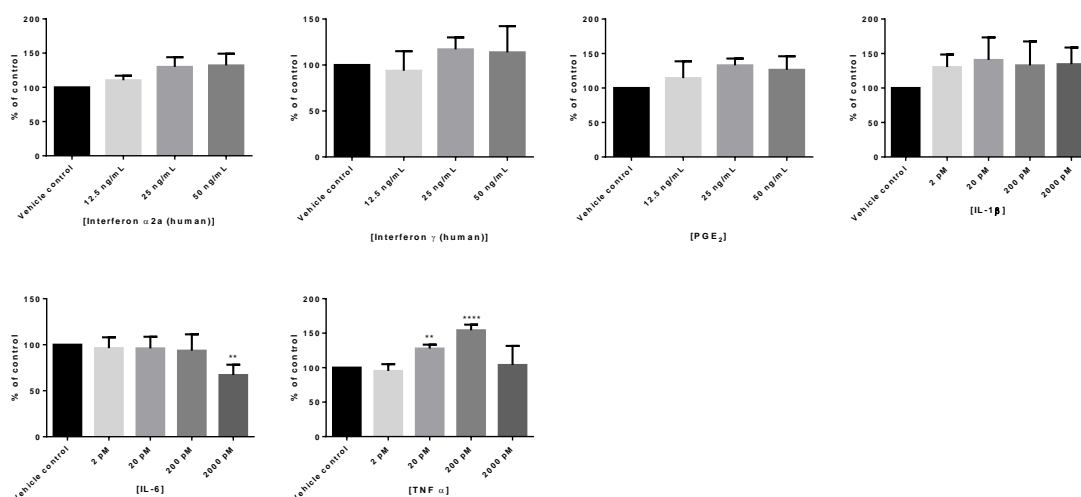


Figure 129 – Free radical production in differentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory cytokines (n = 9)

Annexin V activation as a measurement of apoptosis

Annexin V activation was increased in differentiated SH-SY5Y neuroblastoma cells exposed to IFN- γ (25 ng/mL). Conversely, decreased annexin V activation was observed in these cells exposed to IFN- α_{2A} (12.5 ng/mL and 50 ng/mL), IFN- γ (12.5 ng/mL and 50 ng/mL), IL-1 β (20 pM and 2000 pM), IL-6 (20 pM) and TNF- α (20 pM and 2000 pM).

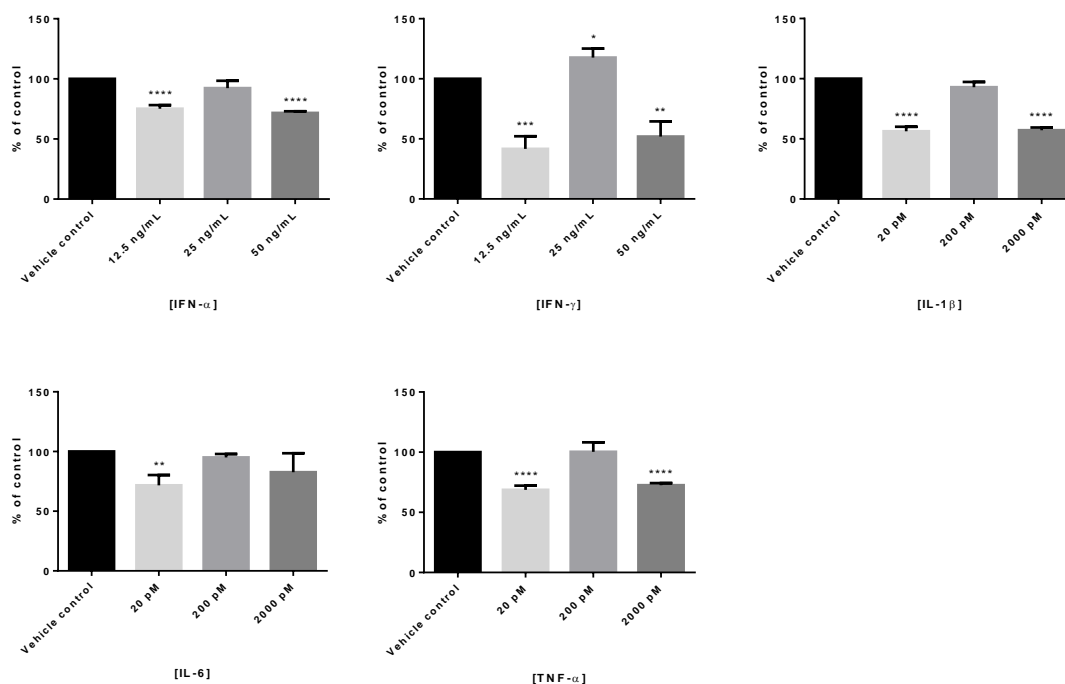


Figure 130 – Annexin V activation in differentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory cytokines (n = 3)

Propidium iodide as a measure of necrosis

Increased necrosis, measured using propidium iodide, was observed after differentiated SH-SY5Y neuroblastoma cells were treated with TNF-α (2000 pM). Decreased necrosis was observed after these cells were treated with IFN-α_{2A} (12.5 ng/mL and 50 ng/mL), IFN-γ (12.5 ng/mL and 50 ng/mL), IL-1β (20 pM and 2000 pM) and TNF-α (20 pM) as seen in figure 131 below.

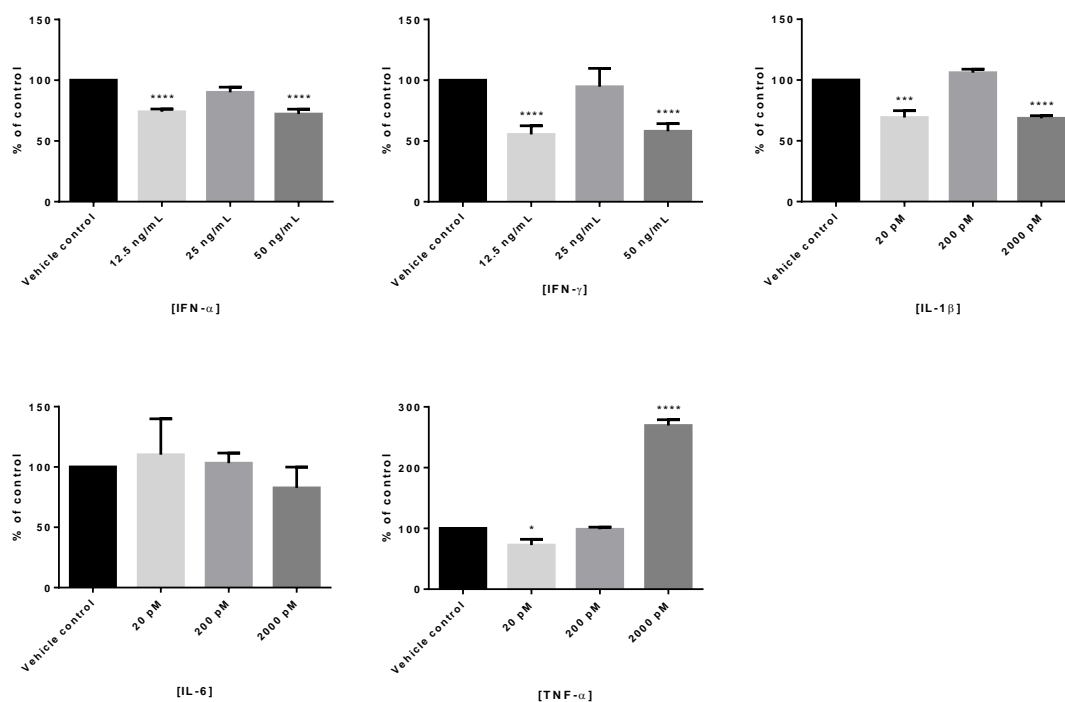


Figure 131 – Necrosis, measured using PI, in differentiated SH-SY5Y neuroblastoma cells exposed to pro-inflammatory mediators (n = 3)

4.4.6 Toxicity of antidepressant medications on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

The effects of antidepressant drugs on the viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Undifferentiated SH-SY5Y neuroblastoma cells

Resazurin

Undifferentiated SH-SY5Y neuroblastoma cells exposed to various antidepressant medications for 24 h resulted in significant decreases in the viability of these cells in a concentration-dependent manner. Amitriptyline (100 μ M and 1000

μM), fluoxetine (100 μM and 1000 μM) and venlafaxine (1000 μM) all significantly decreased the viability of undifferentiated SH-SY5Y neuroblastoma cells by approximately 50%, 80% and 20%, respectively. Conversely, low concentrations of these compounds, that is 10 μM , significantly increased the viability of these cells as seen in figure 132 below.

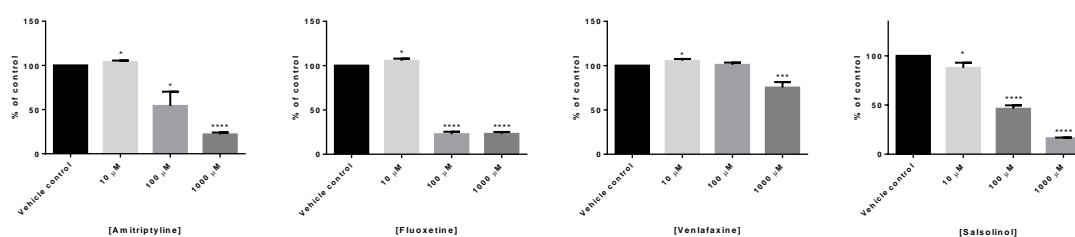


Figure 132 – Changes in the viability of undifferentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline, fluoxetine and venlafaxine (n = 9)

Differentiated SH-SY5Y neuroblastoma cells

Exposure of differentiated SH-SY5Y neuroblastoma cells exposed to antidepressant medications resulted in concentration-dependent decreases in viability. Amitriptyline (1000 μM), fluoxetine (10 μM , 100 μM and 1000 μM) and venlafaxine (100 μM) all significantly decreased the viability of these cells by approximately 80% and 10%, respectively. Amitriptyline (10 μM and 100 μM) both significantly increased the viability of differentiated SH-SY5Y neuroblastoma cells as seen in figure 133 below.

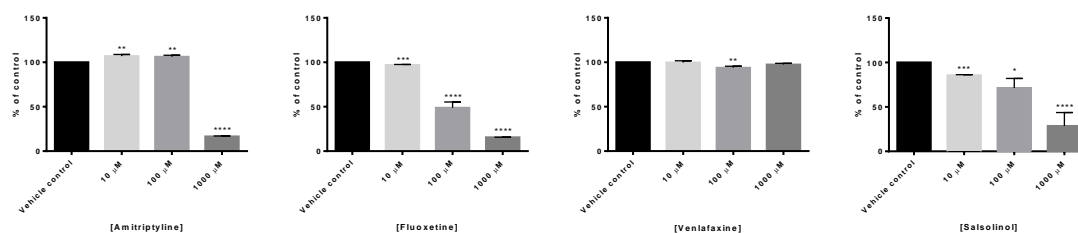


Figure 133 – Changes in the viability of differentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline, fluoxetine and venlafaxine (n = 9)

LDH assay

Undifferentiated SH-SY5Y neuroblastoma cells

No statistically significant changes in total LDH activity were observed in undifferentiated SH-SY5Y neuroblastoma cells exposed to the antidepressants amitriptyline, fluoxetine and venlafaxine as seen in figure 134.

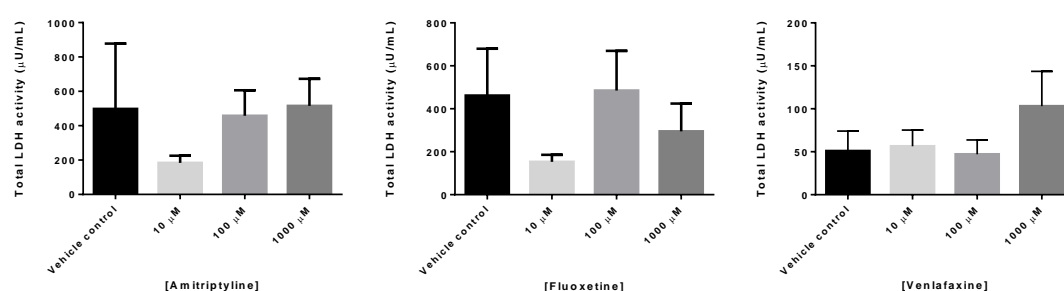


Figure 134 – Total LDH activity relative to cell number after undifferentiated SH-SY5Y neuroblastoma cells were exposed to amitriptyline, fluoxetine and venlafaxine (n = 3)

Differentiated SH-SY5Y neuroblastoma cells

Statistically significant increases in LDH release were observed in a concentration-dependent manner after differentiated SH-SY5Y neuroblastoma cells were exposed to amitriptyline, with 4-fold increases after exposure to 1000 μM . Furthermore, statistically significant increases in LDH release were observed after 24 h exposure to fluoxetine (1000 μM) with 4-fold increases, as seen in figure 135.

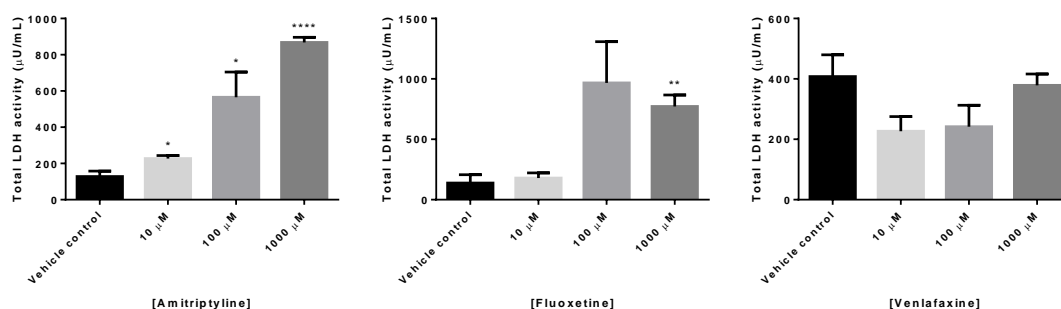


Figure 135 – Total LDH activity relative to cell number after differentiated SH-SY5Y neuroblastoma cells were exposed to amitriptyline, fluoxetine and venlafaxine (n = 3)

Free radical production after exposure to antidepressant medications

DCFH-DA assay

Undifferentiated SH-SY5Y

Statistically significant increases in free radical production was observed after exposure of undifferentiated SH-SY5Y neuroblastoma cells to 1000 μM of amitriptyline, fluoxetine and venlafaxine as measured in the DCFH-DA assay. Two-

fold increases in total LDH activity were observed after undifferentiated SH-SY5Y were exposed to 1000 μ M amitriptyline and fluoxetine as seen in figure 136 below.

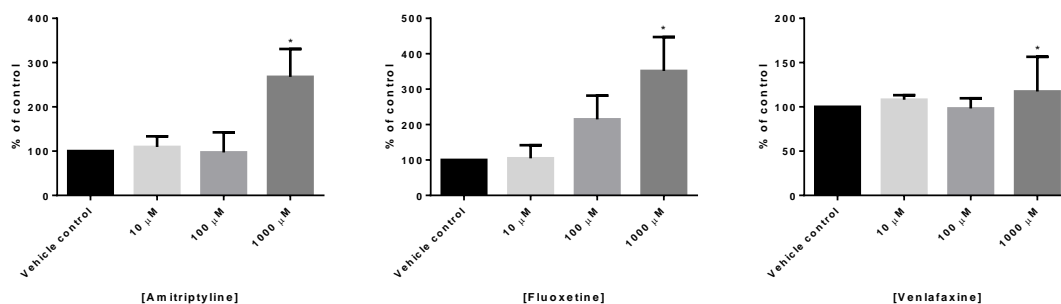


Figure 136 – Free radical production in undifferentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 9)

Differentiated SH-SY5Y neuroblastoma cells

Statistically significant increases in free radical production were observed after exposure of differentiated SH-SY5Y neuroblastoma cells to fluoxetine (1000 μ M), approximately 1-fold higher in comparison to the vehicle control. Although not statistically significant, trending increases in free radical production were observed after these cells were exposed to amitriptyline. Furthermore, statistically significant decreases in free radical production was observed after the exposure of these cells to venlafaxine (100 μ M), however this was modest in nature as seen in figure 137 below.

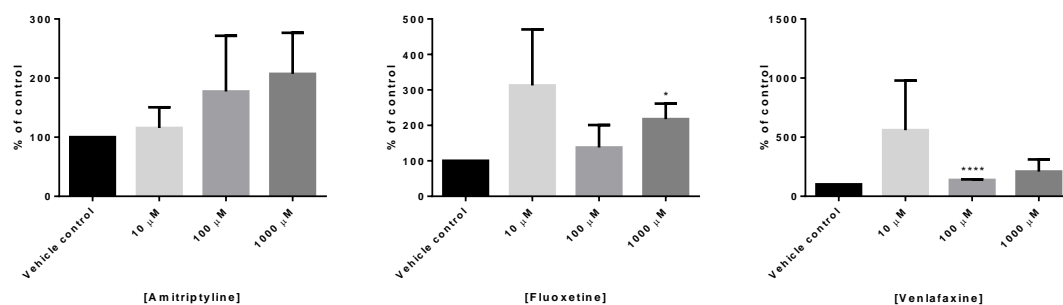


Figure 137 – Free radical production in differentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 9)

DHR 123

Undifferentiated SH-SY5Y neuroblastoma cells

Mitochondrial oxidative stress was measured using DHR 123. It was found the undifferentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline (1000 μ M), fluoxetine (100 μ M and 1000 μ M) and venlafaxine (100 μ M and 1000 μ M) showed increased production of mitochondrial superoxide, 7-fold, 4-fold and 2-fold, respectively, as seen in figure 138. Conversely, exposure to amitriptyline (10 μ M) and venlafaxine (10 μ M) resulted in decreased mitochondrial superoxide production, however, this was modest in nature.

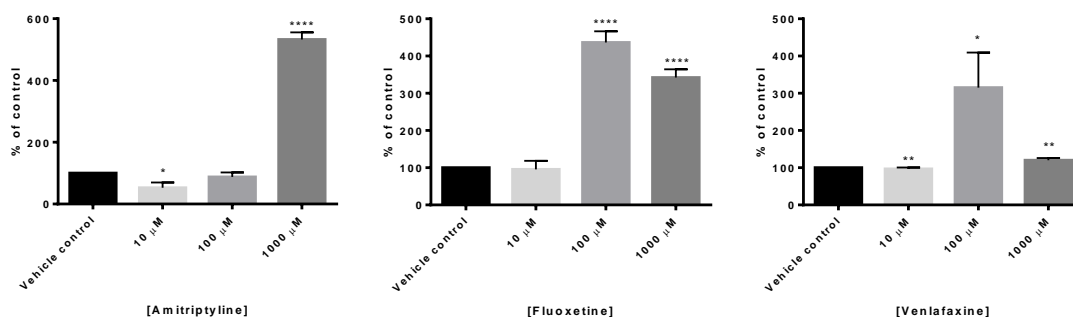


Figure 138 – Mitochondrial free radical production in undifferentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 9)

Differentiated SH-SY5Y neuroblastoma cells

Differentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline (1000 μM), fluoxetine (100 μM and 1000 μM) and venlafaxine (100 μM and 1000 μM) showed increased production of mitochondrial superoxide using the DHR 123 assay as shown in figure 139. Approximate 2-fold increases were observed in cells treated with amitriptyline and venlafaxine and approximate 12-fold increases were observed after exposure to fluoxetine in comparison to cells treated with vehicle control.

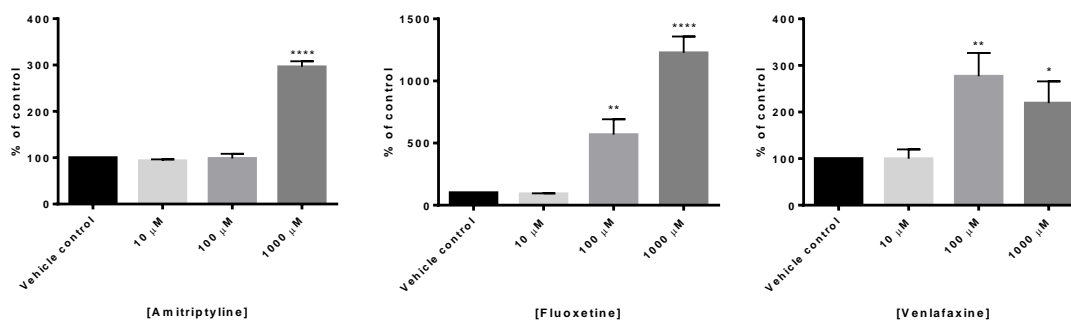


Figure 139 – Mitochondrial free radical production in differentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 9)

Annexin V activation

Undifferentiated SH-SY5Y neuroblastoma cells

Annexin V activation was observed in undifferentiated SH-SY5Y neuroblastoma cells after they were exposed to amitriptyline (1000 μM) and fluoxetine (100 μM and 1000 μM). Increases in the magnitude of 3-fold were observed in comparison to the vehicle control for all three conditions as seen in figure 140.

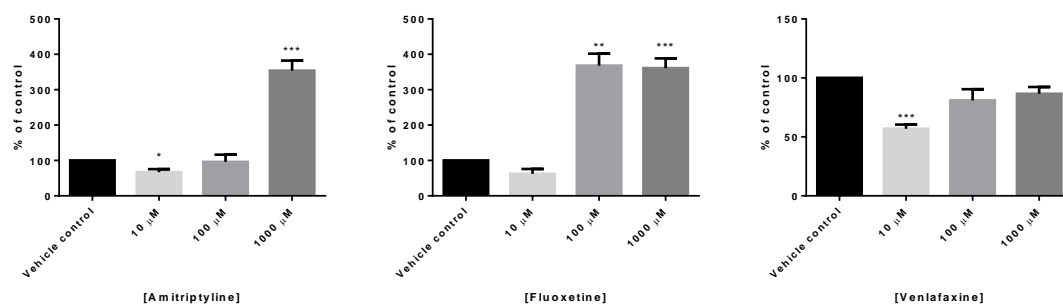


Figure 140 – Annexin V activation in undifferentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 3)

Differentiated SH-SY5Y neuroblastoma cells

Annexin V activation was observed after exposure of differentiated SH-SY5Y neuroblastoma cells to amitriptyline (1000 μ M), fluoxetine (100 μ M and 1000 μ M) and venlafaxine (100 μ M and 1000 μ M) as seen in figure 141. Increases in annexin V activation was in the magnitude of 5-fold after amitriptyline and fluoxetine exposure in comparison to cells treated with vehicle control.

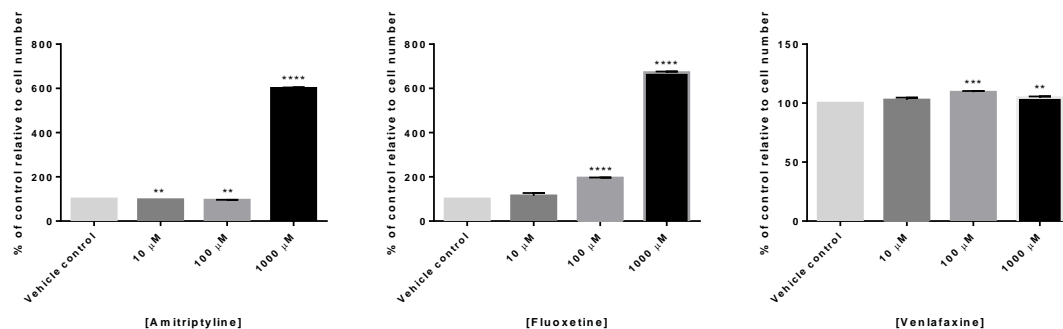


Figure 141 – Annexin V activation in differentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 3)

Necrosis measured using propidium iodide

Undifferentiated SH-SY5Y

Necrosis, using propidium iodide dye, was observed after exposure of undifferentiated SH-SY5Y neuroblastoma cells to amitriptyline (1000 μM) and fluoxetine (100 μM and 1000 μM) as seen in figure 142. Amitriptyline (1000 μM) and fluoxetine (100 μM and 1000 μM) increased necrosis 3-fold in comparison to cells treated with vehicle control.

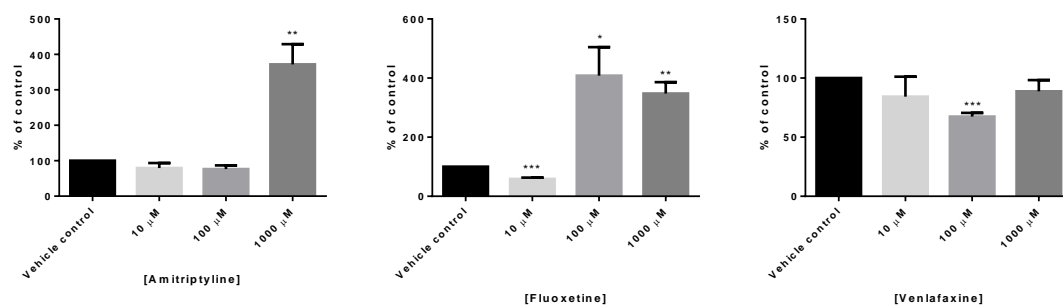


Figure 142 – Necrosis, measured using PI, in undifferentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 3)

Differentiated SH-SY5Y neuroblastoma cells

Necrosis, using propidium iodide dye, was observed after exposure of differentiated SH-SY5Y neuroblastoma cells to amitriptyline (1000 μM) and fluoxetine (10 μM, 100 μM and 1000 μM) as seen in figure 143. Amitriptyline (1000 μM) and fluoxetine (1000 μM) increased necrosis 30-fold and 40-fold, respectively in comparison to cells treated with vehicle control.

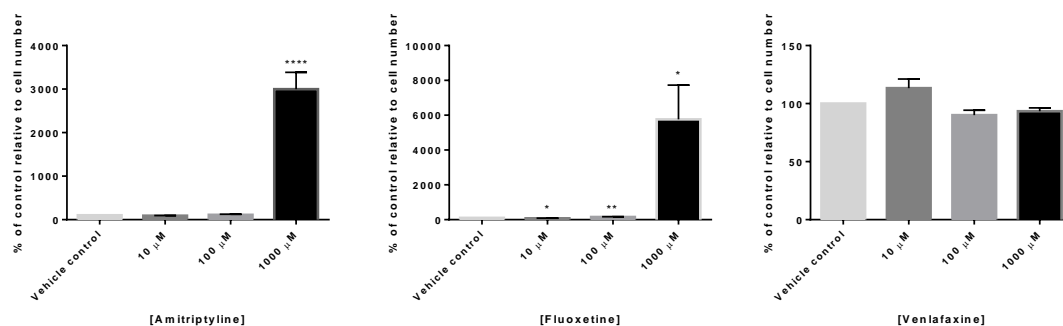


Figure 143 – Necrosis, measured using PI, in differentiated SH-SY5Y neuroblastoma cells after exposure to amitriptyline, fluoxetine and venlafaxine (n = 3)

4.5 Discussion

The aim of this chapter was to develop a number of *in vitro* cell-based models to assess parameters associated with depression and the toxicity of pharmacological compounds. Two models were developed and optimised for this purpose, a neuronal model to assess the neurotoxic effects of coffee constituents and a model of inflammation to assess the effects of coffee constituents.

4.5.1 Development of a model of interferon-induced inflammation

In the current study, the effects of numerous IFNs including human IFN- α 2a, porcine IFN- α , human IFN- β , human IFN- γ , murine IFN- γ and human IFN- λ 3 were assessed for their stimulatory properties in both undifferentiated and PMA differentiated THP-1 human monocytic cells. Very few changes in the parameters assessed were observed in undifferentiated THP-1 cells after exposure to any of the IFNs. Small

increases in viability were observed after undifferentiated THP-1 cells were exposed to 24 h of human IFN- α 2a, human IFN- β , human IFN- γ , murine IFN- γ and human IFN- λ 3. Furthermore, limited increases in pro-inflammatory cytokine production were observed in undifferentiated THP-1 cells contrary to previous reports in literature [273]. Statistically significant increases in IL-6 production were observed in undifferentiated THP-1 cells after they were exposed to 50 ng/mL of IFN- λ 3. Similarly, the only increase in TNF- α concentration was observed after exposure to 12.5 ng/mL and 50 ng/mL of murine IFN- γ and increased IL-1 β concentrations were only observed after exposure to 25 ng/mL porcine IFN- α and 12.5 ng/mL of murine IL-1 β .

Significant changes, in response to IFN stimulus, were observed in IDO activity. IDO activity was measured via the quantification of KYN and Trp and subsequently calculating the ratio of KYN to Trp. Significant increases in IDO activity were observed in undifferentiated THP-1 cells exposed to human IFN- γ , consistent with previously described results [274, 390]. No other treatment altered IDO activity.

PMA differentiated THP-1 cells displayed somewhat different results to those of undifferentiated THP-1 cells. Large, significant increases in the viability of these cells was observed after they were exposed to human IFN- β . Smaller increases in viability were observed after these cells were exposed to porcine IFN- α (400 units/mL), murine IFN- γ (200 and 400 units/mL) and human IFN- λ 3 (50 ng/mL). Furthermore, statistically significant increases in a number of pro-inflammatory cytokines was observed. These included increases in IL-6 concentrations after differentiated THP-1 monocytic cells were exposed to 50 ng/mL of human IFN- γ and IFN- λ 3, increases in

TNF- α after exposure to human IFN- β (25 ng/mL) and mouse IFN- γ (25 ng/mL) and increases in IL-1 β after exposure to human IFN- β (25 and 50 ng/mL) and IFN- λ 3 (25 ng/mL). Furthermore, IDO activity was shown to be increased by both IFN- γ and IFN- α _{2A}, however, the latter produced a much weaker response.

Studies have shown that there is significant differences in the species with regards to IFNs [391]. It has been shown that there is a lack of homology between human IFNs and IFNs from other species [391]. Given this, the results observed in the current study are expected.

As described earlier, inflammation has been shown to play an important role in the pathophysiology of depression [15, 38, 58-60, 123]. Furthermore, IFN therapy has been shown to precipitate symptoms within one week on initiating therapy in human patients [229, 230]. Based on this, the aim of this study was to develop a model of IFN-induced inflammation to use to test compounds of interest.

THP-1 human monocytic cells were chosen as the cell line in the current study for a number of reasons. THP-1 cells are human leukemic monocyte cells and can be differentiated into macrophage and microglial-like cells with the addition of a number of stimuli that will be discussed below [268, 271, 273, 392].

THP-1 cells have been shown to express various enzymes associated with the KP and have the ability to synthesise KMs. THP-1 cells have been shown to produce statistically significant increases in both KYN and QA production after treatment with LPS, IFN- γ or TNF- α through the upregulation of IDO [393] resulting in significant increases in the production of KMs [168]. Additionally, IFN- γ has been shown to induce KMO in this cell lines [393], indicating a favour towards the “neurotoxic arm” of the KP.

Investigations into the differentiated THP-1 cells were then undertaken to identify an optimal model of inflammation. A number of different agents have been investigated in the differentiation of THP-1 cells to induce the change from monocytic phenotype to microglial like phenotype. Amongst these agents are 1, 25-dihydroxyvitamin D₃ (VD₃) and phorbol-12-myristate-13-acetate (PMA) [268, 271]. It has been shown that PMA differentiation results in a closer macrophage phenotype with similarity in surface markers and increased cytokine release [268, 271] and as a result, it has been chosen as the differentiation agent in this study.

The differentiation protocol of THP-1 cells was based on a previously optimised method but required a high level of optimisation due to the potent stimulatory effects of IFN- γ and other IFNs tested. Previous studies have shown that IFN- γ can cause a number of significant changes to monocytic cells. Amongst these are morphological changes, changes in the expression of surface antigens and increases in the production of cytokines [273]. It has been shown that THP-1 cells exposed to IFN- γ resulted in the increased production of IL-1 β , IL-6 and TNF- α [273].

These results suggest PMA differentiated THP-1 cells stimulated with IFN- γ and IFN- α_{2A} provide significant similarities to microglial cells and appear to be appropriate to use as a surrogate microglial cell model to investigate the anti-inflammatory effects of compounds. To further confirm the appropriateness of this model, the effects of antidepressants on inflammatory markers in both IFN- γ and IFN- α_{2A} stimulated THP-1 cells were assessed. These results confirmed that this model was in fact appropriate to assess these factors.

4.5.2 The effects of antidepressant medications on markers of inflammation in differentiated THP-1 cells

The antidepressants amitriptyline, fluoxetine and venlafaxine were all evaluated for their effects on inflammation in IFN- α_{2A} and IFN- γ stimulated differentiated THP-1 cells. All three of these antidepressant medications are known to modulate various parameters of inflammation.

Significant decreases in the viability of differentiated THP-1 cells were observed after exposure to high concentrations amitriptyline, fluoxetine and venlafaxine with the addition of either IFN- α_{2A} or IFN- γ affording some protection from the decrease in viability. Furthermore, the effects of the antidepressants alone and in combination with IFN- α_{2A} and IFN- γ failed to show any significant increases in free radical production with decreases in free radical production in some cases. From these results, it is apparent that the mechanism by which differentiated THP-1 cell display decreased viability is one that is independent to that of free radical production.

The effects of the three antidepressants on parameters of inflammation in IFN- α_{2A} and IFN- γ stimulated THP-1 cells were then assessed. IDO activity was the first parameter addressed with all three of the antidepressants, amitriptyline, fluoxetine and venlafaxine decreasing the activity of IDO to some degree in both IFN- α_{2A} and IFN- γ stimulated THP-1 cells.

The effects of the antidepressant medications on the pro-inflammatory cytokines was also assessed. Similar results were observed for THP-1 cells stimulated with both IFN- α_{2A} and IFN- γ . Pre-treatment with amitriptyline for 24 h prior to either IFN- α_{2A} or IFN- γ stimulus, resulted in a significant decrease in the concentrations of IL-6 at all three concentrations tested along with TNF- α (1000 μ M) after exposure to IFN- α_{2A} . Conversely, increases in IL-1 β concentrations were observed after pre-treatment with all three concentrations tested, along with venlafaxine (1000 μ M) after exposure to IFN- α_{2A} . Similar results were observed with exception to TNF- α concentrations. Differentiated THP-1 cells stimulated with IFN- γ showed increased TNF- α concentrations whereas those stimulated with IFN- α_{2A} showed decreased TNF- α production. Again, venlafaxine (100 μ M) pre-treatment of differentiated THP-1 cells yielded similar results, with the exception of TNF- α concentration, in IFN- γ stimulated cells.

As outlined earlier, numerous classes of antidepressant medications are currently marketed for the treatment of depression including the TCAs [351], SSRIs [351], and SNRIs [351]. The TCA, amitriptyline, decreased parameters of inflammation investigated in the current study.

In the current study, amitriptyline, a TCA, to decrease the concentrations IL-6 and TNF- α produced by differentiated THP-1 cells stimulated with either IFN- γ or IFN- α_{2A} in a concentration-dependent manner. Previous studies have shown amitriptyline to decrease the pro-inflammatory TNF- α , consistent with the findings of the current study but increases IL-6, another pro-inflammatory cytokine in treatment responders *ex vivo* [353]. Furthermore, TNF- α concentrations and IL-1 β concentrations were decreased in rat microglia after treatment with amitriptyline [356-358]. One plausible explanation for the differences in the effects of amitriptyline on the concentrations of IL-6 may be due to the study design. The current study is an *in vitro* study and the study in literature is an *ex vivo* study. To date, there have been no studies assessing the effects of amitriptyline on IDO activity.

Fluoxetine, an SSRI, was assessed for its effects on pro-inflammatory cytokines in the current study. Fluoxetine was shown to decrease the production of IL-6 in both IFN- γ and IFN- α_{2A} stimulated THP-1 cells. Conversely, IL-1 β production was shown to be increased in both models. TNF- α production, however, was increased in the IFN- γ model but decreased in the IFN- α_{2A} model. To date, there have been no studies assessing the effects of fluoxetine on the inflammatory cytokines in *in vitro* models.

The SNRI venlafaxine was evaluated in the current study for its anti-inflammatory effects. Pre-treatment venlafaxine was shown to decrease IL-6 and increase IL-1 β production in both IFN- γ and IFN- α_{2A} stimulated THP-1 cells. TNF- α

production on the other hand was shown to be increased in IFN- γ stimulated cells at 100 μ M but decreased at 1000 μ M and in IFN- α_{2A} stimulated cells.

SNRIs, as their name suggests, inhibit the re-uptake of both serotonin and noradrenaline from the synaptic cleft via the inhibition of SERT and NET [366]. The SNRI venlafaxine has been the widest studied compound in the class and has been shown to have a number of effects on inflammatory mediators. This drug has very little effects on TNF- α levels *in vivo* [360] but decreases pro-inflammatory IL-6 production [359, 365]. The results in the current study are consistent with the results observed in literature with exception to the concentration of TNF- α observed after IFN- γ stimulated cells were treated with 100 μ M of venlafaxine.

The current study, in combination with previous studies, shows that changes to inflammatory mediators occur after inflammatory cells are treated with all classes of antidepressants. This indicates that this effect may play an important role in their mechanisms of action. These results support the neuroinflammatory hypotheses of depression and the role of neuroinflammation in the pathophysiology of depression. However, due to the limited number of studies and the lack of consistency of the observations outlined, further studies are required to fully assess the effects of antidepressant medications on cytokine production.

The current study showed all three antidepressants, amitriptyline, fluoxetine and venlafaxine all decreased IDO activity in a concentration-dependent manner in both

IFN- γ and IFN- α_{2A} stimulated THP-1 cells. Specific studies assessing the effects of antidepressants on IDO activity have yet to be undertaken. A number of other metabolites in the KP have however been assessed. As outlined in the introduction, fluoxetine and amitriptyline increased the concentration of the neuroprotective Trp catabolite, KA and decreased the concentration of the neurotoxic catabolite 3-HK in glial cell cultures in a time-dependent manner. Amitriptyline showed a higher increase in KA than fluoxetine but both antidepressants had relatively the same effect in decreasing the levels of 3-HK [369]. Additionally, fluoxetine and amitriptyline, at a concentration of 10 μ M, were found to strongly induce the expression of both *Kat1* and *Kat2*, enzymes responsible for the biosynthesis of KA [369]. Given their effects on other parameters in the KP, the effects on IDO in the current study appear to be plausible.

In conclusion, this study suggests that PMA differentiated THP-1 cells stimulated with IFN- γ and IFN- α_{2A} are acceptable to assess various inflammatory parameters. Limitations do exist in this model, most notably the lack of increase in the concentrations of IL-1 β and TNF- α . Further optimisation of this model is warranted in the future.

4.5.3 Development of a model of differentiation of SH-SY5Y neuroblastoma cells

As outlined earlier, a number of models of differentiation of SH-SY5Y neuroblastoma cells have been previously described. One such model of interest in depression is a method by which SH-SY5Y neuroblastoma cells are chemically treated with dbcAMP to produce a cell more closely aligned phenotypically with noradrenergic

neurons [284]. In the current study, a previously described method was optimised for use in the current study [284]. dbcAMP was assessed for its effects on the viability of SH-SY5Y neuroblastoma cells using the resazurin reduction assay. It was found that no significant effects on viability occurred with exposure to dbcAMP. Furthermore, it was shown that morphological changes occurred when these cells were treated with 100 μ M dbcAMP. These changes were consistent with a more neuronal phenotype, that is displaying longer and more neurite projections. This was confirmed using both crystal violet staining and a neurite stain. Furthermore, NA concentrations were shown to be significantly increased after treatment with 100 μ M dbcAMP. This optimisation differed quite significantly to the method described by Kume *et al* [284]. The current differentiation method used standard media required to culture SH-SY5Y neuroblastoma cells in comparison to the high cost neuron specific media used in the published study [284]. Furthermore, through the introduction of replacing the dbcAMP 48 h into the process, resulted in a 10-fold lower concentration of dbcAMP exposure to differentiate the cells [284].

4.5.4 Toxicity of kynurenine metabolites on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

As outlined previously, a number of the KMs are known neurotoxins and have been shown to play a role in the pathophysiology of depression [58, 60, 160]. To date, numerous studies have evaluated a number of *in vitro* models on their effectiveness to assess KM toxicity [394]. Studies have utilised SH-SY5Y neuroblastoma cells but have found that these cells are unsuitable for the assessment of KM toxicities [394].

The current study assessed the effects of KMs alone and in combination after 24 h and 72 h exposure to undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells. Consistent with previous studies, significant decreases in viability were shown in undifferentiated SH-SY5Y neuroblastoma cells exposed to the neurotoxic KMs 3-HK and 3-HA at 1000 μ M for 24 h. Again, consistent with literature, no change in viability was observed after undifferentiated SH-SY5Y neuroblastoma cells were treated for 24 h with any concentration of QA. These results were confirmed with the results obtained in the LDH activity. Although not statistically significant, increases in total LDH activity were observed in cells exposed to 1000 μ M 3-HK and 3-HA. Likewise, no changes in LDH activity was observed after undifferentiated SH-SY5Y neuroblastoma cells were exposed to QA (1000 μ M).

To further investigate and confirm the mechanism by which 3-HK and 3-HA alters parameters associated with apoptosis, necrosis and oxidative stress. Increased annexin V activation was observed after undifferentiated SH-SY5Y neuroblastoma cells were exposed to 1000 μ M of 3-HK and 3-HA. When these results are taken into consideration with the corresponding increased trends of mitochondrial oxidative stress and necrosis, it appears that the mechanism by which these neuroactive KMs exert their toxicity towards these cells is via an apoptosis pathway. These results are consistent with literature after a number of studies have shown that these compounds exert their cytotoxicity via apoptotic pathways in a number of cell lines [166, 168, 395, 396]. Given the involvement of oxidative stress in the apoptotic pathway [397], it appears plausible that this may be one mechanism by which apoptosis is induced in these cells.

The effects of 24 h exposure of combinations of the neurotoxic KMs on undifferentiated SH-SY5Y neuroblastoma cells was then investigated as a possible model to assess the toxicity of KMs. It was found that several concentrations of 3-HK and QA, 3-HA and QA and 3-HK, 3-HA and QA afforded very modest decreases in the viability of SH-SY5Y neuroblastoma cells. These results are supported in that only a very small increase in annexin V activation, indicating apoptosis, is observed in undifferentiated SH-SY5Y neuroblastoma cells.

Furthermore, the effects of 72 h exposure of KMs alone and in combination were assessed on the viability of undifferentiated SH-SY5Y neuroblastoma cells. As seen in undifferentiated SH-SY5Y neuroblastoma cells exposed to 3-HK and 3-HA for 24 h, significant decreases in viability were only observed after treatment to 1000 μ M concentrations. Conversely, modest statistically significant increases in viability were observed after undifferentiated SH-SY5Y neuroblastoma cells were QA.

The results observed suggest, consistent with literature, that undifferentiated SH-SY5Y neuroblastoma cells are an unsuitable model to assess the neurotoxicity of KMs. Furthermore, this suggests that undifferentiated SH-SY5Y neuroblastoma cells may not be the best model to use in neurotoxicity studies.

To further investigate the use of SH-SY5Y neuroblastoma cells as a model to use in neurotoxicity studies, dbcAMP differentiated SH-SY5Y neuroblastoma cells were assessed for their appropriateness for KM toxicity studies.

Similar results were observed after dbcAMP differentiated SH-SY5Y neuroblastoma cells were treated with KM for 24 h. Statistically significant decreases in the viability of these cells was observed after exposure to 1000 μ M 3-HK and 3-HA comparable to those of undifferentiated SH-SY5Y neuroblastoma cells. Again, combinations of KMs did not significantly alter the viability of dbcAMP differentiated SH-SY5Y neuroblastoma cells.

Seventy-two hour exposure of KMs to differentiated SH-SY5Y neuroblastoma cells to KMs showed no significant decreases in viability. However, 72 h exposure of differentiated SH-SY5Y neuroblastoma cells to a combination of 3-HK, 3-HA and QA produced a concentration-dependent decrease in viability. These results suggest that dbcAMP differentiated SH-SY5Y neuroblastoma cells provide a more realistic *in vitro* model to assess neurotoxicity.

The difficulty using immortal cells in the evaluation of KM toxicity can be attributed to role of this pathway in tumour cells and immune surveillance [398]. A number of strategies have been undertaken in the past in an attempt to sensitise SH-SY5Y neuroblastoma cells to the neurotoxic effects of KMs [394]. Strategies include glucose deprivation and retinoic acid differentiation [394]. Neither of these strategies were successful in sensitising SH-SY5Y neuroblastoma cells to the toxic effects of KMs [394].

The current study has highlighted that dbcAMP differentiated SH-SY5Y neuroblastoma cells provide a better model to assess the neurotoxic effects of KMs. The direct effects of cytokines were then assessed in both differentiated and undifferentiated SH-SY5Y neuroblastoma cells to further evaluate the appropriateness of this model as a surrogate neuronal cell.

4.5.5 Toxicity of cytokines on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

The effects of numerous pro-inflammatory cytokines and markers of inflammation were assessed for their effects on undifferentiated and differentiated SH-SY5Y neuroblastoma cells. No significant changes to the viability of both undifferentiated and differentiated SH-SY5Y neuroblastoma cells was observed in this study. At best, a modest decrease in viability was observed in undifferentiated SH-SY5Y cells treated with IFN- α and IFN- γ . Similarly, treatment with IFN- α also decreased the viability of differentiated SH-SY5Y neuroblastoma cells.

Studies assessing the effects of these compounds on mechanisms of cell death showed that a number of them are beneficial towards cell survival. This includes the decrease in oxidative stress after undifferentiated SH-SY5Y cells were exposed to IL-1 β , IL-6 and TNF- α . Furthermore, decreased annexin V activation was observed after these cells were exposed to IFN- α , IFN- γ , IL-1 β , IL-6 and TNF- α . These results correspond to those observed using propidium iodide suggesting a decrease in necrosis in these cells after exposure to markers of inflammation.

The effects of markers of inflammation on differentiated SH-SY5Y neuroblastoma cells produced opposing results to those observed in undifferentiated SH-SY5Y neuroblastoma cells. Statistically significant increase in oxidative stress were observed in differentiated SH-SY5Y neuroblastoma cells exposed to TNF- α . Furthermore, non-significant trends of increased oxidative stress is observed in differentiated SH-SY5Y neuroblastoma cells exposed to IFN α , IFN- γ , PGE₂ and IL-1 β . The increases in oxidative stress however do not correspond to increased annexin V activation.

Given the role of these pro-inflammatory markers in the neuroinflammation, neuroprotection and neurotoxicity [399-401], further studies are required to fully elucidate the role of the inflammatory markers in neurotoxicity. Given that dbcAMP differentiated SH-SY5Y neuroblastoma cells are more susceptible to the toxic effects of these mediators than their undifferentiated counterpart, suggests that they may be more appropriate for the use in neurotoxicity studies.

4.5.6 Toxicity of antidepressant medications on undifferentiated and differentiated SH-SY5Y neuroblastoma cells

To further evaluate the use of undifferentiated and differentiated SH-SY5Y neuroblastoma cells as a model for neurotoxicity studies, the toxicity of various antidepressant medications including amitriptyline, fluoxetine and venlafaxine were investigated.

Significant differences between the viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells were observed especially at lower concentrations. Significant decreases in viability were observed in undifferentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline (100 and 1000 μ M), fluoxetine (100 and 1000 μ M) and venlafaxine (1000 μ M). In contrast, the viability of differentiated SH-SY5Y neuroblastoma cells also decreased but to a lesser degree than that of the undifferentiated SH-SY5Y neuroblastoma cells. Amitriptyline only decreased the viability of differentiated SH-SY5Y neuroblastoma cells at 1000 μ M, fluoxetine 100 μ M decreased the viability of differentiated SH-SY5Y neuroblastoma cells to a lesser degree and 1000 μ M venlafaxine had no effect on the viability of SH-SY5Y neuroblastoma cells.

Further studies were undertaken to assess if the mechanisms of cell death varied between undifferentiated and differentiated SH-SY5Y neuroblastoma cells. Significant increases in oxidative stress were observed after undifferentiated SH-SY5Y neuroblastoma cells exposed to 1000 μ M of amitriptyline, fluoxetine and venlafaxine. Conversely, only statistically increases in oxidative stress were seen after differentiated SH-SY5Y neuroblastoma cells were treated with 1000 μ M of fluoxetine. However, mitochondrial oxidative stress was increased consistently with the viability results in both undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells. Furthermore, corresponding increases in annexin V activation and necrosis were observed in both undifferentiated and differentiated SH-SY5Y neuroblastoma cells. These results suggest that mitochondrial oxidative stress induced apoptosis is the main mechanism by which antidepressants exert their toxic effects on both undifferentiated and differentiated SH-SY5Y cells. Furthermore, the data shows that differentiated SH-

SY5Y neuroblastoma cells are affected less than their undifferentiated counterparts suggesting that differentiated SH-SY5Y neuroblastoma cells provide a more accurate representation of human neurons.

These results are consistent with literature. Studies have shown, that at high concentrations, amitriptyline is toxic to primary rat neurons, HT22-cells and PC-12 cells in concentrations comparable to the current study [402, 403]. Furthermore, the level of toxicity at 100 μ M concentrations is more closely aligned to the result observed in the dbcAMP differentiated SH-SY5Y neuroblastoma cells in comparison to those observed in the undifferentiated phenotype [402]. Amitriptyline has also been shown to elicit these effects via an apoptotic mechanism of cell death [402]. Likewise, this is consistent with the findings of the current study with both annexin V activation and alteration to the membrane potential, consistent with apoptosis [388], present in both undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to amitriptyline.

Fluoxetine has previously been shown to cause cytotoxic effects in a concentration-dependent manner in HT22-cells and PC-12 cells [403]. Again, the degree of cytotoxicity observed was consistent with the results obtained in the current study [403]. To date, no studies have assessed the neurotoxicity of venlafaxine. The current study suggests that venlafaxine is far less neurotoxic than amitriptyline and fluoxetine.

Significant phenotypic differences were observed in the current study between dbcAMP differentiated and undifferentiated SH-SY5Y neuroblastoma cells. This study indicates the dbcAMP differentiated SH-SY5Y cells may be used as a surrogate neuronal-like cell and may be more appropriate to evaluate the neurotoxicity of compounds.

4.6 Conclusion

The aim of the current study was to develop two *in vitro* models as alternatives for using human primary cells. The first of these was a surrogate microglial-like cell model and the second a surrogate neuronal-like model. This study was successful in validating the use of an IFN-induced inflammatory model using PMA differentiated THP-1 human monocytic cells. Furthermore, the second model, a surrogate neuronal-like cell model was again successfully valid as a more neuronal phenotype than undifferentiated SH-SY5Y neuroblastoma cells. These models provided the essential features of the cell lines required, although, with any *in vitro* model, limitations do exist. One such limitation is that in the current study, the antidepressants investigated in the study amitriptyline, fluoxetine and venlafaxine, were only used in the original state however, *in vivo*, these three drugs undergo extensive metabolism to a number of active metabolites. Further studies assessing the effects of these metabolites on the viability of the neuronal-like cells is warranted.

Chapter 5 – The effects of key bioactive coffee constituents of parameters of inflammation and neurotoxicity

5.1 Introduction

As outlined in chapter one, coffee is a very popular beverage with numerous bioactive constituents [1]. This study has already shown that caffeinated coffee displays possible antidepressant-like effects in an *in vivo* model of LPS-induced inflammatory depression along with caffeine however, this was not seen with decaffeinated coffee. Caffeine however produced a much smaller protective effect suggesting that caffeine in combination with other bioactive coffee constituents is required to observe the full potential protective effects. These results align with the observations of the epidemiological studies [3, 4, 6-8, 11]. Chapter three then highlighted that one of the bioactive coffee constituents, FA, was decreased after the decaffeination process. Given this and the previous literature outlined in chapter one on the potential mechanisms by which these compounds may be beneficial in depression, the effects of key bioactive coffee constituents, alone and in combination with caffeine, were further investigated for their potential mechanisms of action in depression using the *in vitro* models developed in chapter four.

Coffee contains many bioactive constituents including numerous polyphenol and alkaloid compounds [124]. Polyphenols have been shown to have numerous biological effects including beneficial effects on cardiovascular and metabolic disorders, inflammation and cancer, oxidative stress, cerebral ischaemia, obesity and functioning of the brain. With specific regards to the brain, polyphenols, in general, have been shown to prevent neuroinflammation along with possessing antioxidant properties and

the ability to modulate neurotransmitter levels [32]. Furthermore, the alkaloids present in coffee, caffeine and trigonelline have also shown a number of beneficial effects in both depression and parameters associated with the pathophysiology of depression [3, 4, 6-8, 11, 34]. The specific coffee constituents of interest in the current study, caffeine, CA, CGA, FA, PA and trigonelline have been described in depth in section 1.2.2.

Two *in vitro* cell-based models were developed and outlined in chapter four for the evaluation of the effects of coffee constituents on markers of inflammation and the toxicity of these compounds on neuronal cells. A surrogate microglial-cell model was developed in chapter four using IFN-stimulated PMA differentiated THP-1 cells. PMA differentiated THP-1 monocytic cells have been widely used as a surrogate microglial- or macrophage-like cell in literature [268, 271, 273, 379, 392]. These cells have been shown to display many of the key features of macrophages and microglial cells as presented in chapter 4.

The model of PMA differentiated THP-1 human monocytic cells used in the current study was stimulated with either IFN- γ or IFN- α_{2A} to produce an activated microglial-like cell model. A number of key features were observed in this model including increased IL-6 production, increased PGE₂ production and increased IDO activity. All three of these markers are well-known to be associated with neuroinflammation and observed in activated microglial cells [33]. Furthermore, these three markers are known biomarkers associated with depression [60, 229, 230, 404]. As with any model, this model has its limitations. Two other inflammatory cytokines that are normally secreted from microglial cells in their activated state, IL-1 β and TNF- α ,

were not significantly raised in this model. Although these markers were not raised in the current model, the fact that other important markers including IL-6, IDO and PGE₂ were suggests that this model is appropriate for the preliminary evaluation of the effects of coffee constituents, both alone and in combination with caffeine, on markers of inflammation.

A second important parameter to evaluate is the effects of coffee constituents on the viability of neuronal cells. As highlighted in chapter one, the beneficial effects of coffee are observed in a J shaped relationship [3, 4]. That is, at high concentrations coffee has negative health effects, namely increased risk of suicide and anxiety [5, 21, 22, 28]. One possible pathway by which this occurs may be via neuronal cell toxicity.

The neuronal-like model outlined in chapter four was used in the current study to evaluate the effects of key bioactive coffee constituents on viability. SH-SY5Y neuroblastoma cells are routinely used in neurotoxicology studies [277, 337] but to date these compounds have not been tested in this cell line. Furthermore, given the differences in the phenotypes of undifferentiated and differentiated SH-SY5Y neuroblastoma cells, both cell lines were used. Literature reports that undifferentiated SH-SY5Y neuroblastoma cells are a predominantly dopaminergic phenotype [405] and the dbcAMP differentiated SH-SY5Y neuroblastoma cells were shown to be noradrenergic in nature in both the study undertaken in chapter four and in literature [284].

A number of studies have investigated the protective properties of the key biologically active constituents of coffee against known toxins of SH-SY5Y neuroblastoma cells, but the direct effects of these compounds on both undifferentiated and differentiated SH-SY5Y cells have yet to be determined. Given this and the human data suggesting the potential harms of coffee consumption, particularly at high concentrations, the effects of these compounds were assessed in the two models outlined above.

It is expected that this study will provide information relating to the effects of coffee constituents, both alone and in combination with caffeine, on markers of inflammation in a surrogate microglial model. Literature suggests that the pathophysiology of depression is multifactorial and involves components of inflammation, oxidative stress and alterations to Trp catabolism [60]. Coffee, a well-accepted and consumed beverage, has been shown to have antidepressant-like activity and a number of the key bioactive constituents to have anti-inflammatory and antioxidant activities [52, 57, 88, 94, 96, 97]. To date, however, these effects have never been evaluated in a differentiated monocytic model. Furthermore, the effects of these compounds on Trp catabolism have yet to be investigated. As a result, this study investigated key bioactive coffee constituents on IFN- γ and IFN- α -induced inflammation in differentiated THP-1 human monocytic cells.

Furthermore, this study will also provide an insight into the effects that key bioactive coffee constituents have on the two phenotypically different neuronal-like cells, that is undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells.

It is proposed that this component of the study will provide information on the potential harms of coffee consumption, particularly at high concentrations, in light of the epidemiological studies.

There is a large amount of recently published literature that has implicated coffee as having antidepressant-like activity. The focus of this study is firstly to evaluate firstly evaluate the toxicity of key bioactive coffee constituents on the viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells and THP-1 human monocytic cells. Secondly, the effects of these compounds on parameters associated with inflammation will be evaluated in an IFN- γ and IFN- α stimulated THP-1 human monocytic cell-based model.

5.2 Hypothesis aims and objectives

5.2.1 Hypothesis

The primary hypothesis of this study is that key bioactive coffee constituents including caffeine, CA, CGA, FA, PA and trigonelline will alter inflammatory response in IFN- γ and IFN- α_{2A} stimulated THP-1 human monocytic cells as a surrogate model of activated microglial cells.

The secondary hypothesis of this study is that the key bioactive coffee constituents including caffeine, CA, CGA, FA, PA and trigonelline will not reduce the viability of undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells as models of dopaminergic and noradrenergic neurons.

5.2.2 Aims and objectives

The primary aim of this study was to evaluate the anti-inflammatory properties of key bioactive coffee constituents on IFN- γ and IFN- α_{2A} stimulated THP-1 monocytic cells as a surrogate model of activated microglial cells.

This aim will be achieved through the following objectives:

- Assessing the effects of coffee constituents, alone and in combination with caffeine, on viability, free radical production, IL-6, IL-1 β , TNF- α and IDO activity.

The secondary aim of this study was to assess the effects of key bioactive coffee constituents on the viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells as models of phenotypically different neuronal-like cells.

This aim will be achieved through the following objectives:

- Assessing the effects of coffee constituents on the viability of undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells; and
- Assessing the effects of coffee constituents on specific cell death mechanism including oxidative stress, apoptosis and necrosis; and
- Assessing the effects of coffee constituents on cAMP concentrations to indicate if the effects are at a receptor level or signal transduction level in undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells.

5.3 Materials and methods

5.3.1 The effects of key bioactive coffee constituents, alone and in combination with caffeine, on markers of inflammation in a surrogate microglial cell model

Routine cell culture

THP-1 cells

THP-1 human monocytic cells were grown and maintained according to the methods outlined in section 4.3.4.

Differentiation of THP-1 human monocytic cells

The differentiation of THP-1 cells was undertaken according to the methods described in section 4.3.1. Briefly, THP-1 cells were seeded at 3×10^5 cells/mL of trypan blue excluding cells in 24 or 96-well microtitre plates. 50 ng/mL of PMA was then added to each well and then left for the 5 days for the differentiation process to occur. The THP-1 cells were then used for the assays described below.

Preparation of working solutions

Stock solutions of all compounds of interest including caffeine (PCCA, Australia), CA (Sigma-Aldrich, St Louis, MO, USA), CGA (Sigma-Aldrich, St Louis, MO, USA), FA (Sigma-Aldrich, St Louis, MO, USA), PA (Sigma-Aldrich, St Louis, MO, USA), trigonelline (Sigma-Aldrich, St Louis, MO, USA) and salsolinol (Sigma-Aldrich, St Louis, MO, USA) were made up to a concentration of 10 mM in sterile PBS and were further diluted in media to produce the working solutions. Human IFN- γ (ORF genetics, Iceland) and human IFN- α_{2a} (PeproTech, NJ, USA) were made up to a

concentration of 100 µg/mL in PBS containing 0.1% BSA (Sigma-Aldrich, St Louis, MO, USA) and were further diluted in media to produce the working solutions. PMA (Sigma-Aldrich, St Louis, MO, USA) was made up in DMSO to a concentration of 2 mM and was further diluted in media to produce the working solutions. All stock solutions were stored at -20°C and working solutions prepared fresh on the day of the experiment.

The effects of key bioactive constituents on the viability of differentiated THP-1 cells
Resazurin

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.1. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.1 and 22 µL or 55 µL of a 440 µM of resazurin was added to each well of a 96 or 24 well plate respectively and the plates incubated for 4 h. The reduction of resazurin to resorufin was determined by fluorescence.

The effects of key bioactive coffee constituents on the production of free radicals in differentiated THP-1 cells

2',7'-dichlorofluorescein assay

The DCFH-DA assay was performed according to the methods outlined in section 4.3.1. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.1. The spent media was then replaced with serum free media containing 10 µM of DCFH-DA and the plates incubated for 30 min and the fluorescence subsequently read.

The effects of key bioactive coffee constituents on IDO activity in differentiated THP-1 cells

IDO activity was assessed using the HPLC protocol outlined in section 2.3.8. Briefly, the concentrations of Trp and KYN were quantified in spent supernatant, using HPLC, and the ratio of KYN to Trp calculated and used as a measure of IDO activity.

Quantification of TNF- α concentrations

TNF- α concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the TNF- α human ELISA kit (PeproTech, Rocky Hill, NJ, USA) was used to quantify TNF- α concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

Quantification of IL-6 concentrations

IL-6 concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the IL-6 human ELISA kit (PeproTech, Rocky Hill, NJ, USA) was used to quantify IL-6 concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

Quantifications of IL-1 β concentrations

IL-1 β concentrations were quantified using the protocol outlined in section 4.3.1. Briefly, the IL-1 β human ELISA kit (Abcam, Melbourne, Australia) was used to quantify IL-1 β concentrations present in spent supernatants and was performed according to the manufacturer's recommendations.

5.3.2 The effect of key bioactive coffee constituents on the viability on neuronal-like cells

Routine cell culture

Human neuroblastoma cells (SH-SY5Y) were obtained from Sigma-Aldrich (St Louis, MO, USA). Cells were grown and maintained at 37°C and 5% CO₂ in complete Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Mulgrave, Victoria, Australia) containing 4.5 g/L of glucose, L-glutamine, 110 mg/L of sodium pyruvate, phenol red, 10% foetal bovine serum (Bovogen, Australia) and 0.1 mg/1mL of gentamicin (Gibco, Mulgrave, Victoria, Australia).

Differentiation of SH-SY5Y neuroblastoma cells

Differentiation of SH-SY5Y neuroblastoma cells was undertaken as previously described in section 4.3.3. Briefly, cells were plated and 100µM of dbcAMP added. A subsequent media change, with media containing 100 µM of dbcAMP, was done 48 h later and the cells left to differentiate for a further 24 h, with a total differentiation time of 72 h.

Resazurin proliferation assay

The resazurin reduction viability assay was performed according to the methods outlined in section 4.3.1 with minor modifications. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2 and the media was replaced with fresh media containing 44 µM of resazurin was

added to each well and the plates incubated for 3 h. The reduction of resazurin to resorufin was determined by fluorescence.

LDH assay

The LDH assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2. The LDH cytotoxicity kit (Cayman Chemical, Michigan, USA) was used to determine LDH leakage and was performed according to the manufacturer's instructions.

Free radical formation

DCFH-DA assay

The DCFH-DA assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2. The spent media was then replaced with serum free media containing 10 μ M of DCFH-DA and the plates incubated for 30 min and the fluorescence subsequently read.

DHR 123 assay

The DHR 123 assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2. The spent media was then replaced with

serum free media containing 25 μ M of DHR 123 and the plates incubated for 30 min and the fluorescence subsequently read.

MitoSOX Red

MitoSOX Red was used to measure mitochondrial superoxide formation. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier. Following incubation for 24 h, medium above the cells was replaced with media containing MitoSOX Red reagent (5 μ M) for 10 minutes at 37°C protected from light. Cells were then washed twice with PBS and fluorescence due to the oxidized dye (excitation 510 nm; emission 580 nm) was measured using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Apoptosis

Annexin V activation

The Annexin V-FITC assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure annexin V activation and was performed according to the manufacturer's instructions.

Caspase-3 measurement

Caspase activation has been identified as playing an important role in apoptosis. There are two pathways of caspase activation that have been identified to date – the cell

surface death receptor and the mitochondrial-initiated pathway. Specifically, caspase-3 is activated in mitochondrial-initiated pathway and is widely accepted as a marker of apoptosis [406, 407].

This assay uses a specific caspase-3 substrate, N-Ac-DEVD-N'-MC-R110, which upon cleavage by active caspase-3, generates a highly fluorescent product that can be measured using excitation and emission wavelengths of 485 and 535 nm respectively [408].

The Caspase-3 fluorescence assay kit purchased from Sapphire Bioscience (Cayman Chemical, Michigan, USA) consisting of Caspase-3 substrate N-Ac-DEVD-N'-MC-R110, active caspase-3 standard, caspase-3 inhibitor N-Ac-Asp-Glu-Val-Asp-CHO, caspase-3 dithiothreitol (1 M), caspase-3 assay buffer and cell-based assay lysis buffer and used.

Caspase-3 activation was used as index of apoptosis. Cells were seeded at a density of 2×10^5 cells/mL, incubated for 24 h and then treated as described earlier in section 5.3.1. Following incubation for a further 24 h, caspase-3 activity was determined using a caspase-3 fluorescence assay kit (Cayman Chemicals, Michigan, USA). All steps were performed according to the manufacturer's instructions. Briefly, the culture medium of treated cells in 96-well microtitre plates was aspirated and 200 μ L of caspase-3 assay buffer added to each well and the microtitre plate subsequently centrifuged at $400 \times g$ for 10 min using an Eppendorf 5810 R centrifuge. The

supernatant was then aspirated and 100 μ L of the cell-based assay lysis buffer added to each well. The plate was then left to incubate at room temperature for 30 minute on an orbital shaker. After incubation, the plate was then centrifuged at 400 x *g* for 10 min using an Eppendorf 5810 R centrifuge and 90 μ L of supernatant transferred to a black 96-well microtitre plate and 10 μ L of the caspase-3 assay buffer and 100 μ L of the caspase-3 substrate solution were added to the wells and the plates were then incubated at 37°C for 30 minutes. The fluorescence intensity was then read at excitation 485 nm and emission 535 nm using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland).

Necrosis

Propidium iodide fluorimetry

The propidium iodide assay was performed according to the methods outlined in section 4.3.4. Briefly, cells were plated and treated with the appropriate compound according to the methods described in section 5.3.2. The Annexin V-FITC apoptosis kit (Cayman Chemical, Michigan, USA) was used to measure propidium iodide fluorescence and was performed according to the manufacturer's instructions.

Cyclic AMP assay

The Cyclic AMP EIA kit (Cayman Chemical, Michigan, USA), was used in this study. This assay is a competitive EIA and is based on the competition between free cAMP and a cAMP-acetylcholinesterase conjugate for a limited number of cAMP-specific binding sites. The amount of cAMP-acetylcholinesterase able to bind will be

inversely proportional to the amount of cAMP in the sample. An acetylcholinesterase substrate is then added to each well to enzymatically produce a yellow product that is then measured spectrophotometrically at 412 nm. The intensity of the product is inversely proportional to the concentration of free cAMP in the sample.

The cyclic AMP EIA assay kit (Cayman Chemicals, Michigan, USA) was used in this study and contained cyclic AMP EIA antiserum, cyclic AMP AChE Tracer, cyclic AMP EIA standard, EIA buffer concentrate (10X), wash buffer concentrate (400X), polysorbate 20, mouse anti-rabbit IgG coated plate, Ellman's reagent, acetic anhydride, potassium hydroxide, EIA tracer dye and EIA antiserum dye.

To determine the effects of 24 h exposure of bioactive coffee constituents on cAMP concentrations in undifferentiated and differentiated SH-SY5Y neuroblastoma cells, cells were treated according to the protocols outlined in section 5.3.2 above. All steps were performed according to the manufacturer's instructions. Briefly, all buffers, samples and assay specific reagents were prepared according to the manufacturer's instructions. The appropriate amount of EIA buffer, cAMP EIA standards, samples, cAMP AChE tracer and cAMP EIA antiserum were added to the appropriate wells of the pre-coated plate and the plate incubated for 18 h at 4°C. Following the appropriate incubation, the plate was developed by adding 200 µL of freshly reconstituted Ellman's reagent to each well along with 5 µL of tracer to the total activity wells. The plate was then left to develop for 2 h on an orbital shaker protected from light. UV absorbance was read using a Tecan Infinite M200 Pro (Tecan, Mannedorf, Switzerland) at 405 nm.

5.3.3 Statistical analysis

One-way ANOVA with Tukey's post hoc test (where significance was observed) were used in this study and were performed using GraphPad InStat version 3.06 (2003) with $P < 0.05$ (*,#), $P < 0.01$ (**,##), and $P < 0.001$ (***,###). All graphs were drawn using Graphpad Prism v6 (San Diego, USA).

5.4 Results

5.4.1 The effects of key bioactive coffee constituents, alone and in combination with caffeine, on markers of inflammation in a surrogate microglial cell model

The effects of key bioactive coffee constituents on the proliferation of undifferentiated and differentiated THP-1 human monocytic cells

Resazurin proliferation assay

The resazurin reduction assay was used to assess the effects of key bioactive coffee constituents on the proliferation of undifferentiated and differentiated THP-1 human monocytic cells.

Undifferentiated THP-1 monocytic cells

The exposure of undifferentiated THP-1 cells to various concentrations of the key bioactive coffee constituents resulted in an alteration in their proliferation in a number of cases. CGA (10 μ M, 100 μ M and 1000 μ M) and PA (1000 μ M) significantly decreased the proliferation of undifferentiated THP-1 human monocytic cells. Conversely, PA (10 μ M and 100 μ M) and trigonelline (100 μ M and 1000 μ M) both increased the proliferation of undifferentiated THP-1 human monocytic cells. All changes in viability were modest in nature with exception to the decreases in viability caused by PA 1000 μ M, where a 50% decrease in viability was observed, as seen in figure 144 below.

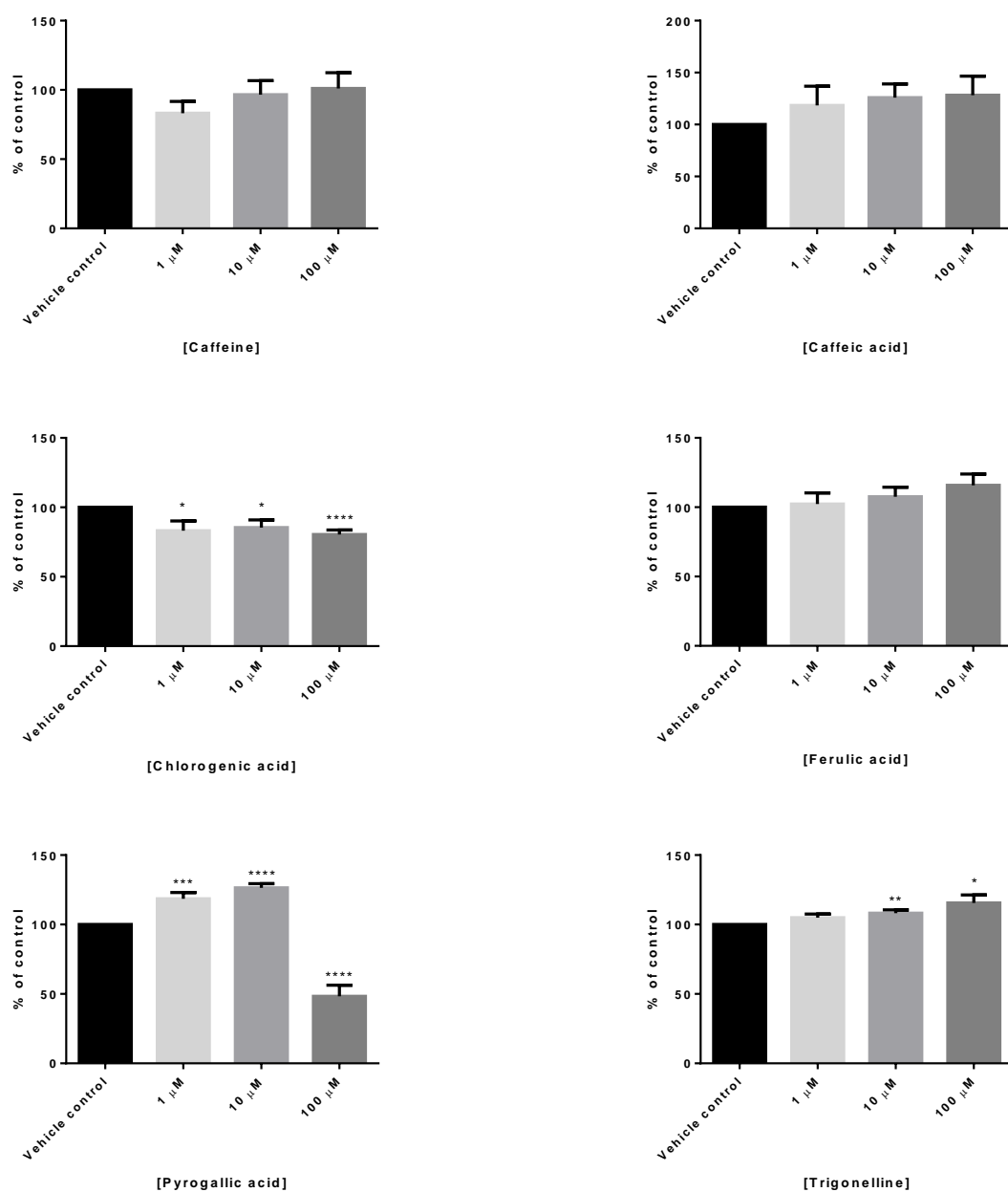


Figure 144 – Viability of undifferentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents using the resazurin reduction assay (n = 9)

Differentiated THP-1 monocytic cells

The exposure of differentiated THP-1 human monocytic cells to key bioactive coffee constituents altered the proliferation of these cells. Caffeine (1 μ M, 10 μ M and 100 μ M), CGA (100 μ M) and PA (100 μ M) all significantly decreased the proliferation of the differentiated THP-1 human monocytic cells. Conversely, CA (100 μ M) and FA (1 μ M and 10 μ M) both increased the proliferation of THP-1 human monocytic cells as seen in figure 145 below. All changes observed were modest in nature.

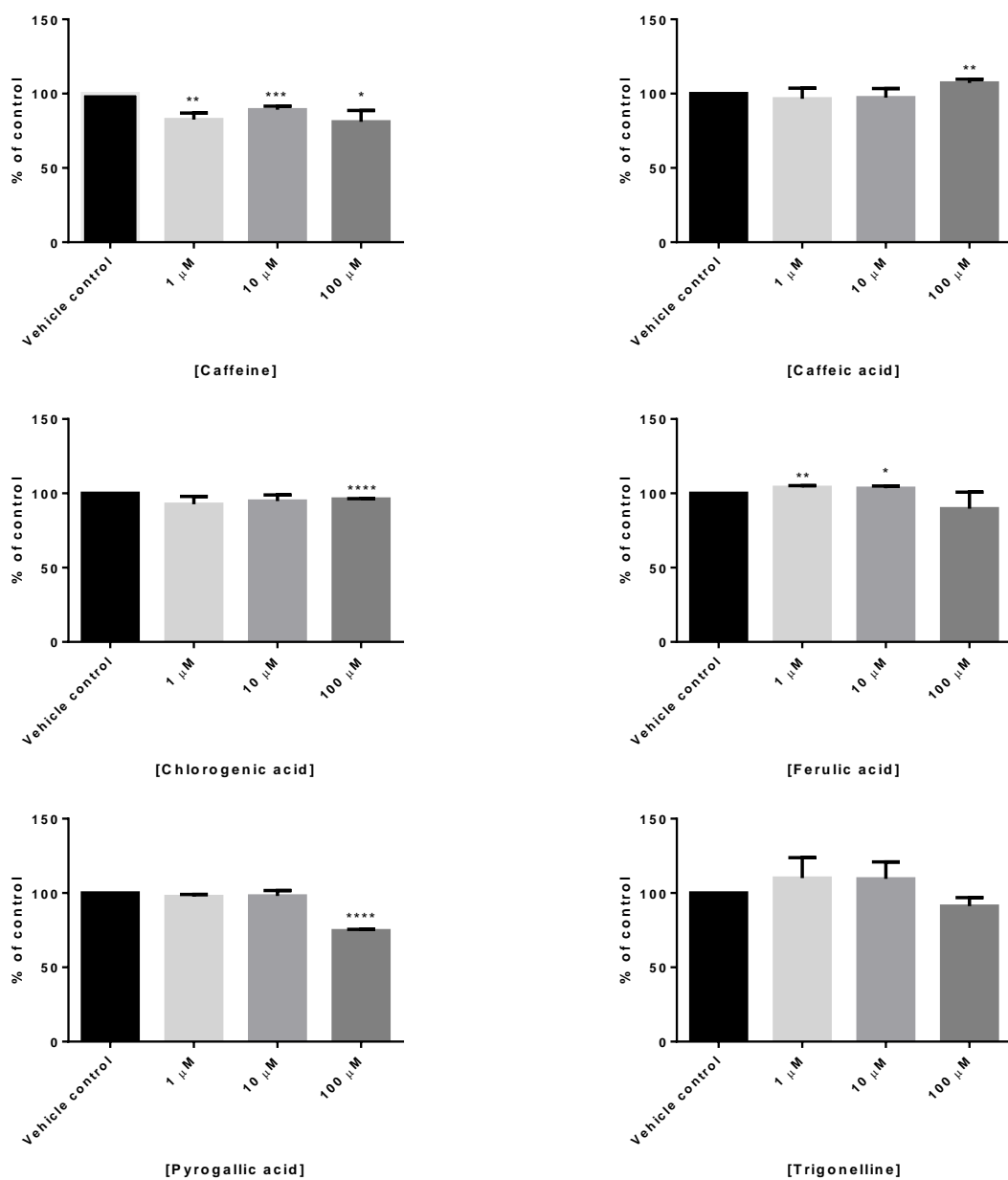


Figure 145 – Viability of differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents using the resazurin reduction assay (n = 9)

Changes in the proliferation of differentiated THP-1 human monocytic cells were observed after these cells were exposed to key bioactive coffee constituents in

combination with caffeine (100 μ M) for 24 h. An increase in the proliferation of differentiated THP-1 human monocytic cells was observed in cells treated with CA + 100 μ M caffeine (100 μ M). Conversely, a decrease in proliferation was observed in cells exposed to CGA + 100 μ M of caffeine (100 μ M) and PA + 100 μ M caffeine (1 μ M and 10 μ M) as seen in figure 146 below. All changes observed were modest in nature.

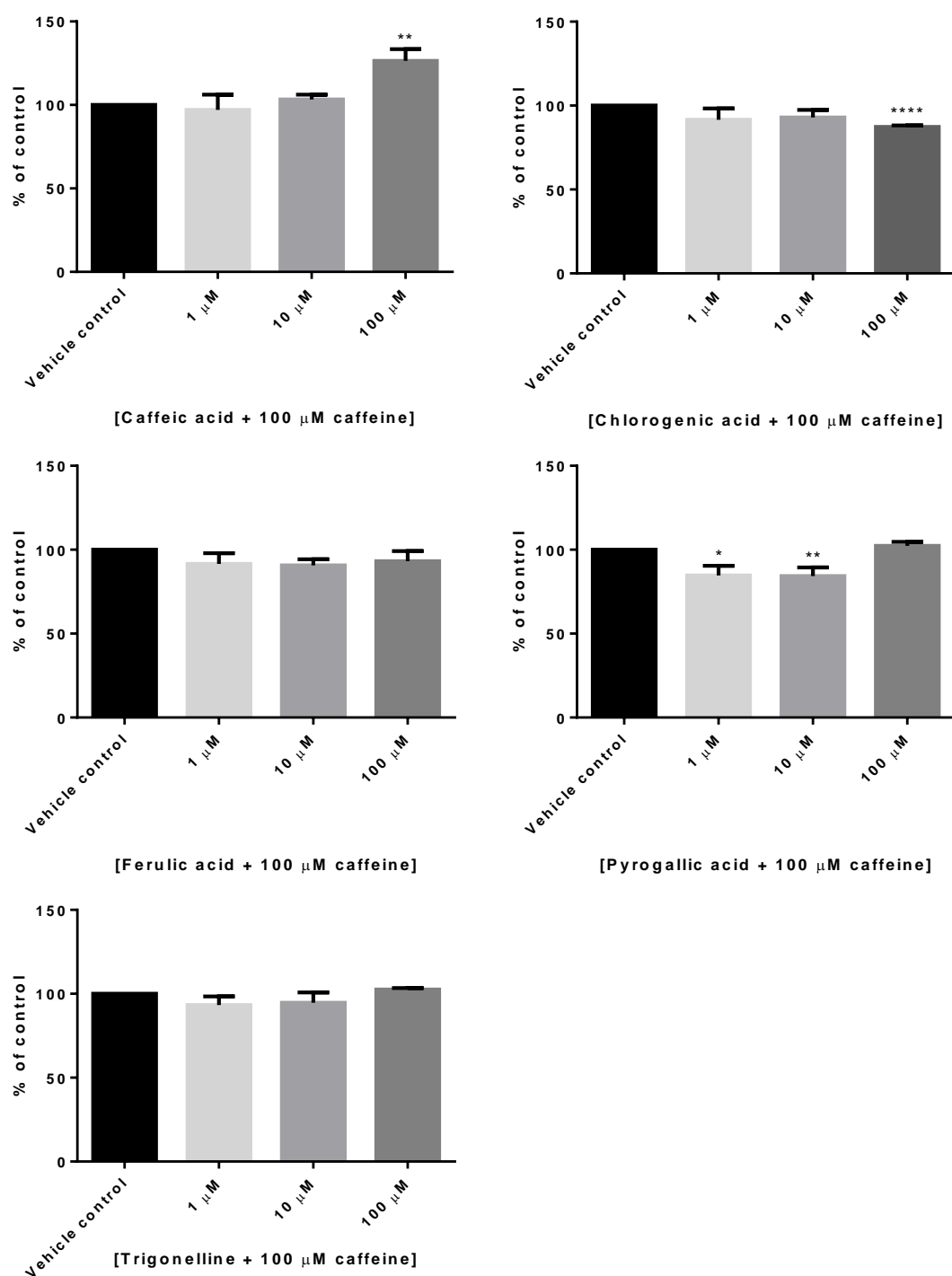


Figure 146 – Viability of differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents in combination with caffeine using the resazurin reduction assay

Modest decreases in the viability of differentiated THP-1 human monocytic cells pre-treated with several of key bioactive coffee constituents and then treated with IFN- γ was observed. Decreases in viability were observed after these cells were pre-treated with caffeine (100 μ M) and trigonelline (1 μ M). Conversely, pre-treatment with caffeic acid (100 μ M) and subsequent treatment with IFN- γ afforded a modest increase in viability in comparison to the vehicle control as seen in figure 147 below.

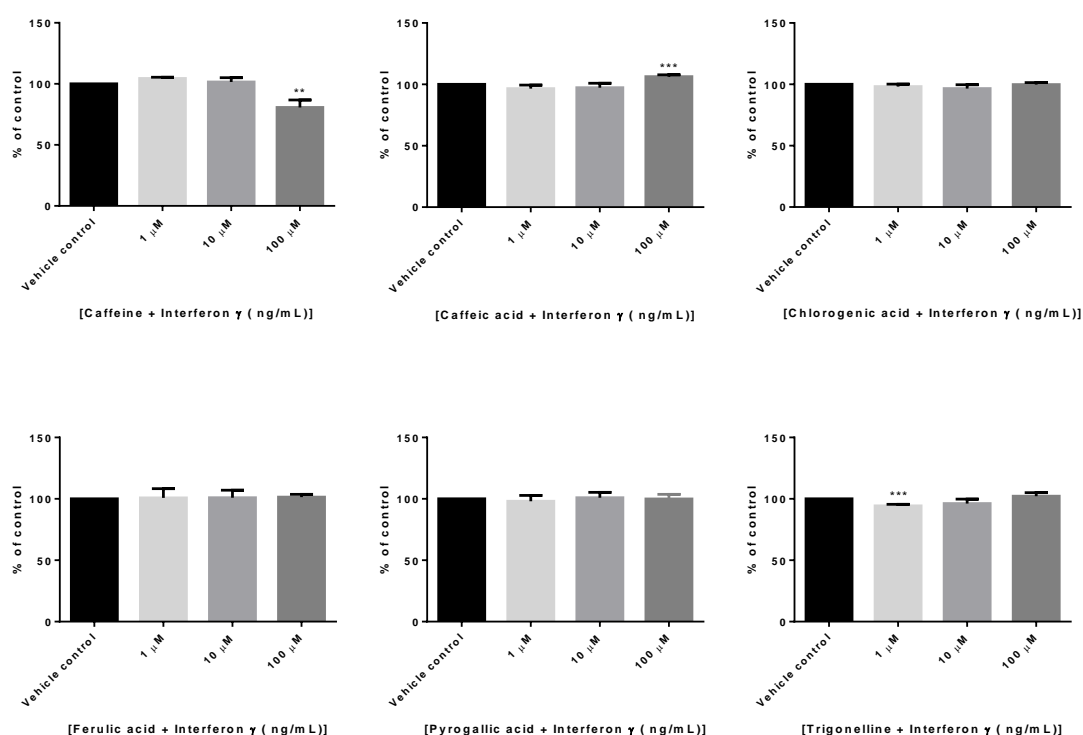


Figure 147 – Viability of differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents and interferon gamma using the resazurin reduction assay (n = 9)

No changes in the viability of differentiated THP-1 cells pre-treated with key bioactive coffee constituents in combination with 100 μ M of caffeine and treated with human IFN- γ was observed as seen in figure 148 below.

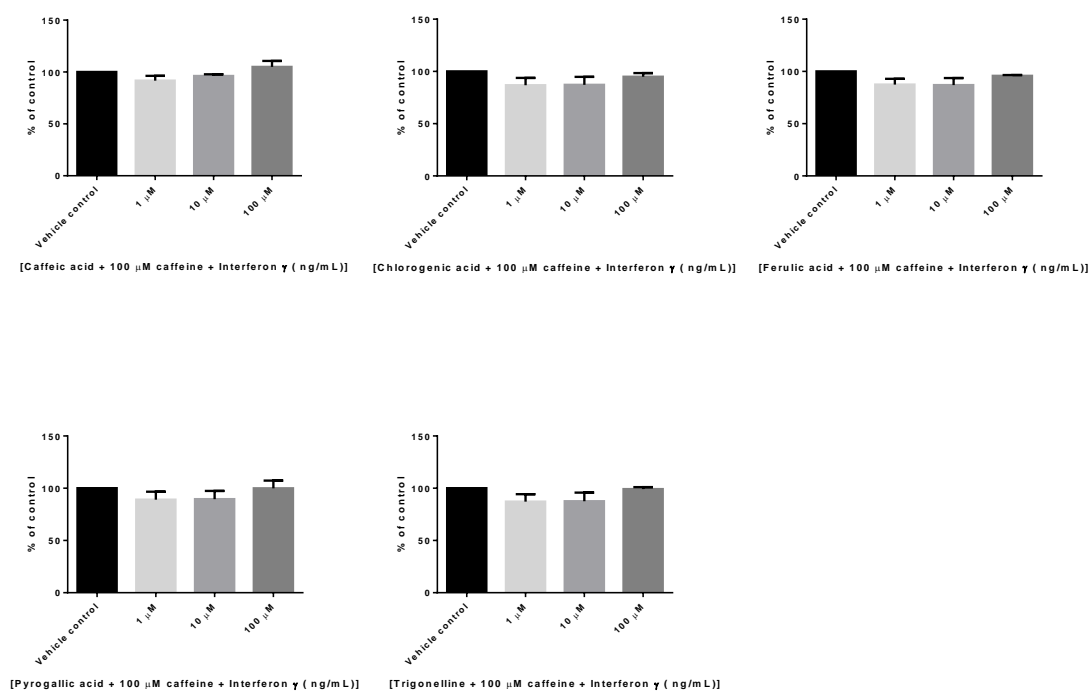


Figure 148 – Viability of differentiated THP-1 cells after 24 h pre-treatment with key bioactive coffee constituents in combination with 100 μ M caffeine and treatment with interferon gamma using the resazurin reduction assay (n = 9)

A modest decrease in the viability of differentiated THP-1 cells pre-treated with caffeine (1 μ M) and PA (1 μ M, 10 μ M and 100 μ M) and treated with IFN- α_{2A} was observed in comparison to the vehicle control as seen in figure 149 below.

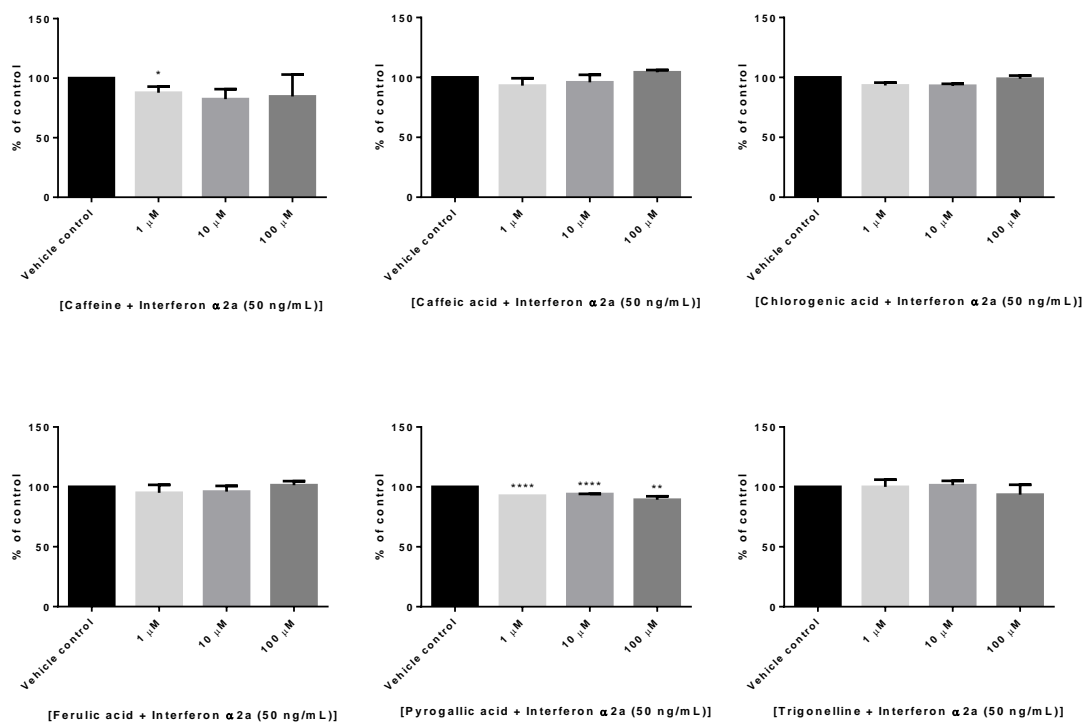


Figure 149 – Viability of differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents and interferon alpha using the resazurin reduction assay (n = 9)

A very small decrease in the viability of THP-1 cells pre-treated with CGA (1 μ M and 10 μ M) and subsequently treated with human IFN- α _{2A} in comparison to the vehicle control was observed. No other changes to viability were observed as seen in figure 150 below.

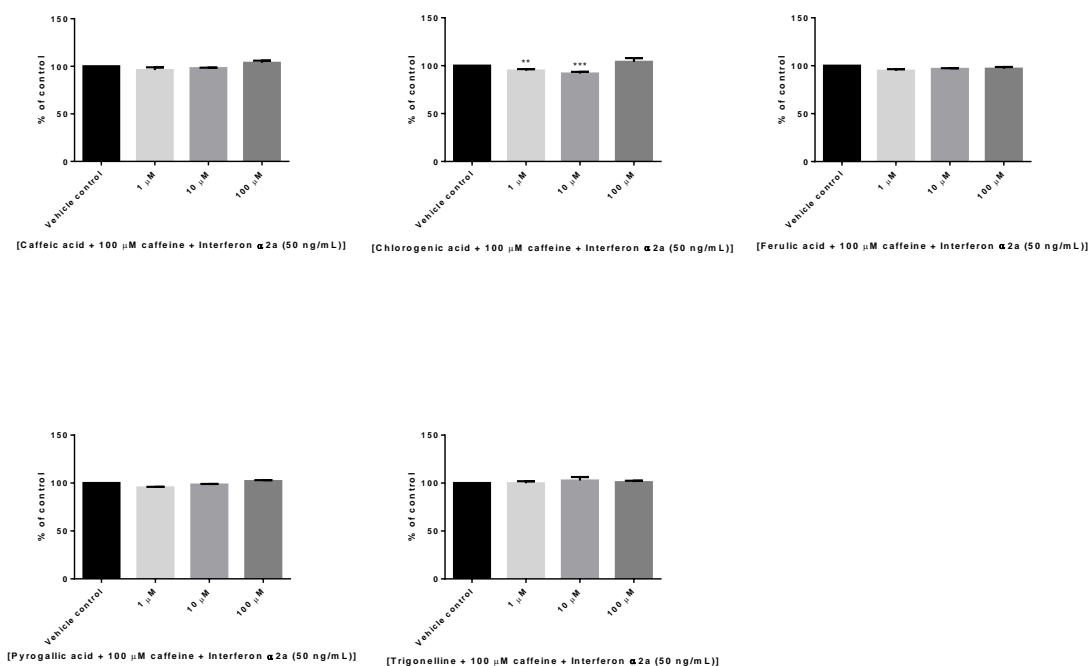


Figure 150 – Viability of differentiated THP-1 cells after 24 h pre-treatment with key bioactive coffee constituents in combination with 100 μ M caffeine and treatment with interferon alpha using the resazurin reduction assay (n = 9)

The effects of key bioactive coffee constituents on free radical production in differentiated THP-1 human monocytic cells

DCFH-DA assay

The effect of 24 h exposure of key bioactive coffee constituents on the production of free radicals in differentiated THP-1 human monocytic cells was evaluated using DCFH-DA. A number of changes were observed. Caffeine (1 μ M, 10 μ M and 100 μ M), FA (1 μ M, 10 μ M and 100 μ M), PA (100 μ M) and trigonelline (100 μ M) were shown to significantly decrease free radical production in differentiated THP-1 human monocytic cells. Conversely, CA (1 μ M, 10 μ M and 100 μ M), CGA (10 μ M) and PA (1 μ M and 10 μ M) were shown to significantly increase the production of free

radicals as seen in figure 151. Approximate 50% reductions in free radical production were observed in differentiated THP-1 cells was observed after treatment with caffeine, FA, PA and trigonelline and modest increases were observed in cells treated with CA and CGA as seen in figure 151 below.

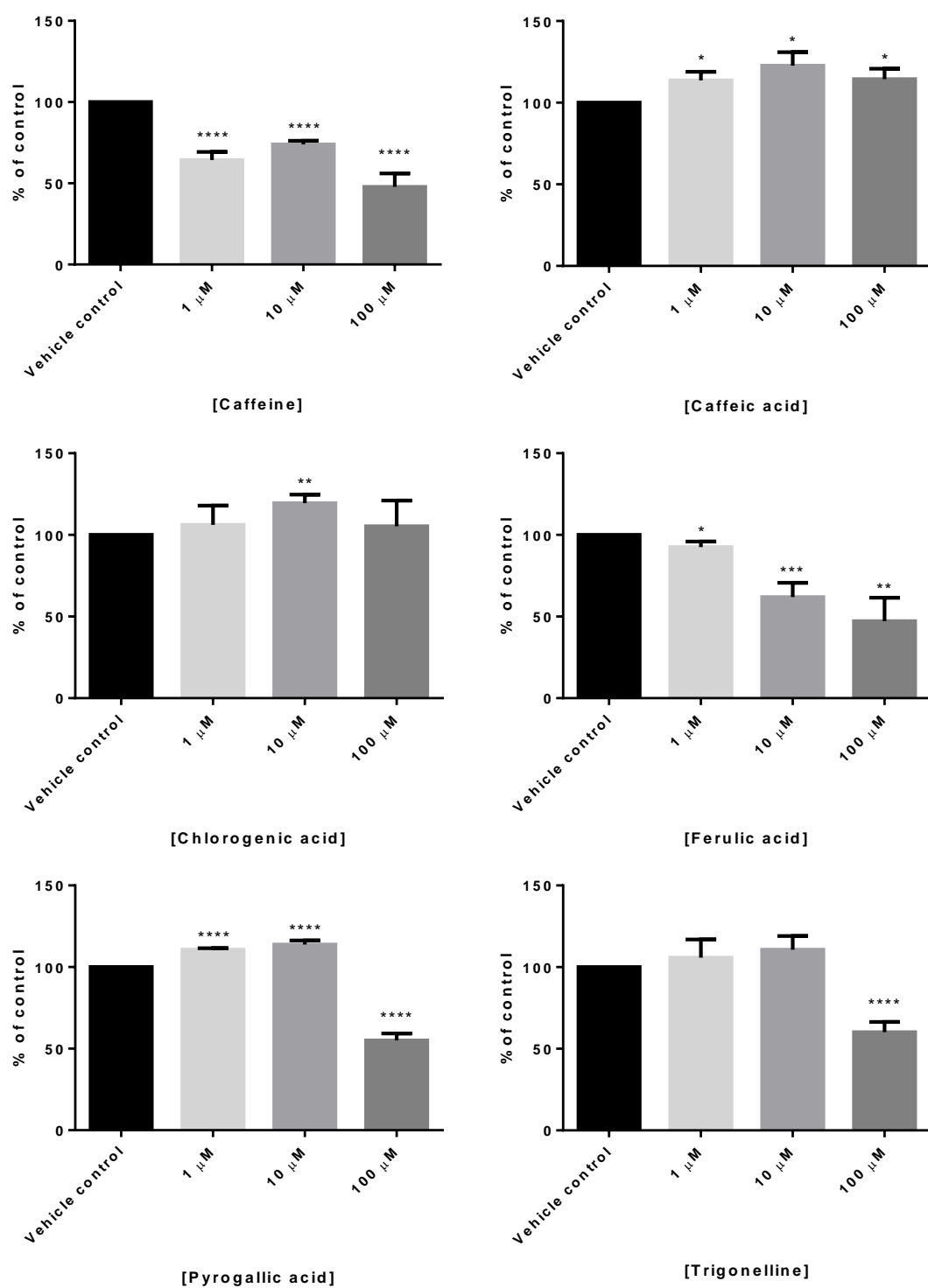


Figure 151 – Free radical production in differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents using the DCFH-DA assay (n = 9)

Differences in free radical production were observed in differentiated THP-1 human monocytic cells that have had 24 h exposure to key bioactive coffee constituents in combination with 100 μ M caffeine. Decreases in free radical production were observed after exposure to CA + 100 μ M caffeine (100 μ M) and CGA + 100 μ M caffeine (100 μ M). Conversely, increases in free radical production were observed after exposure to CGA + 100 μ M caffeine (10 μ M), FA + 100 μ M caffeine (1 μ M) and trigonelline + 100 μ M caffeine (1 μ M, 10 μ M and 100 μ M). All changes were modest in nature as seen in figure 152.

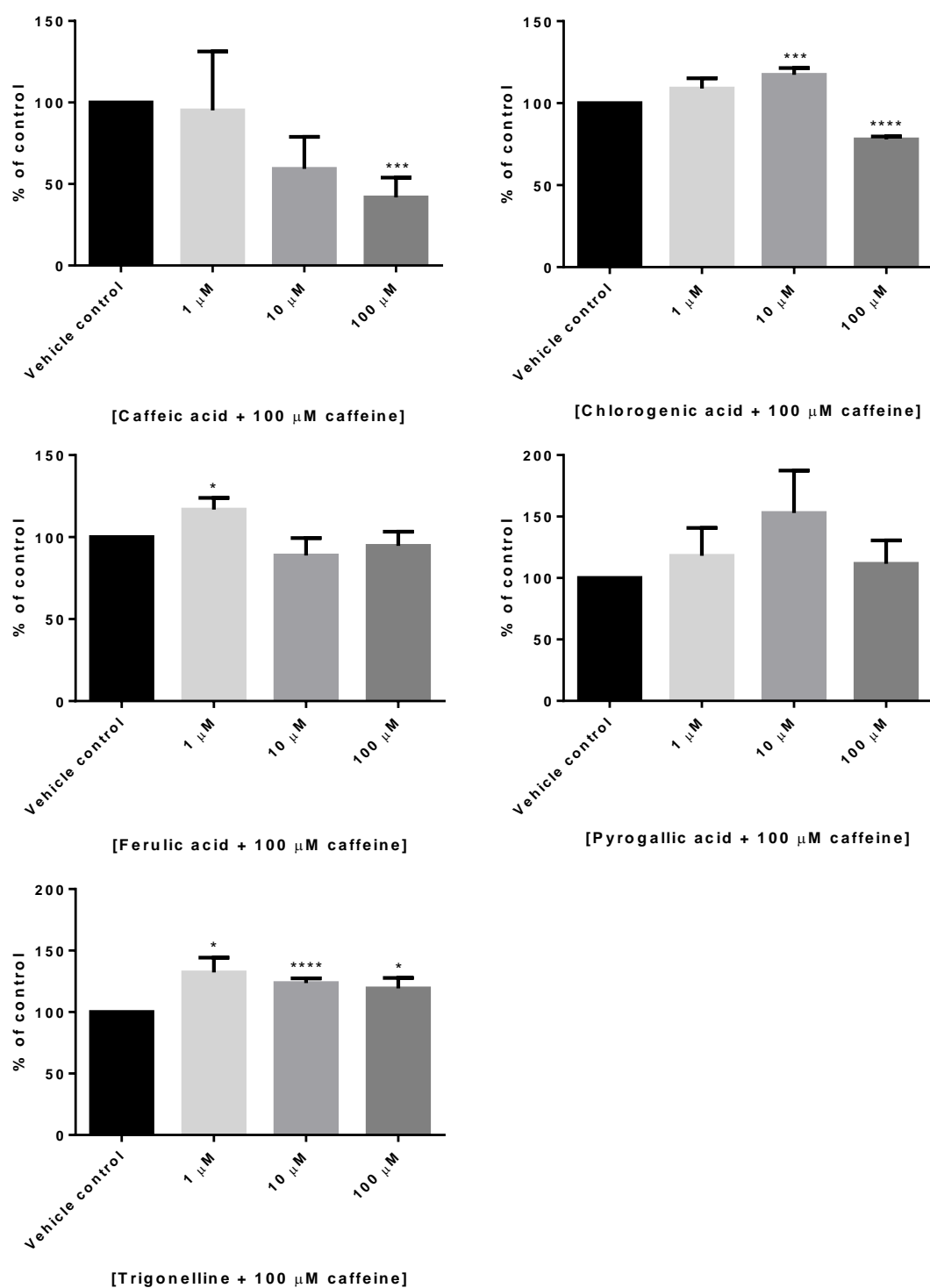


Figure 152 – Free radical production in differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents in combination with caffeine using the DCFH-DA assay

An increase in the free radical production in differentiated THP-1 cells pre-treated with caffeine (10 μ M) and subsequently treated with human IFN- γ in comparison to the vehicle control was observed. This increase was in the magnitude of approximately 50% higher than vehicle control. No other changes to free radical production was observed with other treatments as seen in figure 153 below.

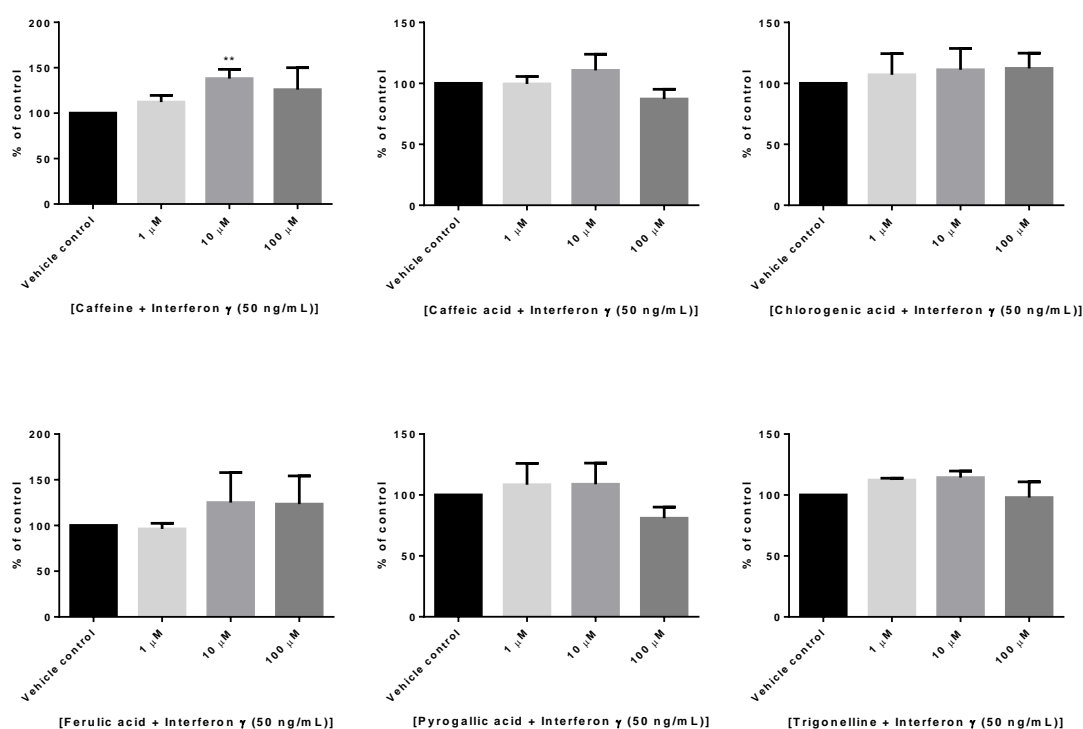


Figure 153 – Free radical production in differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents using the DCFH-DA assay (n = 9)

A significant decrease in free radical production was observed after differentiated THP-1 cells were pre-treated with PA (100 μ M) and subsequently treated with IFN- γ in comparison to the vehicle control. Conversely, pre-treatment of THP-1 cells with trigonelline (1 μ M) in combination with 100 μ M caffeine and treated with IFN- γ afforded an increase in the free radical production of approximately 50% in comparison to the vehicle control as seen in figure 154 below.

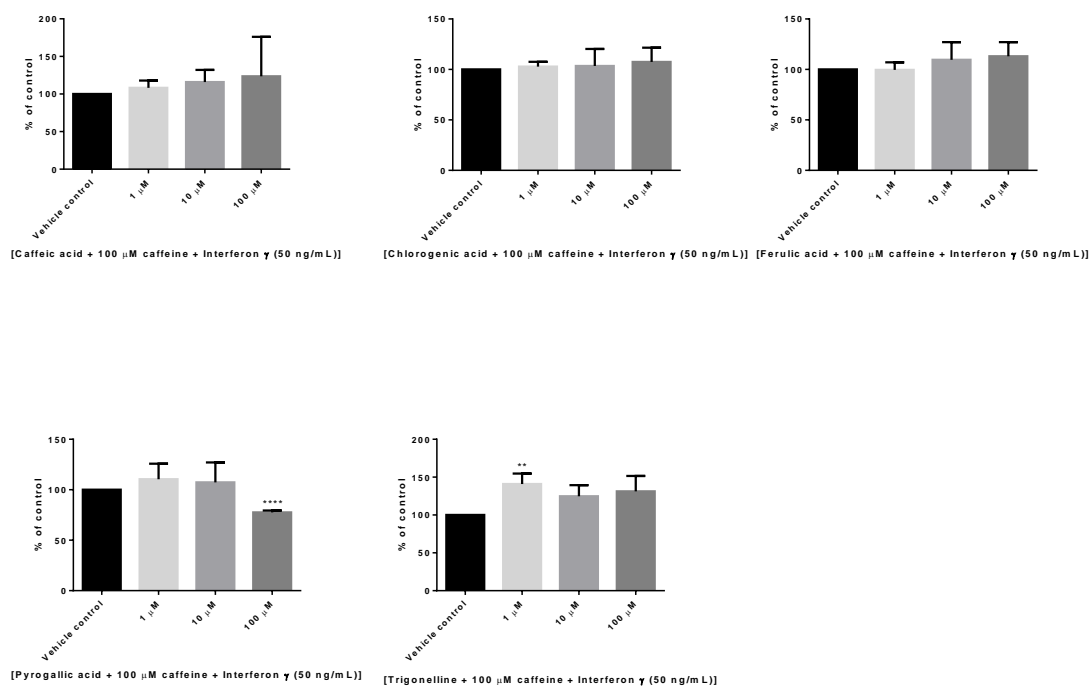


Figure 154 – Free radical production in differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents in combination with caffeine using the DCFH-DA assay

An increase in the free radical production in differentiated THP-1 cells pre-treated with caffeine (1 μM) and PA (1 μM) and subsequently treated with human IFN-γ in comparison to the vehicle control was observed. This increase was in the magnitude of approximately 25% higher than vehicle control. No other changes to free radical production were observed with other treatments as seen in figure 155 below.

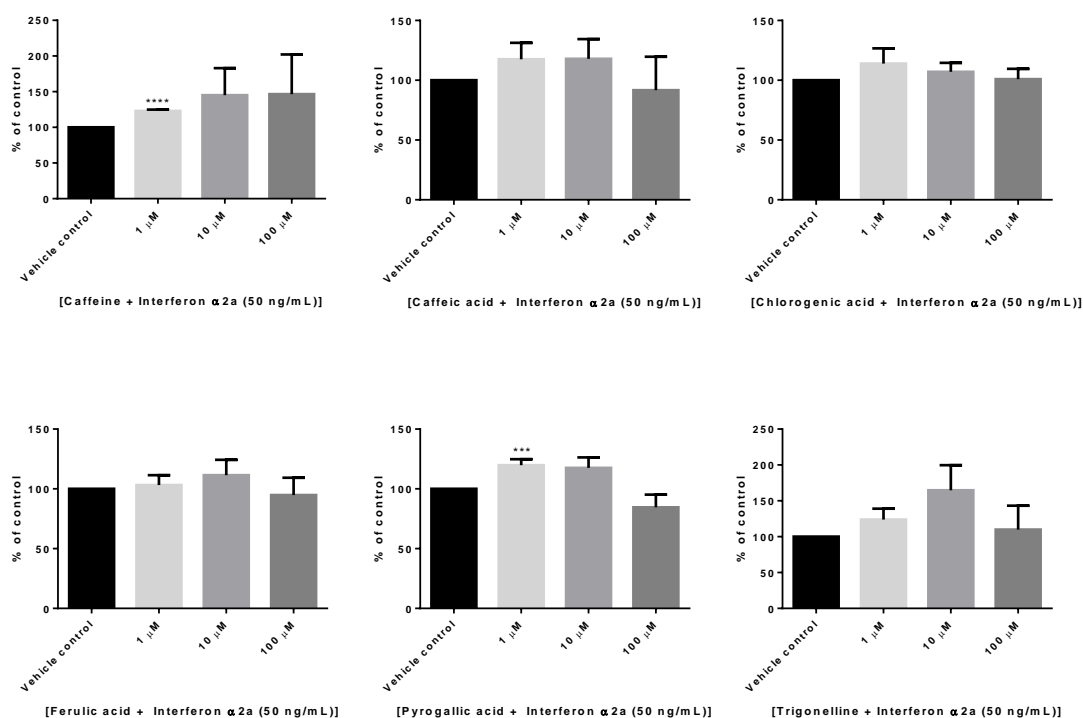


Figure 155 – Free radical production in differentiated THP-1 cells after 24 h pre-treatment to key bioactive coffee constituents and interferon alpha using the DCFH-DA assay (n = 9)

A significant increase in free radical production was observed after differentiated THP-1 cells were pre-treated with FA (1 μ M) and subsequently treated with IFN- α _{2A} in comparison to the vehicle control. This afforded an increase in the free radical production of approximately 20% in comparison to the vehicle control as seen in figure 156 below.

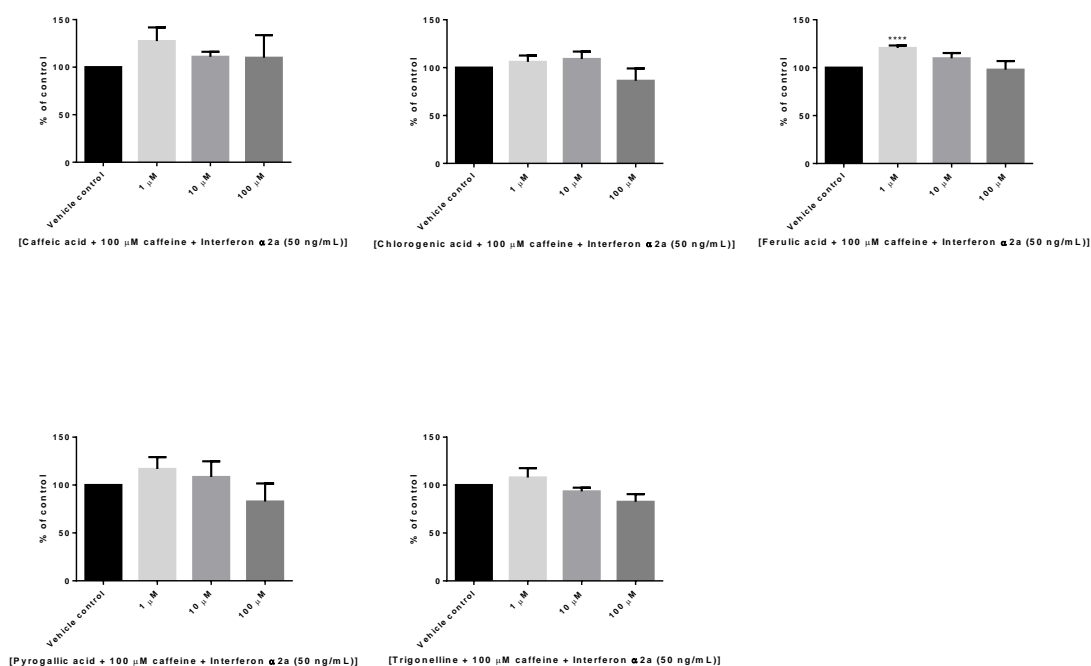


Figure 156 – Free radical production in differentiated THP-1 cells after 24 h exposure to key bioactive coffee constituents in combination with caffeine using the DCFH-DA assay

The effects of key bioactive coffee constituents on markers of inflammation in interferon gamma stimulated differentiated THP-1 monocytic cell

Caffeine

IDO activity

IDO activity was measured by quantifying Trp and KYN levels and subsequently taking the ratio of KYN to Trp as a measure of the activity of the enzyme. All three concentrations (1 μ M, 10 μ M and 100 μ M) of caffeine assessed showed significant inhibitory effects on the activity of IDO. Decreases in the magnitude of approximately 7-fold was observed, as seen in figure 157.

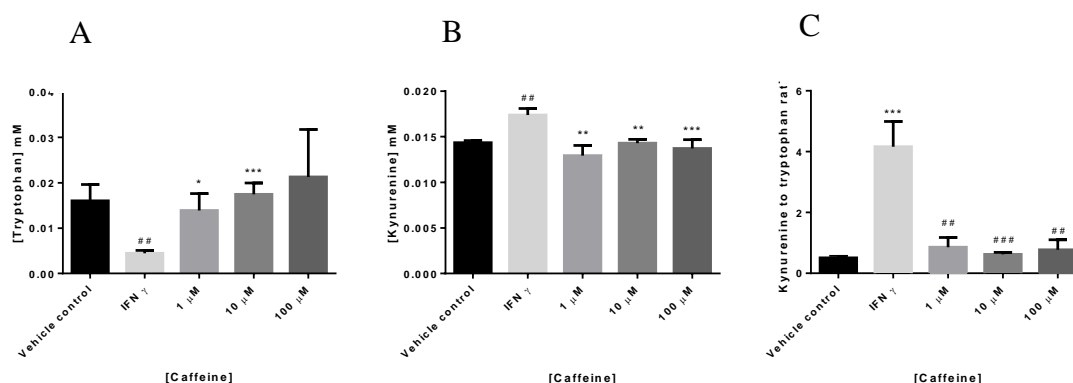


Figure 157 – a) The effects of caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

In the current model, statistically significant increases in pro-inflammatory cytokines are only observed in IL-6 concentrations after treatment with IFN- γ , however, trends of increase are observed in IL-1 β concentrations and a trend of decrease observed in TNF- α concentrations. Statistically significant decreases in IL-6 concentrations were observed after IFN- γ stimulated cells were exposed to caffeine (1 μ M and 10 μ M). Trends in the increase in TNF- α concentrations were observed after 24 h exposure to caffeine however these were not statistically significant. IL-6 concentrations decreased by approximately half and returned to the levels seen in cells treated with vehicle, as seen in figure 158 below.

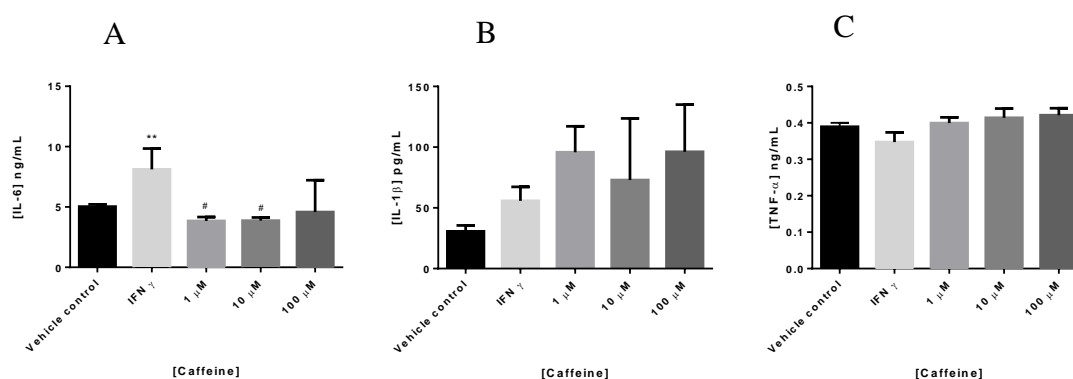


Figure 158 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeic acid

IDO activity

24 h pre-treatment of differentiated THP-1 human monocytic cells with CA afforded corrections to the human IFN- γ depletion of Trp, caused an increase in KYN and a resultant increase in IDO activity. CA was shown to significantly increase the Trp concentration present in the samples at 1 μ M and 100 μ M with an observed trending increase in cells treated with 10 μ M. Furthermore, corresponding decreases in KYN concentration will all three concentrations of CA tested afforded a statistically significant decrease in comparison to IFN- γ alone. This subsequently resulted in a significant decrease in IDO activity at all three concentrations tested by approximately 3-fold as seen in figure 159.

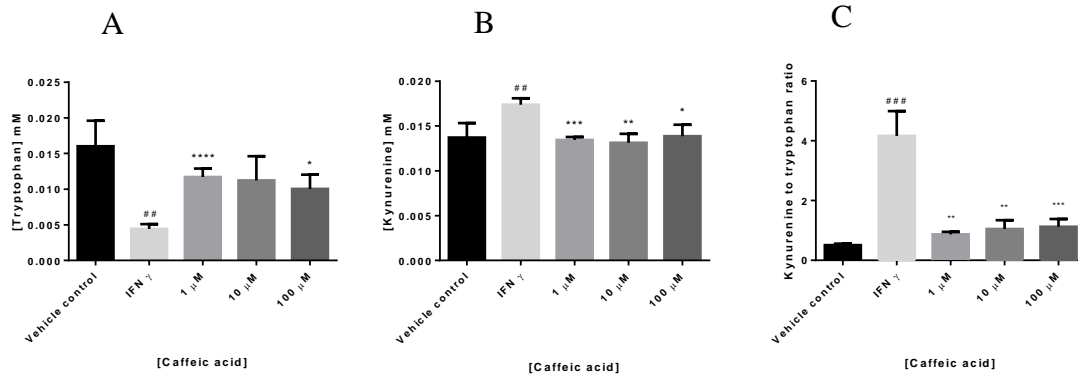


Figure 159 - a) The effects of caffeic acid exposure of interferon gamma-induced tryptophan concentrations; b) The effects of caffeic acid exposure on interferon gamma-induced kynurenine concentrations; c) The effects of caffeic acid exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CA resulted in the decrease in IL-6 concentrations after exposure to 10 μM and increases in TNF-α concentrations at 1 μM, 10 μM and 100 μM in comparison to IFN-γ stimulus alone as seen in figure 160.

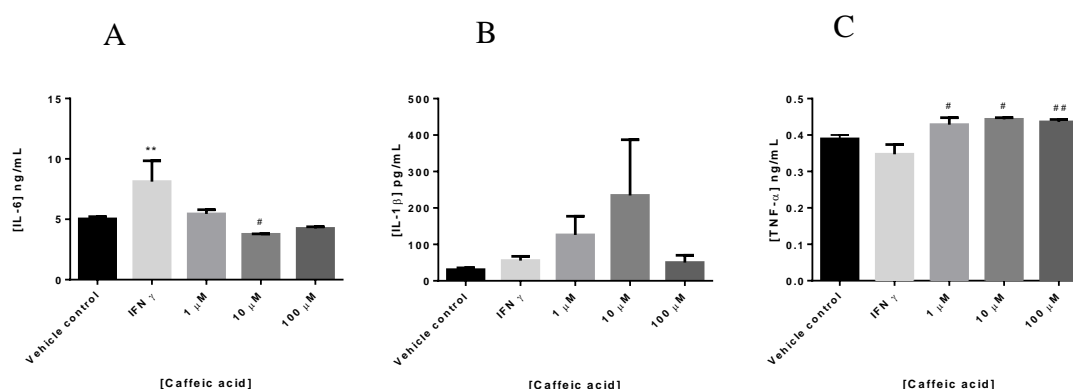


Figure 160 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with caffeic acid on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus

Chlorogenic acid

IDO activity

CGA was shown to significantly increase the Trp concentration present in the samples at all three concentrations tested. Furthermore, corresponding statistically significant decreases in KYN concentration was observed at 1 μ M with 1 μ M and 100 μ M trending towards significance in comparison to IFN- γ alone. This subsequently resulted in a significant decrease in IDO activity at all three concentrations tested by approximately 5-fold as seen in figure 161.

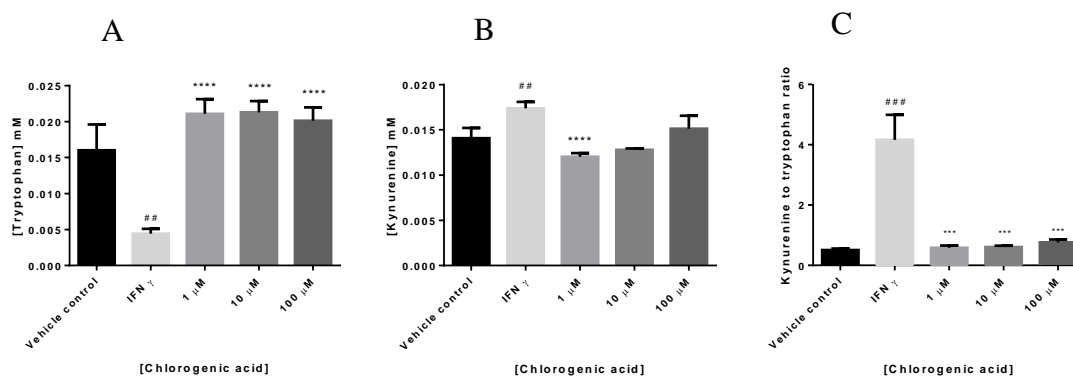


Figure 161 - a) The effects of chlorogenic acid exposure of interferon gamma-induced tryptophan concentrations; b) The effects of chlorogenic acid exposure on interferon gamma-induced kynurenine concentrations; c) The effects of chlorogenic acid exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CGA resulted in the non-significant decrease in IL-6 concentrations after exposure to all three concentrations tested. Non-significant increase in IL-1 β were observed along with significant increases in TNF- α concentrations at 1 μ M, 10 μ M and 100 μ M in comparison IFN- γ stimulus alone as seen in figure 162.

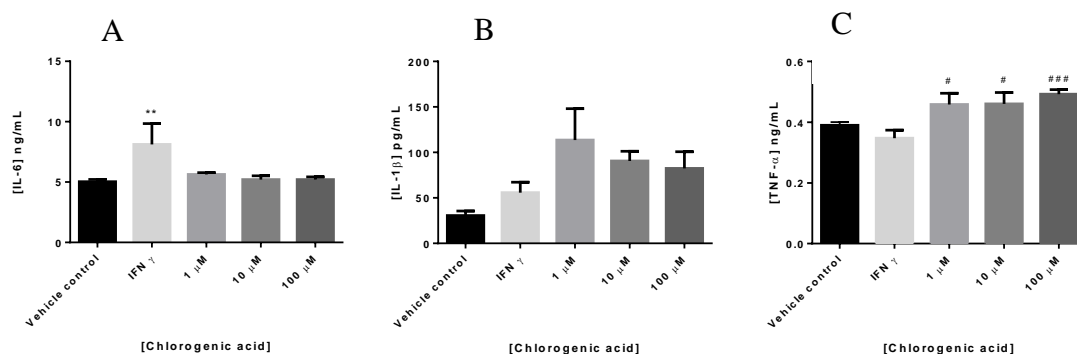


Figure 162 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with chlorogenic acid on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Ferulic acid

IDO activity

FA was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested. Statistically significant decreases in KYN concentration were however observed at all three concentrations tested in comparison to IFN-γ alone. This subsequently resulted in a significant decrease in IDO activity at all three concentrations tested in comparison to IFN-γ alone by approximately 50% as seen in figure 163.

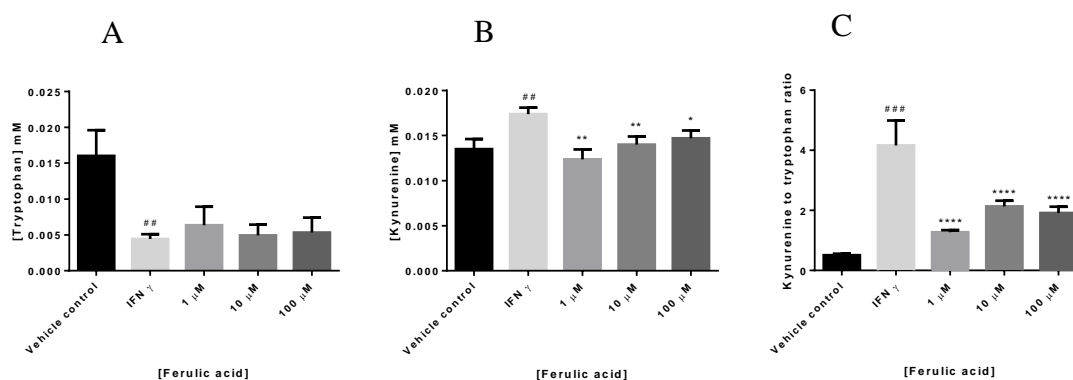


Figure 163 - a) The effects of ferulic acid exposure of interferon gamma-induced tryptophan concentrations; b) The effects of ferulic acid exposure on interferon gamma-induced kynurenine concentrations; c) The effects of ferulic acid exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with FA resulted in the non-significant decrease in IL-6 concentrations after exposure to all three concentrations tested. Significant increases in IL-1 β were observed at 10 μ M and 100 μ M along with significant increases in TNF- α concentrations at 1 μ M, 10 μ M and 100 μ M in comparison to IFN- γ stimulus alone as seen in figure 164.

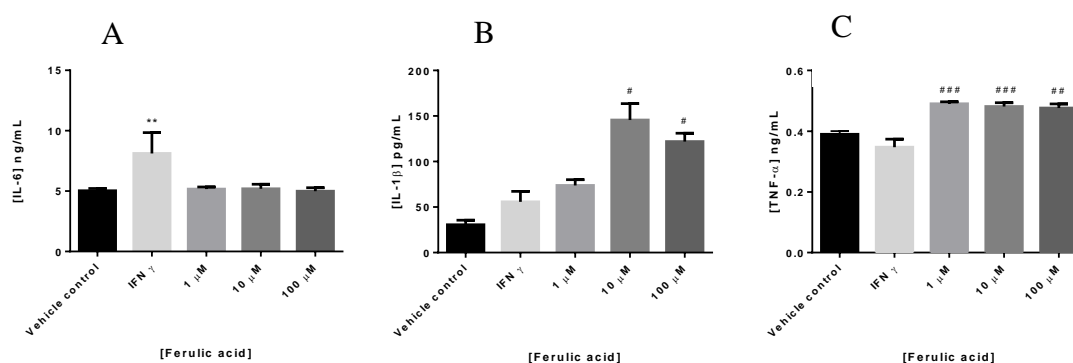


Figure 164 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with ferulic acid on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Pyrogalllic acid

IDO activity

PA was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested however a trending decrease was observed. Statistically significant decreases in KYN concentration were however observed at 10 μ M and non-significant decreases at 1 μ M and 100 μ M in comparison to IFN- γ alone. This subsequently resulted in a significant increase in IDO activity at all three concentrations tested in comparison to IFN- γ alone as seen in figure 165.

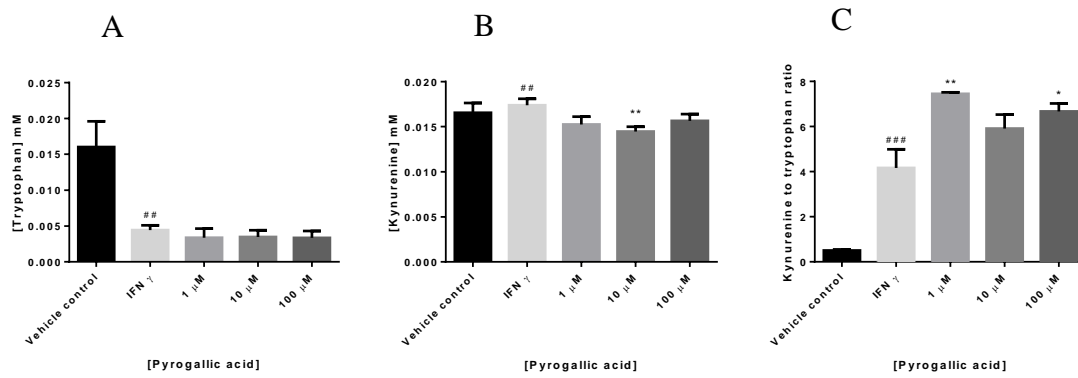


Figure 165 - a) The effects of pyrogallol acid exposure of interferon gamma-induced tryptophan concentrations; b) The effects of pyrogallol acid exposure on interferon gamma-induced kynurenine concentrations; c) The effects of pyrogallol acid exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with PA resulted in the non-significant decrease in IL-6 concentrations in an apparent concentration-dependent manner after exposure to all three concentrations tested. Furthermore, non-significant decreases in IL-1 β were observed at 10 μ M and 100 μ M along with significant increases in TNF- α concentrations at 1 μ M, 10 μ M and 100 μ M in comparison to IFN- γ stimulus alone as seen in figure 166.

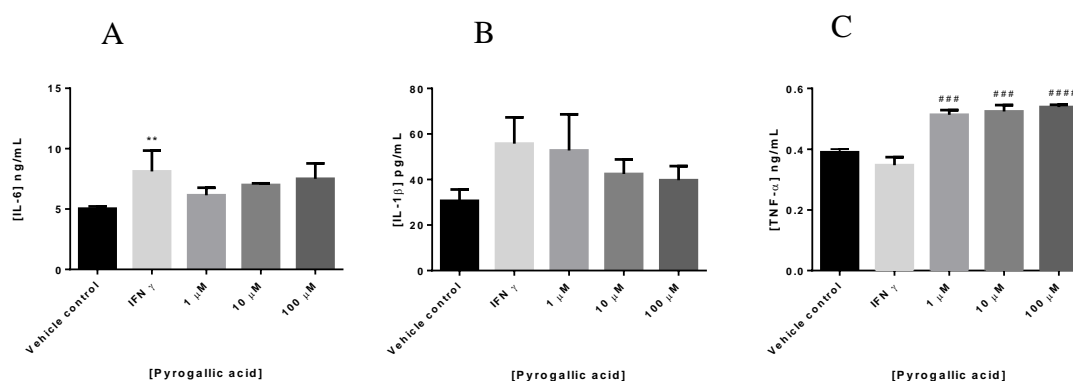


Figure 166 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with pyrogalllic acid on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Trigonelline

IDO activity

Trigonelline was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested however, statistically significant increases in KYN concentration were observed at all three concentrations tested in comparison to IFN- γ alone. This however did not lead to any change in IDO activity in comparison to IFN- γ alone as seen in figure 167.

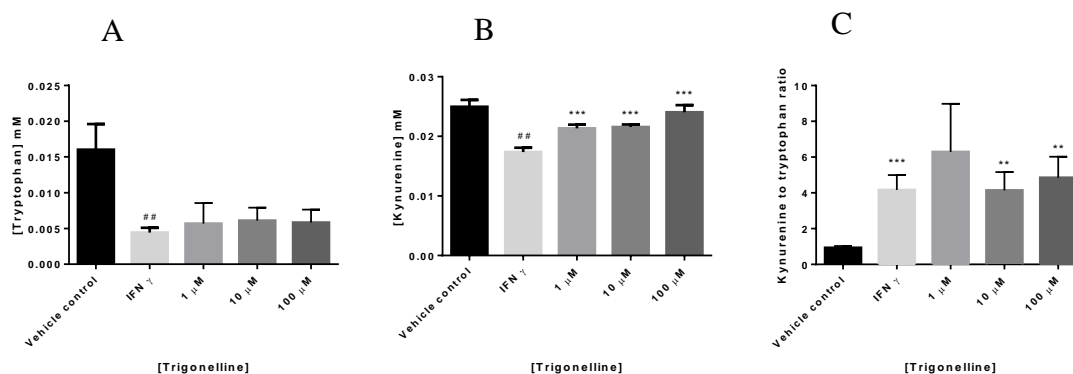


Figure 167 - a) The effects of trigonelline exposure of interferon gamma-induced tryptophan concentrations; b) The effects of trigonelline exposure on interferon gamma-induced kynurenine concentrations; c) The effects of trigonelline exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with trigonelline resulted in the non-significant increase in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, non-significant increases in IL-1 β were observed at all three concentrations tested with significant increases in TNF- α concentrations, of approximately 50%, at 1 μ M, 10 μ M and 100 μ M in comparison to IFN- γ stimulus alone as seen in figure 168 below.

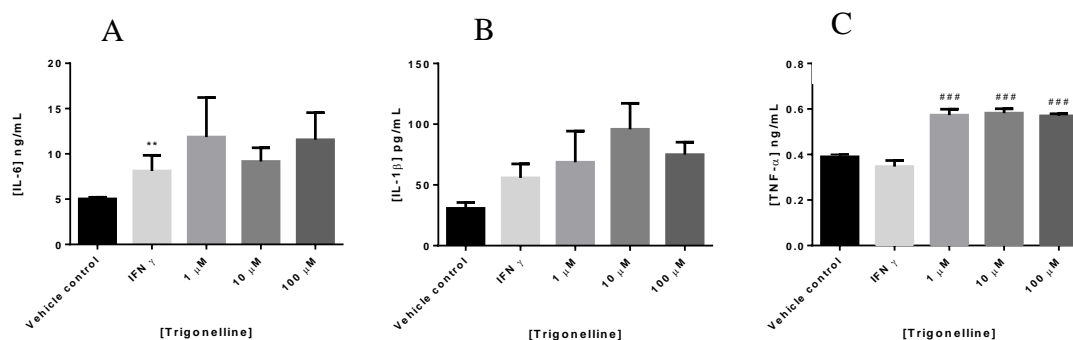


Figure 168 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with trigonelline on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + caffeic acid

IDO activity

CA in combination with 100 μ M caffeine was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested. Non-significant increases in KYN concentration were however observed at all three concentrations tested in comparison to IFN- γ alone. This subsequently resulted in no change in IDO activity at all three concentrations tested in comparison to IFN- γ alone as seen in figure 169 below.

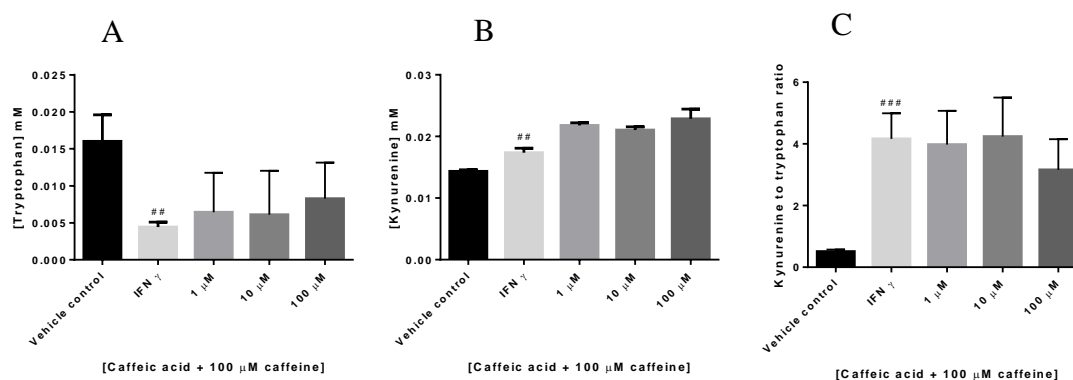


Figure 169 - a) The effects of caffeic acid in combination with 100 μM caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of caffeic acid in combination with 100 μM caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of caffeic acid in combination with 100 μM caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CA in combination with caffeine (100 μM) resulted in the non-significant concentration-dependent decrease in IL-6 concentrations after exposure to 10 μM and 100 μM. Furthermore, significant increases in IL-1β concentrations, an approximate 1000-fold increase, were observed at all three concentrations tested as well as significant increases in TNF-α concentrations, by approximately 30%, at 1 μM, 10 μM and 100 μM in comparison to IFN-γ stimulus alone as seen in figure 170 below.

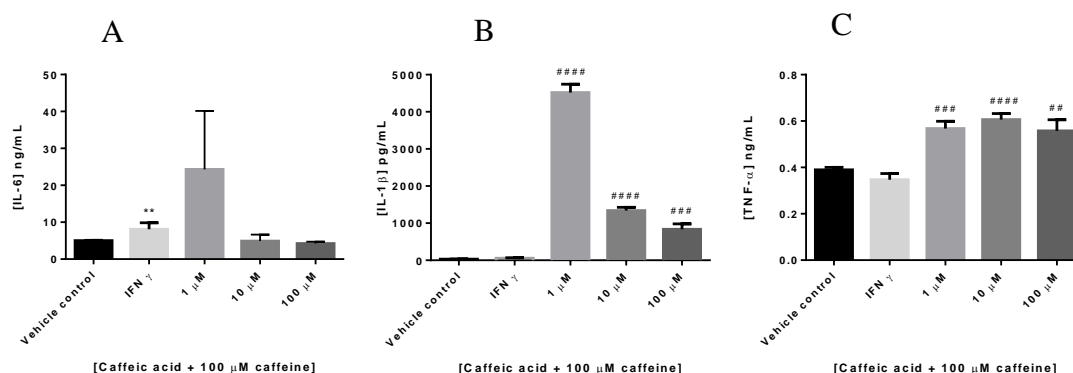


Figure 170 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with caffeic acid in combination with 100 μM caffeine on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + chlorogenic acid

IDO activity

CGA in combination with 100 μM caffeine was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested however trending decreases were observed. Significant decreases in KYN concentration were however observed at 10 μM and 100 μM in comparison to IFN-γ alone. This subsequently resulted in no change in IDO activity at all three concentrations tested in comparison to IFN-γ alone as seen in figure 171 below.

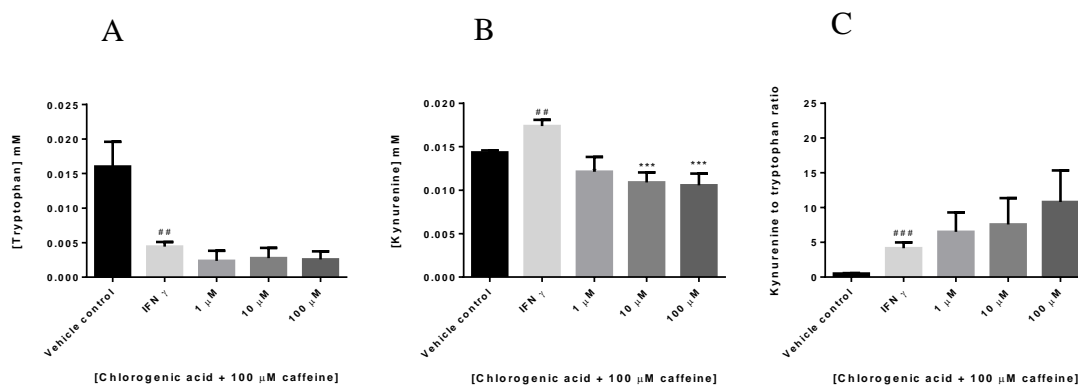


Figure 171 - a) The effects of chlorogenic acid in combination with 100 μ M caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of chlorogenic acid in combination with 100 μ M caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of chlorogenic acid in combination with 100 μ M caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CGA in combination with 100 μ M caffeine resulted in the non-significant concentration-dependent changes in IL-6 concentrations after exposure to 100 μ M. Statistically significant decreases in IL-6 concentrations were observed after cells were pre-treated with 1 μ M ($p < 0.01$) and 10 μ M ($p < 0.01$). Furthermore, significant increases in IL-1 β , in the magnitude of approximately 1000-fold, were observed at all three concentrations tested as well as significant increases in TNF- α concentrations, by approximately 30%, at 1 μ M, 10 μ M and 100 μ M in comparison to IFN- γ stimulus alone as seen in figure 172 below.

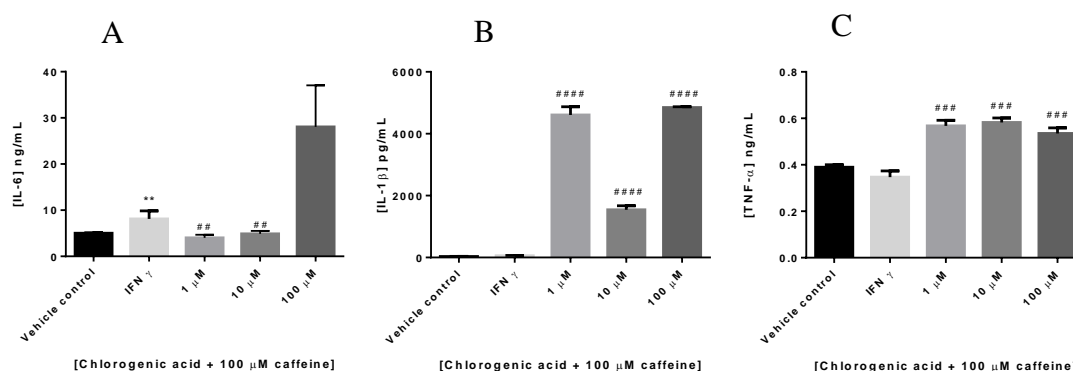


Figure 172 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with chlorogenic acid in combination with 100 μM caffeine on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + ferulic acid

IDO activity

FA in combination with 100 μM caffeine was shown to not significantly alter the Trp concentration present in the samples at all three concentrations tested however trending decreases were observed. Significant decreases in KYN concentration were however observed at 10 μM and 100 μM in comparison to IFN-γ alone. This subsequently resulted in no change in IDO activity at all three concentrations tested in comparison to IFN-γ alone as seen in figure 173 below.

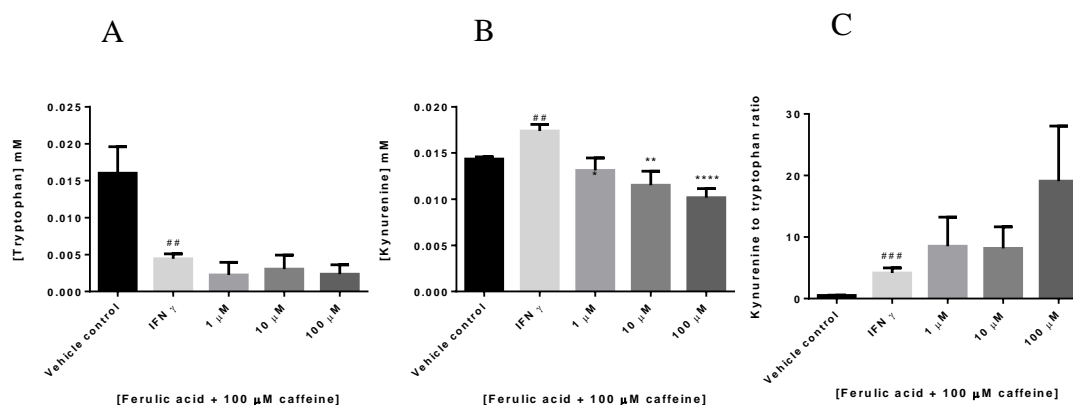


Figure 173 - a) The effects of ferulic acid in combination with 100 μ M caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of ferulic acid in combination with 100 μ M caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of ferulic acid in combination with 100 μ M caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with FA in combination with 100 μ M caffeine resulted in the non-significant changes in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant decreases in IL-1 β concentrations, approximately 40-fold decreases, were observed at all three concentrations tested as well as significant increases in TNF- α concentrations, by approximately 30%, at 1 μ M, 10 μ M and 100 μ M in comparison to IFN- γ stimulus alone as seen in figure 174 below.

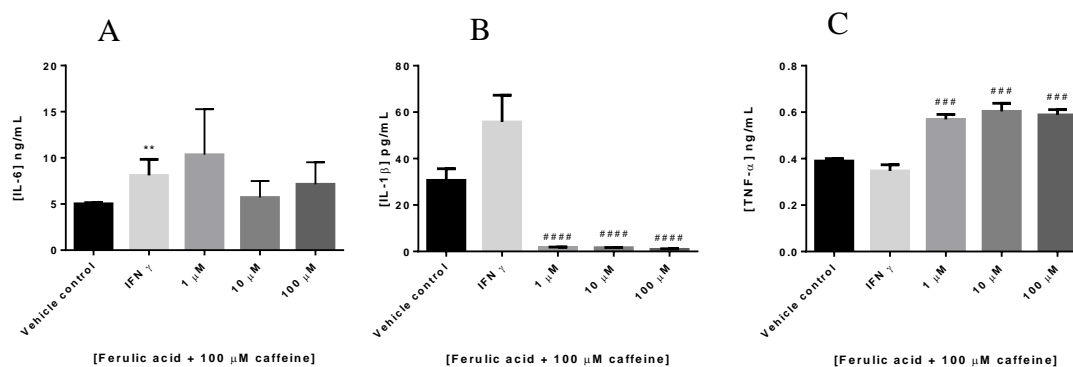


Figure 174 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with ferulic acid in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + pyrogalllic acid

IDO activity

PA in combination with 100 μ M caffeine was shown to significantly increase Trp concentrations at 1 μ M and cause a trending decrease at 10 μ M and 100 μ M, however this was not statistically significant. Significant decreases in KYN concentration were however observed at 10 μ M and 100 μ M in comparison to IFN- γ alone. This however resulted in no change in IDO activity at all three concentrations tested in comparison to IFN- γ alone as seen in figure 175 below.

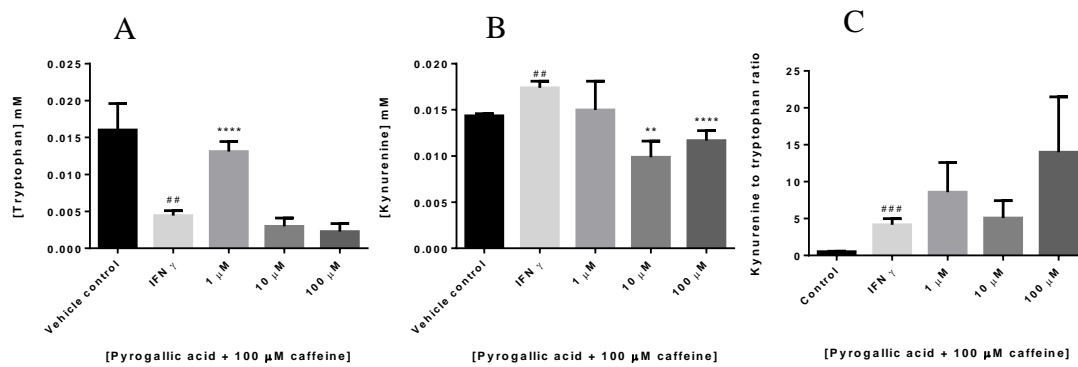


Figure 175 - a) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with PA in combination with 100 μM caffeine resulted in the significant decrease in IL-6 concentrations after exposure to 100 μM. Furthermore, significant decreases in IL-1β concentrations, approximately 40-fold decreases, were observed at all three concentrations tested as well as significant increases in TNF-α concentrations, by approximately 30%, at 1 μM, 10 μM and 100 μM in comparison to IFN-γ stimulus alone as seen in figure 176 below.

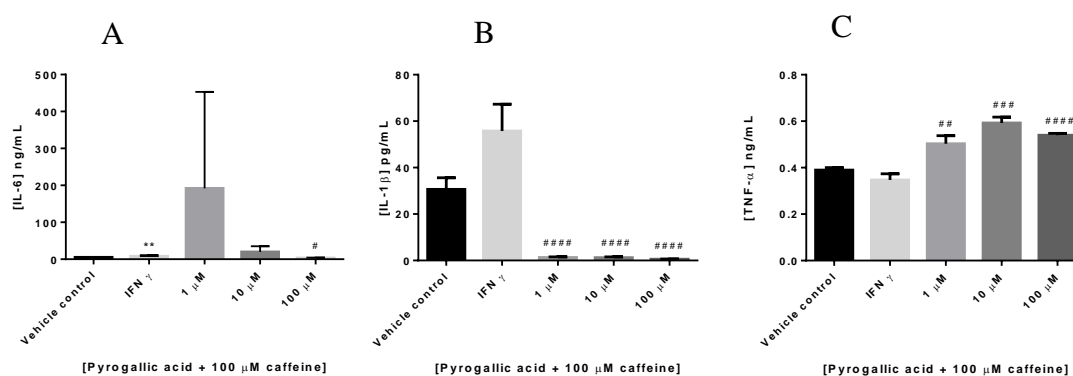


Figure 176 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with pyrogalllic acid in combination with 100 μM caffeine on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + trigonelline

IDO activity

Trigonelline in combination with 100 μM caffeine was shown to non-significantly decrease Trp concentrations at all three concentrations tested. Significant decreases in KYN concentration were however observed at all three concentrations tested in a concentration-dependent manner in comparison to IFN-γ alone. This however resulted in no change in IDO activity at all three concentrations tested in comparison to IFN-γ alone as seen in figure 177.

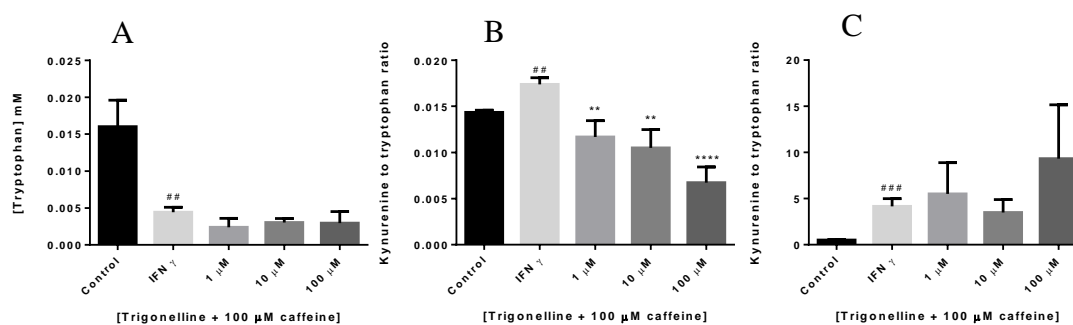


Figure 177 - a) The effects of trigonelline in combination with 100 μM caffeine exposure of interferon gamma-induced tryptophan concentrations; b) The effects of trigonelline in combination with 100 μM caffeine exposure on interferon gamma-induced kynurenine concentrations; c) The effects of trigonelline in combination with 100 μM caffeine exposure on interferon gamma-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with trigonelline in combination with 100 μM caffeine resulted in the significant decrease in IL-6 concentrations, approximately 3-fold, after exposure to all three concentrations tested. Furthermore, significant decreases in IL-1β concentrations, approximately 40-fold, were observed at all three concentrations tested however no change in TNF-α concentrations was observed in comparison to IFN-γ stimulus alone as seen in figure 178 below.

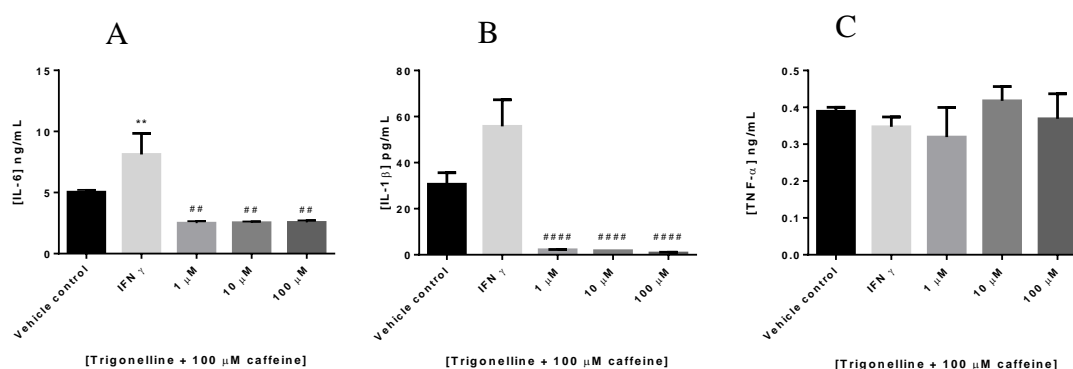


Figure 178 – The effects of 24 h exposure of interferon gamma-stimulated THP-1 human monocytic pre-treated with trigonelline in combination with 100 μM caffeine on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

The effects of key bioactive coffee constituents on markers of inflammation in interferon α_{2A} stimulated differentiated THP-1 monocytic cell

Caffeine

IDO activity

Caffeine was shown to significantly increase Trp concentrations at 10 μM. Exposure to 1 μM and 100 μM resulted in a non-significant increase. Significant increases in KYN concentration were however observed at all three concentrations tested in a concentration-dependent manner in comparison to IFN- α_{2A} alone. This resulted in an approximate 3-fold decrease in IDO activity at all three concentrations tested in comparison to IFN- α_{2A} alone as seen in figure 179.

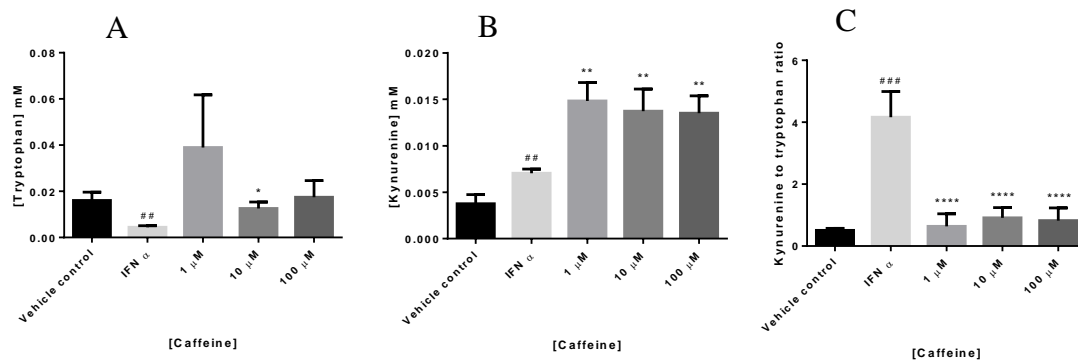


Figure 179 - a) The effects of caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with caffeine resulted in the significant decrease in IL-6 concentrations, by approximately 50%, after exposure to all three concentrations tested. Furthermore, significant increases in IL-1 β concentrations, in the magnitude of approximately 20-fold increases, were observed at all three concentrations tested as well as increases in TNF- α concentrations by approximately 30% increases, in comparison to IFN- α_{2A} stimulus alone as seen in figure 180.

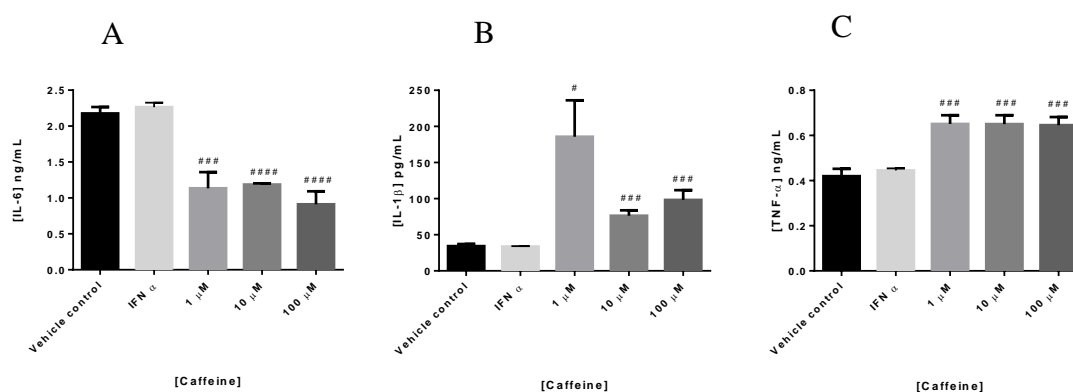


Figure 180 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with caffeine on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeic acid

IDO activity

CA was shown to significantly increase Trp concentrations at all three concentrations tested. Significant increases in KYN concentration were observed at 1 μM in comparison to IFN-α_{2A} alone. This resulted in an approximate 5-fold decrease in IDO activity at all three concentrations tested in comparison to IFN-α alone as seen in figure 181 below.

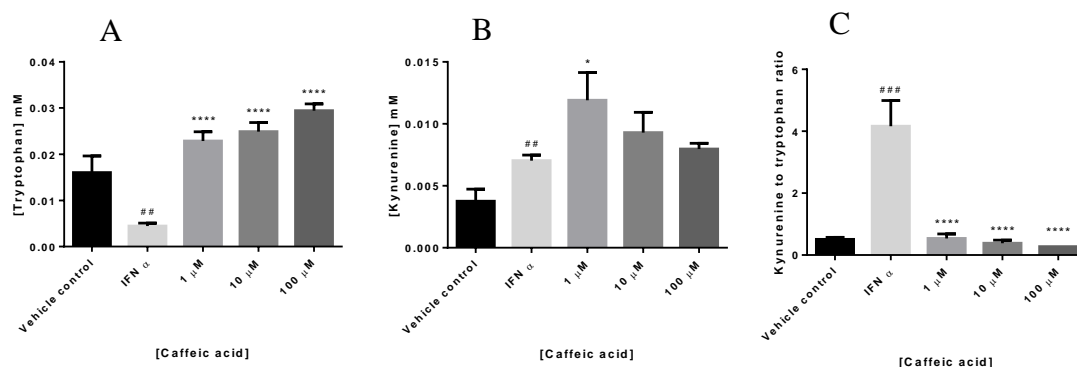


Figure 181 - a) The effects of caffeic acid exposure of interferon alpha-induced tryptophan concentrations; b) The effects of caffeic acid exposure on interferon alpha-induced kynurenine concentrations; c) The effects of caffeic acid exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CA resulted in the significant decrease of approximately 50% of IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant increases in IL-1 β concentrations, approximately 3-fold in magnitude, were observed at all three concentrations tested as well as increases in TNF- α concentrations by approximately 20% increases in comparison to IFN- α_{2A} stimulus alone as seen in figure 182 below.

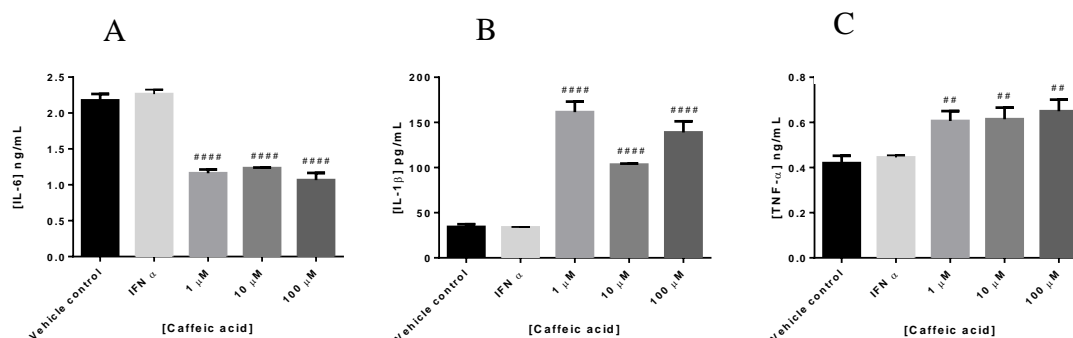


Figure 182 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with caffeic acid on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Chlorogenic acid

IDO activity

CGA was shown to significantly increase Trp concentrations at all three concentrations tested. Significant increases in KYN concentration were observed at all concentrations tested in comparison to IFN- α_{2A} alone. This resulted in an approximate 5-fold increase in IDO activity at all three concentrations tested in comparison to IFN- α_{2A} alone as seen in figure 183 below.

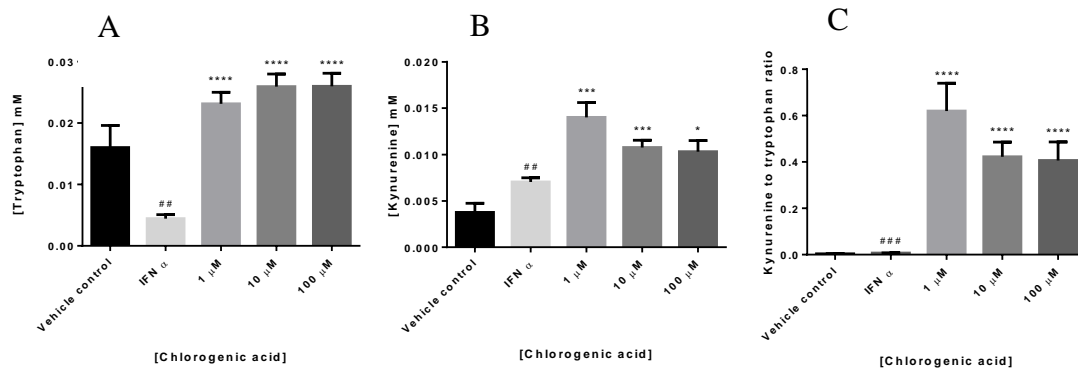


Figure 183 - a) The effects of chlorogenic acid exposure of interferon alpha-induced tryptophan concentrations; b) The effects of chlorogenic acid exposure on interferon alpha-induced kynurenine concentrations; c) The effects of chlorogenic acid exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CGA resulted in the significant decrease in IL-6 concentrations, approximately 50%, after exposure to all three concentrations tested. Furthermore, significant concentration-dependent increases in IL-1 β concentrations, approximately 2-fold in magnitude were observed at all three concentrations tested as well as increases in TNF- α concentrations by approximately 20% increase, in comparison to IFN- α_{2A} stimulus alone as seen in figure 184 below.

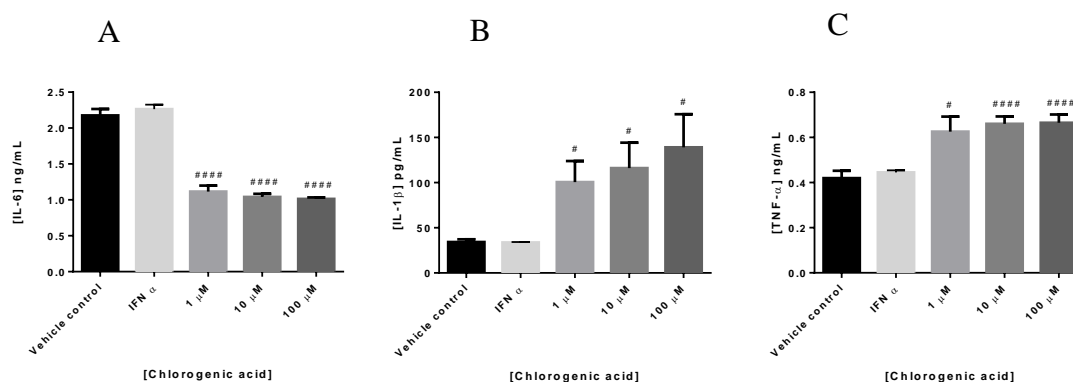


Figure 184 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with chlorogenic acid on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Ferulic acid

IDO activity

FA was shown to have no effect on Trp concentrations at all three concentrations tested. Non-significant increases in KYN concentrations were observed at all concentrations tested in comparison to IFN- α_{2A} alone. This resulted in a concentration-dependent decrease in IDO activity at all three concentrations tested in comparison to IFN- α_{2A} alone as seen in figure 185 below.

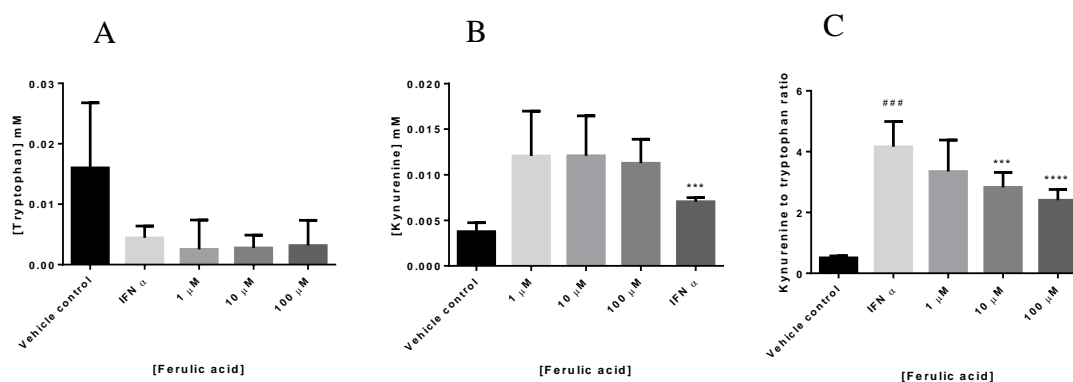


Figure 185 - a) The effects of ferulic acid exposure of interferon alpha-induced tryptophan concentrations; b) The effects of ferulic acid exposure on interferon alpha-induced kynurenine concentrations; c) The effects of ferulic acid exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with FA resulted in the significant decrease in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant concentration-dependent increases in IL-1β concentrations were observed at all three concentrations tested as well as an increase in TNF-α concentrations in comparison to IFN-α_{2A} stimulus alone as seen in figure 186 below.

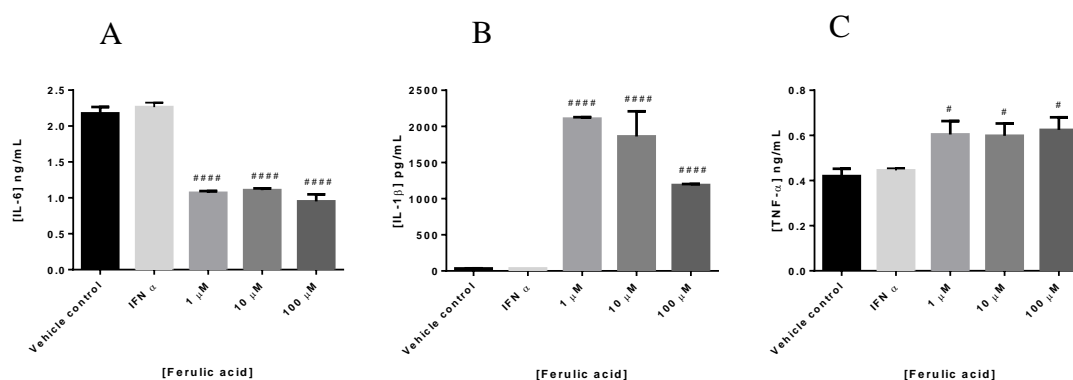


Figure 186 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with ferulic acid on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Pyrogalllic acid

IDO activity

PA was shown to significantly increase Trp concentrations at 10 μM and 100 μM. PA (1 μM) also increased the concentration of Trp however this was not statistically significant. Significant increases in KYN concentration were observed at 1 μM and 10 μM in comparison to IFN-α_{2A} alone. This resulted in a decrease in IDO activity at all three concentrations tested in comparison to IFN-α_{2A} alone however only decrease caused by 10 μM and 100 μM were statistically significant as seen in figure 187 below.

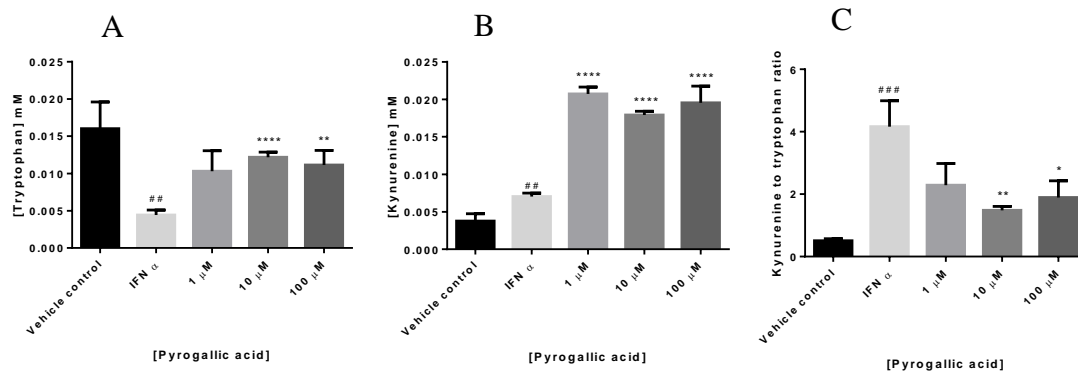


Figure 187 - a) The effects of pyrogallallic acid exposure of interferon alpha-induced tryptophan concentrations; b) The effects of pyrogallallic acid exposure on interferon alpha-induced kynurenine concentrations; c) The effects of pyrogallallic acid exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with PA resulted in the significant decrease in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant increases in IL-1 β concentrations were observed at 1 μ M and 10 μ M as well as significant increases in TNF- α concentrations in comparison to IFN- α_{2A} stimulus alone as seen in figure 188 below.

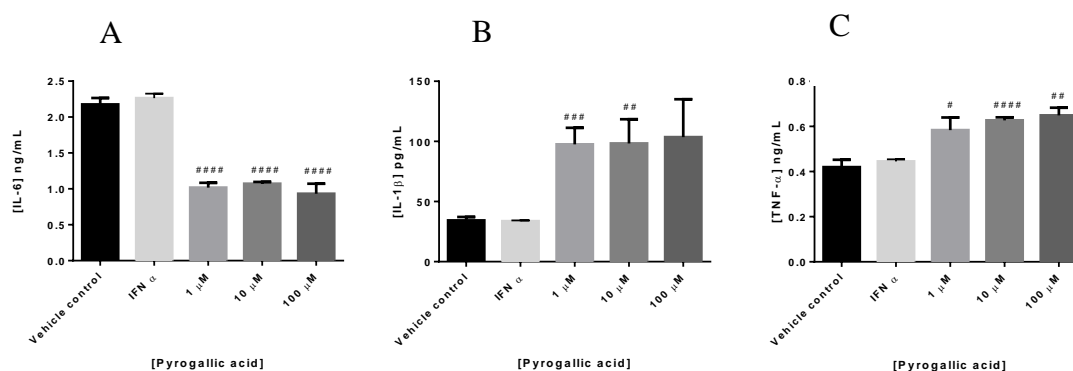


Figure 188 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with pyrogallol acid on a) IL-6; b) IL-1β; and c) TNF-α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Trigonelline

IDO activity

Trigonelline was shown to significantly increase Trp concentrations at 10 μM. Trigonelline (1 μM and 100 μM) also increased the concentration of Trp however this was not statistically significant. Significant increases in KYN concentration were observed at all concentrations tested in comparison to IFN-α_{2A} alone. This resulted in a decrease in IDO activity at all three concentrations tested in comparison to IFN-α_{2A} alone however the only decrease caused by 100 μM was statistically significant as seen in figure 189 below.

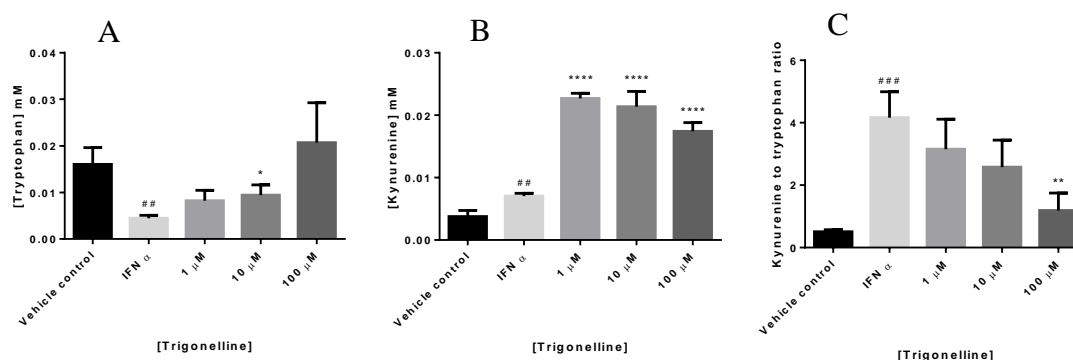


Figure 189 - a) The effects of trigonelline exposure of interferon alpha-induced tryptophan concentrations; b) The effects of trigonelline exposure on interferon alpha-induced kynurenine concentrations; c) The effects of trigonelline exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with trigonelline resulted in the significant decrease in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant increases in IL-1 β concentrations were observed at 1 μ M and 100 μ M as well as significant increases in TNF- α concentrations at all concentrations tested were observed in comparison to IFN- α_{2A} stimulus alone as seen in figure 190 below.

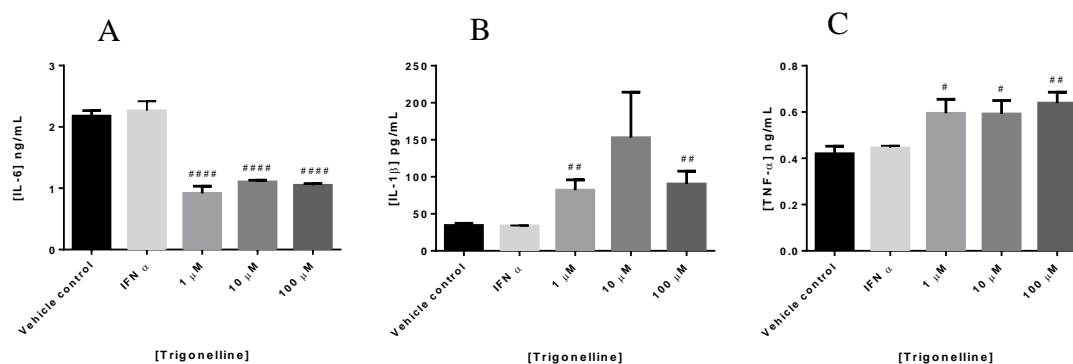


Figure 190 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with pyrogalllic acid on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + caffeic acid

IDO activity

CA in combination with 100 μ M caffeine was shown to have no effect on Trp concentrations in comparison to IFN- α_{2A} alone. Significant increases in KYN concentration were observed at 1 μ M and 100 μ M in comparison to IFN- α_{2A} alone. This resulted in no change in IDO activity at any of the concentrations tested in comparison to IFN- α_{2A} alone as seen in figure 191 below.

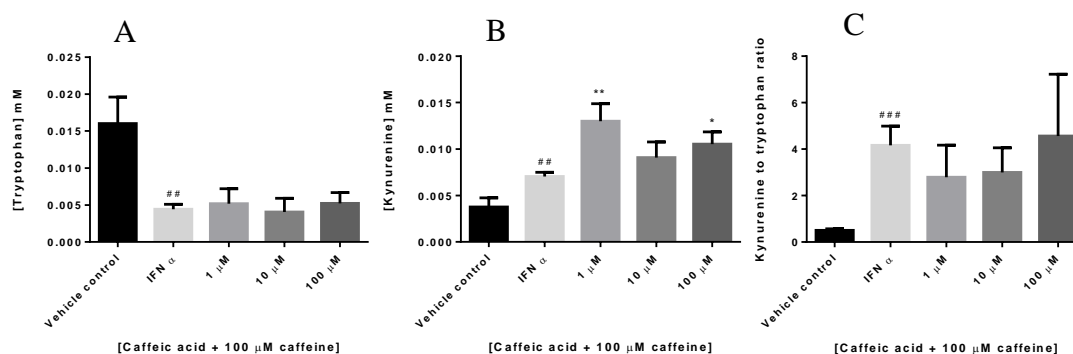


Figure 191 - a) The effects of caffeic acid in combination with 100 μ M caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of caffeic acid in combination with 100 μ M caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of caffeic acid in combination with 100 μ M caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CA in combination with 100 μ M caffeine resulted in the significant increase in IL-6 concentrations after exposure to all three concentrations tested. Furthermore, significant decreases in IL-1 β concentrations were observed at 100 μ M and 1000 μ M however no significant changes in TNF- α concentrations were observed in comparison to IFN- α_{2A} stimulus alone as seen in figure 192 below.

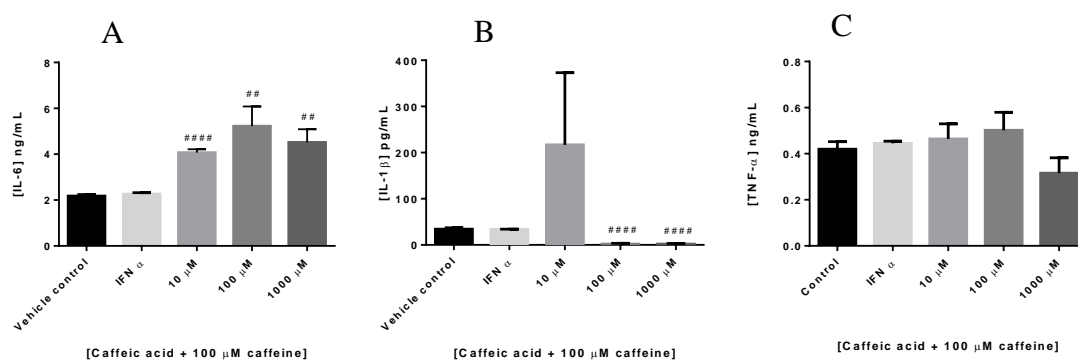


Figure 192 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with caffeic acid in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + chlorogenic acid

IDO activity

CGA in combination with 100 μ M caffeine was shown to have no effect on Trp concentrations in comparison to IFN- α_{2A} alone. Significant increases in KYN concentration were observed at all concentrations tested in comparison to IFN- α_{2A} alone. This resulted in a significant decrease in IDO activity after exposure to 100 μ M was a statistically significant decrease in comparison to IFN- α_{2A} alone as seen in figure 193 below.

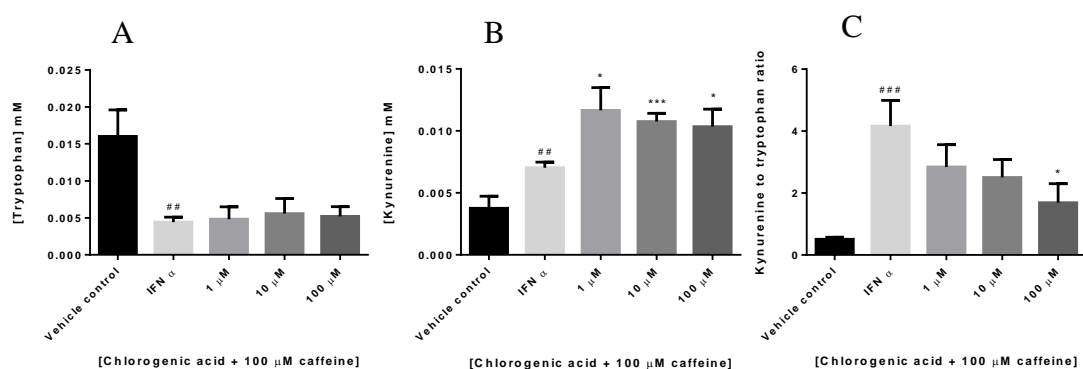


Figure 193 - a) The effects of chlorogenic acid in combination with 100 μM caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of chlorogenic acid in combination with 100 μM caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of chlorogenic acid in combination with 100 μM caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with CGA in combination with 100 μM caffeine resulted in the significant increase in IL-6 concentrations after exposure to 10 μM. Furthermore, significant decreases in IL-1β concentrations were observed at all three concentrations tested however no significant changes in TNF-α concentrations were observed in comparison to IFN-α_{2A} stimulus alone as seen in figure 194 below.

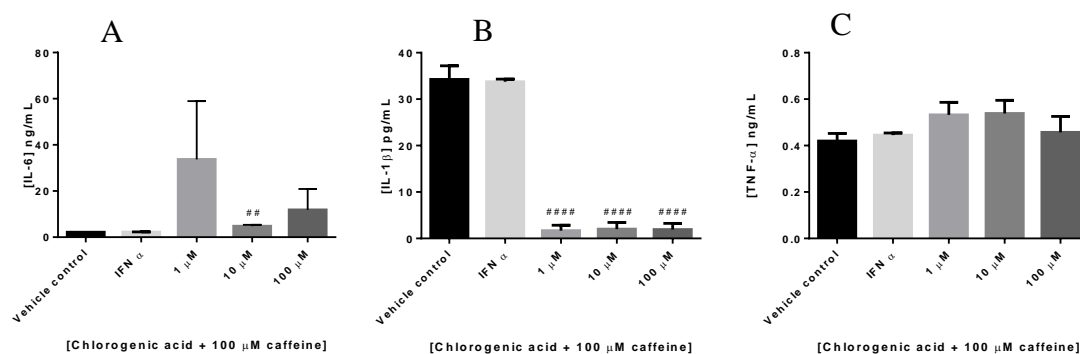


Figure 194 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with chlorogenic acid in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN- α stimulus.

Caffeine + ferulic acid

IDO activity

FA in combination with 100 μ M caffeine was shown to have no effect on Trp concentrations in comparison to IFN- α_{2A} alone. Significant increases in KYN concentration were observed at 1 μ M in comparison to IFN- α_{2A} alone. This resulted in a significant decreases in IDO activity at 1 μ M only in comparison to IFN- α_{2A} alone as seen in figure 195 below.

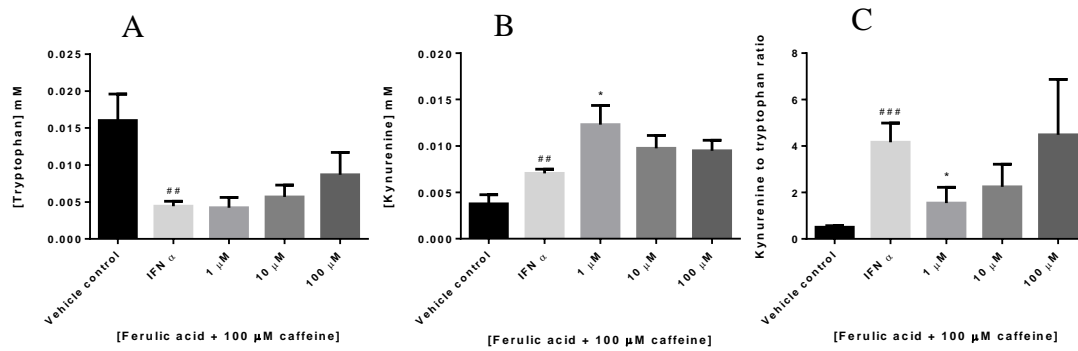


Figure 195 - a) The effects of ferulic acid in combination with 100 μ M caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of ferulic acid in combination with 100 μ M caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of ferulic acid in combination with 100 μ M caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with FA in combination with 100 μ M caffeine resulted significant increases in IL-6 concentrations after exposure to 10 μ M and 100 μ M. Furthermore, significant decreases in IL-1 β concentrations were observed at all three concentrations tested however no significant changes in TNF- α concentrations were observed in comparison to IFN- α_{2A} stimulus alone as seen in figure 196 below.

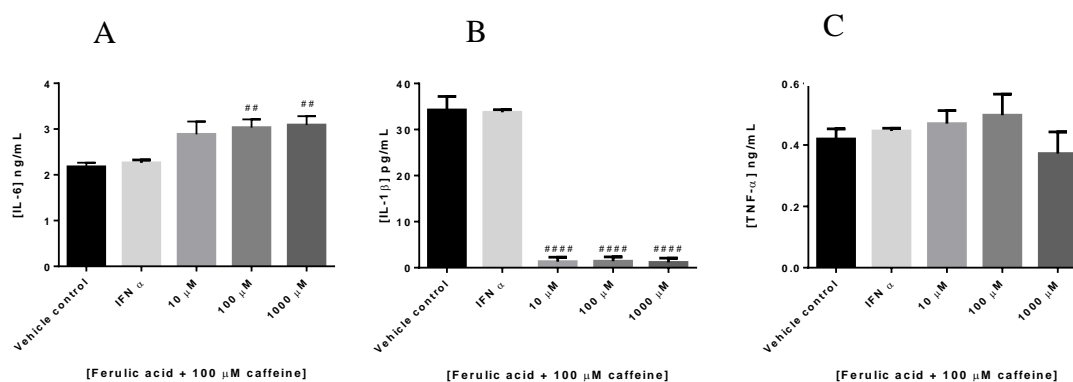


Figure 196 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with ferulic acid in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + pyrogalllic acid

IDO activity

PA in combination with 100 μ M caffeine was shown to have no effect on Trp concentrations in comparison to IFN- α_{2A} alone. No significant changes in KYN concentration were observed at any of the concentrations tested in comparison to IFN- α_{2A} alone. This resulted in significant decreases in IDO activity at 10 μ M and 100 μ M in comparison to IFN- α_{2A} alone as seen in figure 197 below.

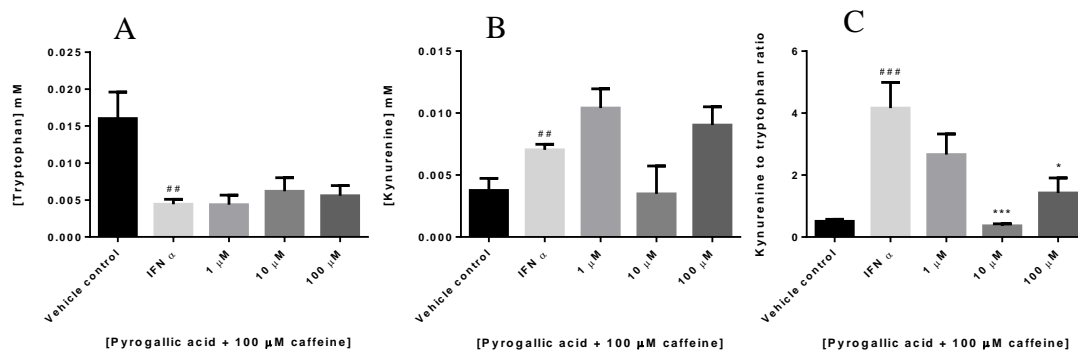


Figure 197 - a) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of pyrogallallic acid in combination with 100 μM caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with PA in combination with 100 μM caffeine resulted in the significant increase in IL-6 concentrations after exposure to all concentrations tested. Furthermore, significant decreases in IL-1β concentrations were observed at all three concentrations tested however no significant changes in TNF-α concentrations were observed in comparison to IFN-α_{2A} stimulus alone as seen in figure 198 below.

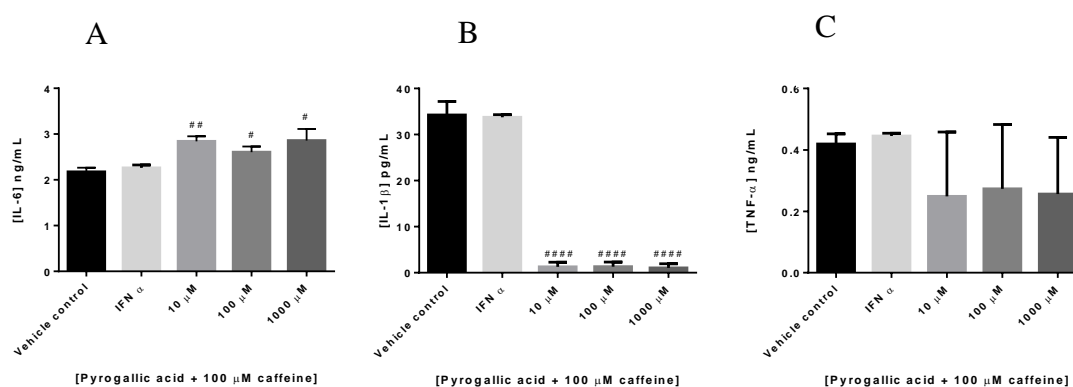


Figure 198 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with pyrogallallic acid in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

Caffeine + trigonelline

IDO activity

Trigonelline in combination with 100 μ M caffeine was shown to increase Trp concentrations after exposure to 10 μ M in comparison to IFN- α_{2A} alone. Significant increases in KYN concentration were observed at 1 μ M in comparison to IFN- α_{2A} alone. This resulted in a decrease in IDO activity at all concentrations tested in comparison to IFN- α_{2A} alone as seen in figure 199 below.

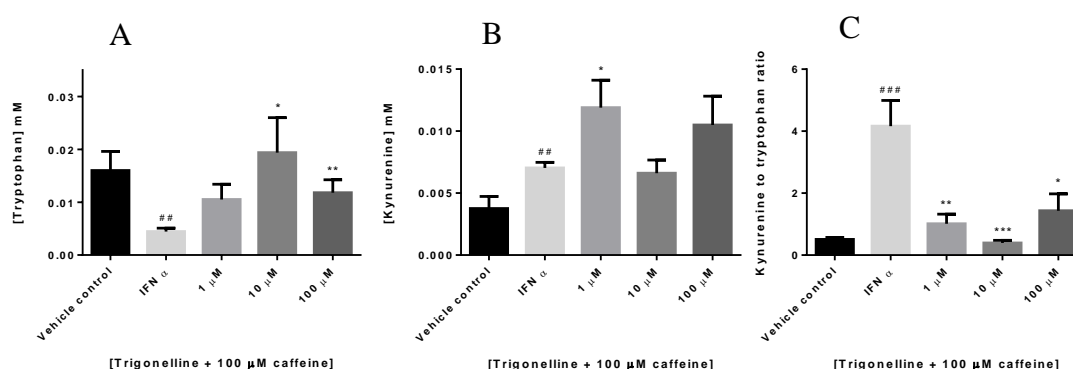


Figure 199 - a) The effects of trigonelline in combination with 100 μM caffeine exposure of interferon alpha-induced tryptophan concentrations; b) The effects of trigonelline in combination with 100 μM caffeine exposure on interferon alpha-induced kynurenine concentrations; c) The effects of trigonelline in combination with 100 μM caffeine exposure on interferon alpha-induced IDO activity where # indicates in comparison to the vehicle control and * indicates in comparison to IFN stimulus

Cytokines

Pre-treatment of differentiated THP-1 cells with trigonelline in combination with 100 μM caffeine resulted in the significant increase in IL-6 concentrations after exposure to 1 μM. Furthermore, significant decreases in IL-1β concentrations were observed at all three concentrations tested however no significant changes in TNF-α concentrations were observed in comparison to IFN-α_{2A} stimulus alone as seen in figure 200 below.

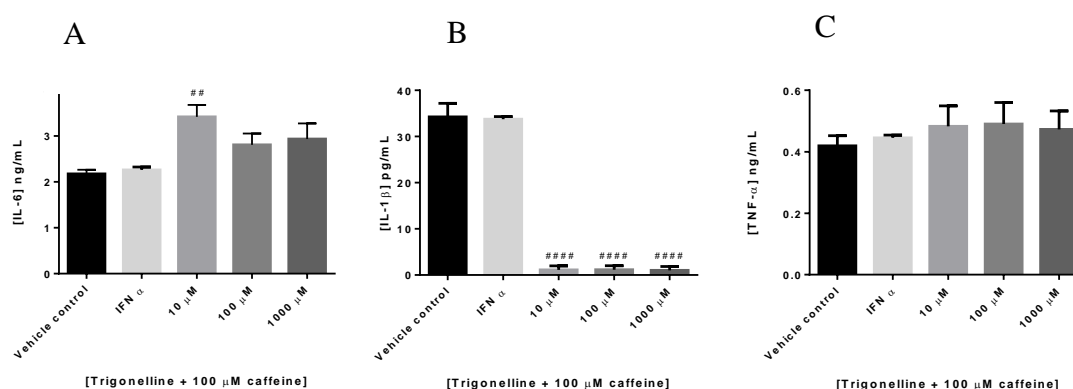


Figure 200 – The effects of 24 h exposure of interferon alpha-stimulated THP-1 human monocytic pre-treated with trigonelline in combination with 100 μ M caffeine on a) IL-6; b) IL-1 β ; and c) TNF- α where * indicates in comparison to the vehicle control and # indicates in comparison to IFN stimulus.

5.4.2 The effect of key bioactive coffee constituents on the viability on neuronal-like cells

Decreased viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells Resazurin reduction assay

The viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells after 24 h exposure to key biologically active constituents of coffee was evaluated using the resazurin reduction assay and was confirmed using the LDH cytotoxicity assay. Decreased viability was observed after 24 h exposure to various coffee constituents in both undifferentiated and differentiated SH-SY5Y neuroblastoma cells. Exposure of undifferentiated SH-SY5Y neuroblastoma cells to 1000 μ M of CA, CGA, PA and the positive control salsolinol resulted in a statistically significant decrease in viability as seen in figure 201. Exposure of differentiated SH-SY5Y neuroblastoma cells to 1000 μ M of caffeine, CA, CGA, PA and the positive control salsolinol resulted

in a statistically significant decrease in viability as seen in figure 201. Statistically significant differences were seen between undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to coffee constituents. Caffeine (1000 μ M) was shown to only reduce the viability of differentiated SH-SY5Y neuroblastoma cells, while CA (1000 μ M) and CGA (1000 μ M) decreased viability to a greater degree in undifferentiated SH-SY5Y neuroblastoma cells ($p < 0.05$). CA and CGA (1000 μ M) decreased the viability in undifferentiated and differentiated SH-SY5Y cells by approximately 70 and 50% and 70 and 40% respectively. Furthermore, PA (1000 μ M) decreased the viability of both undifferentiated and differentiated SH-SY5Y neuroblastoma cells by approximately 60%.

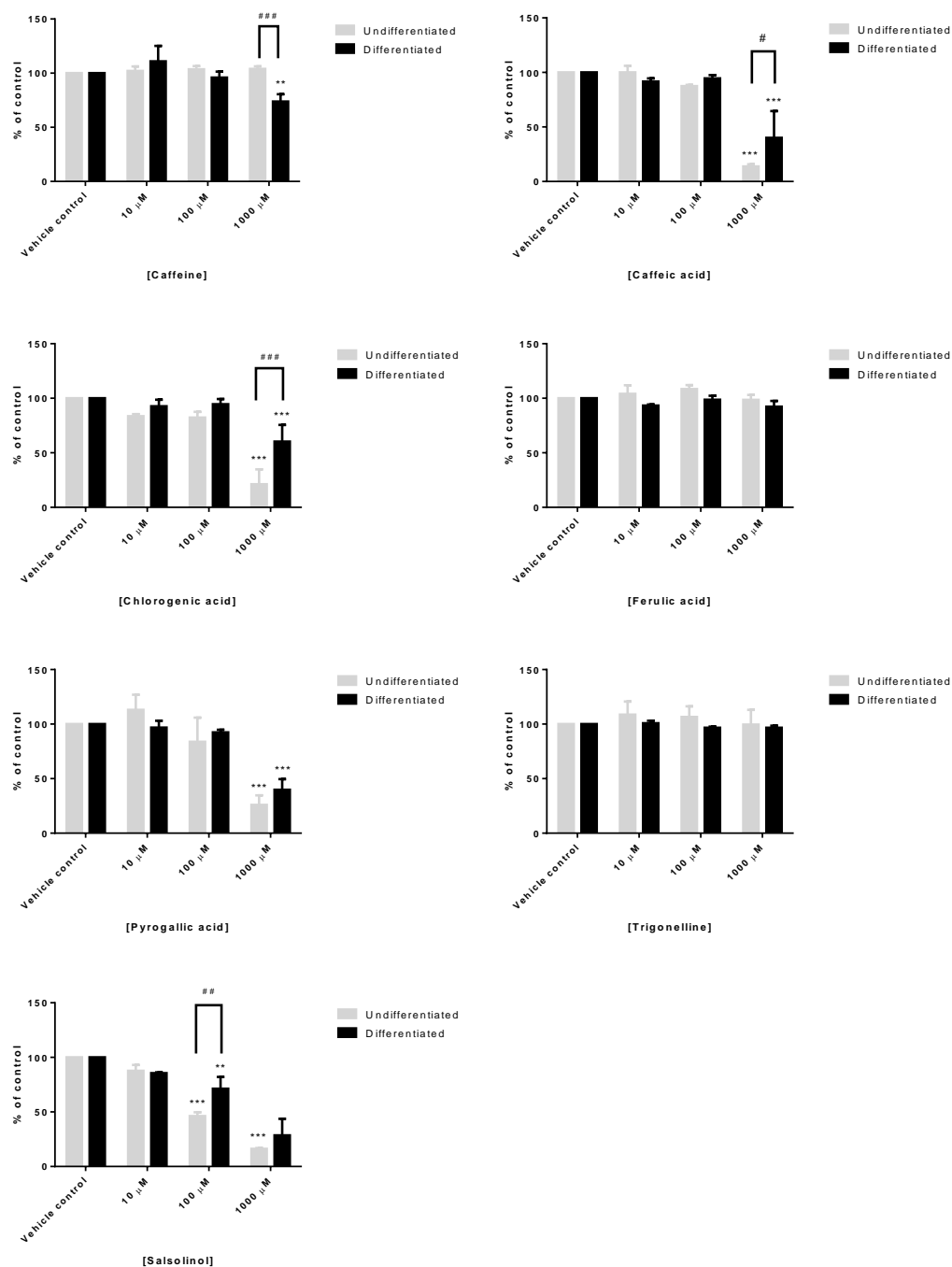


Figure 201 - Viability of undifferentiated and differentiated SH-SY5Y neuroblastoma cells after 24 h exposure to bioactive coffee constituents using the resazurin reduction assay

Statistically significant increases in LDH activity were seen in differentiated SH-SY5Y neuroblastoma cells after 24 h exposure to PA (1000 μ M) and the positive control salsolinol ($p < 0.001$). Increases after exposure to PA were approximately 30-fold higher than the total activity observed in comparison to the vehicle control. Likewise, statistically significant increases in LDH activity were observed after undifferentiated SH-SY5Y neuroblastoma cells were treated with CA, CGA and PA ($p < 0.001$), approximately 12-fold, 10-fold and 20-fold in nature, respectively. Furthermore, statistically significant differences between undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to 1000 μ M of caffeine, CGA, FA and trigonelline for 24 h were observed as seen in figure 202.

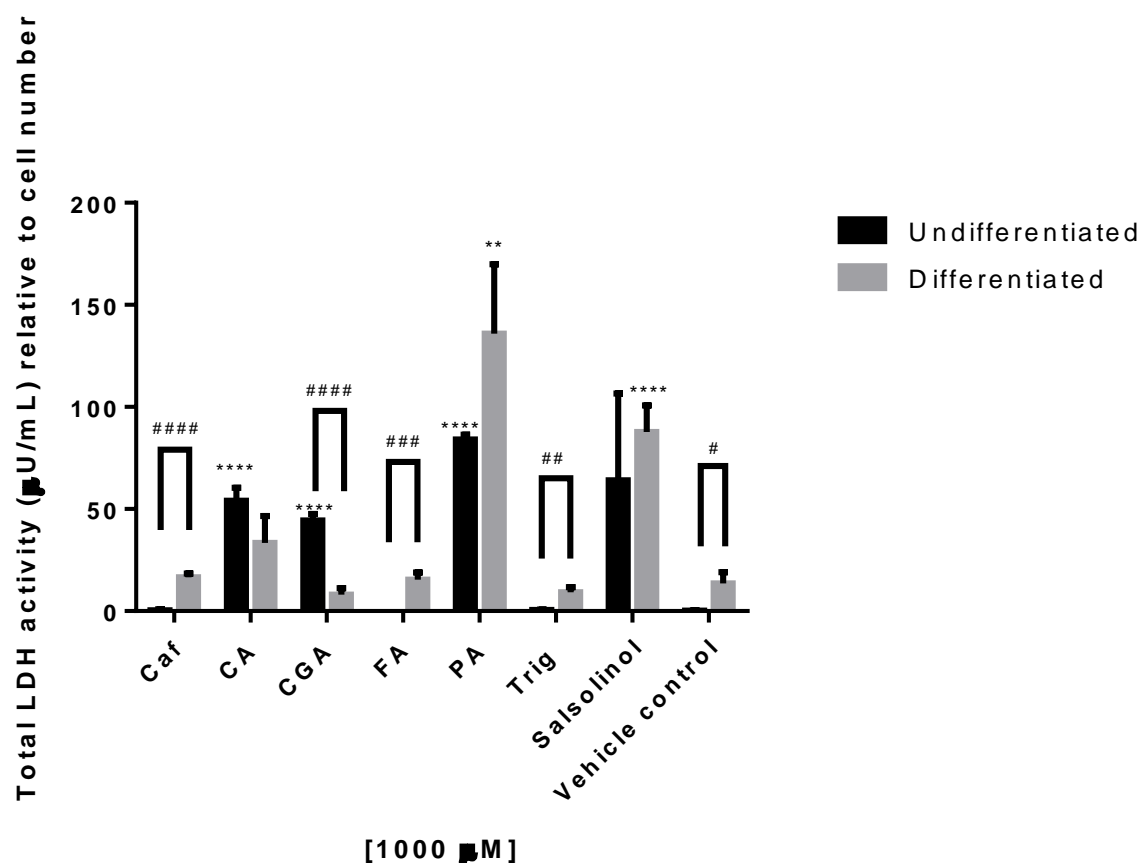


Figure 202 - Total LDH activity relative to cell number of undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to bioactive coffee constituents

Exposure of undifferentiated and differentiated SH-SY5Y neuroblastoma cells on the production of free radicals

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA)

The 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay and dihydrohodamine-1,2,3 (DHR) were used to assess the effects of biologically active coffee constituents on oxidative stress. Exposure of 1000 µM of CA, CGA and PA to

undifferentiated SH-SY5Y neuroblastoma cells for 24 h resulted in increases in oxidative stress ($p < 0.05$), approximately 6-fold, 6-fold and 2-fold in nature, as measured by DCFH-DA. In differentiated SH-SY5Y neuroblastoma cells 24 h exposure to 1000 μM of PA resulted in increased free radical production compared to vehicle control ($p < 0.05$). The increased free radical production effects were not seen in differentiated SH-SY5Y neuroblastoma cells exposed to 1000 μM of CA and CGA for 24 h as seen in figure 203.

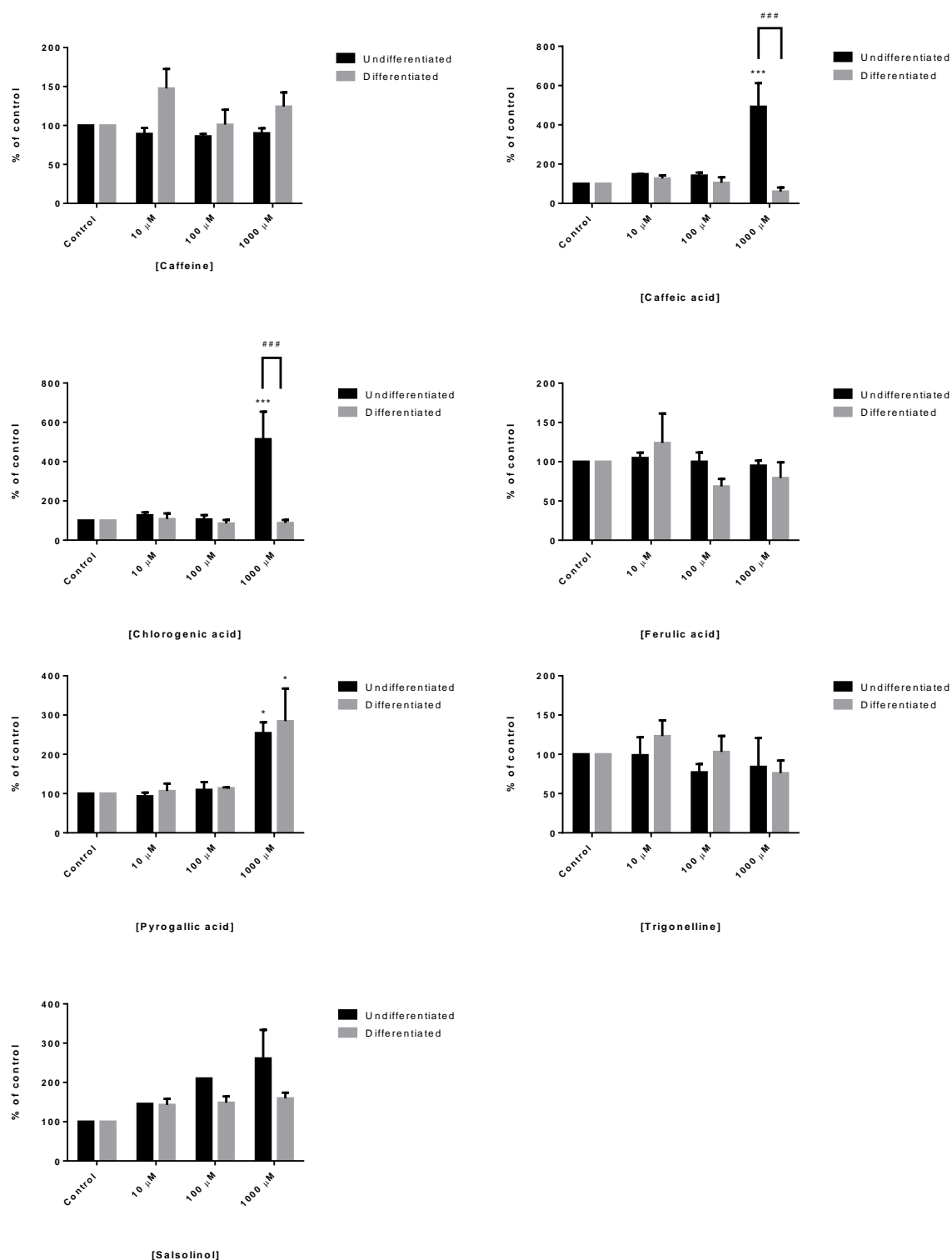


Figure 203 - Measurement of oxidative stress using the DCFH-DA assay in undifferentiated and differentiated SH-SY5Y neuroblastoma cells

Statistically significant increases in intracellular oxidative stress using the dihydrorhodamine assay were shown in undifferentiated SH-SY5Y neuroblastoma cells exposed to 1000 μ M of CA, CGA, PA and the positive control (all $p < 0.0001$) with approximate 7-fold, 5-fold and 4-fold increases, respectively. Likewise, statistically significant increases in intracellular oxidative stress were observed in differentiated SH-SY5Y neuroblastoma cells exposed to CA ($p < 0.001$), and PA ($p < 0.0001$) although to a lesser degree than that observed in undifferentiated SH-SY5Y neuroblastoma cells as seen in figure 204. CA and PA only increased intracellular oxidative stress in differentiated SH-SY5Y cells approximately 3-fold and 2-fold and showed significantly lower production than their undifferentiated counterparts.

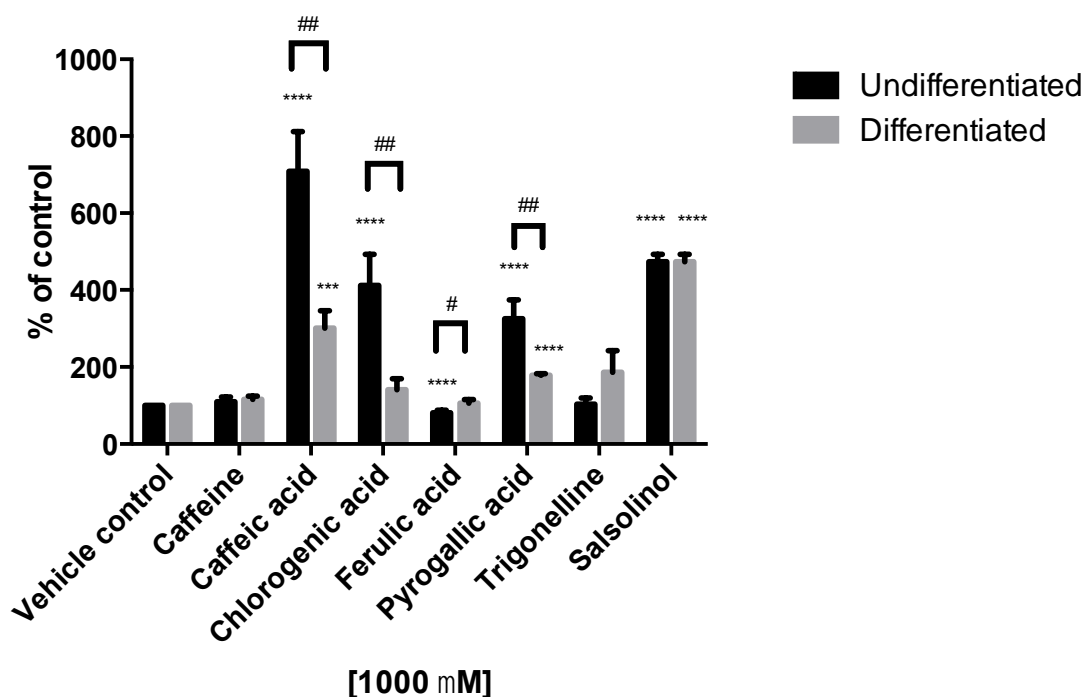


Figure 204 - Measurement of oxidative stress using the DHR 123 assay in undifferentiated and differentiated SH-SY5Y neuroblastoma cells (n = 9)

MitoSOX Red

In addition to the other marker of oxidative stress, DCFH-DA and DHR 123, MitoSOX red was used as a further indicator for oxidative stress. MitoSOX red is a probe specific for mitochondrial oxidative stress. Undifferentiated SH-SY5Y neuroblastoma cells showed greater mitochondrial oxidative stress than their differentiated counterpart. Furthermore, CA, CGA and PA all increased mitochondrial free radical production as seen in figure 205.

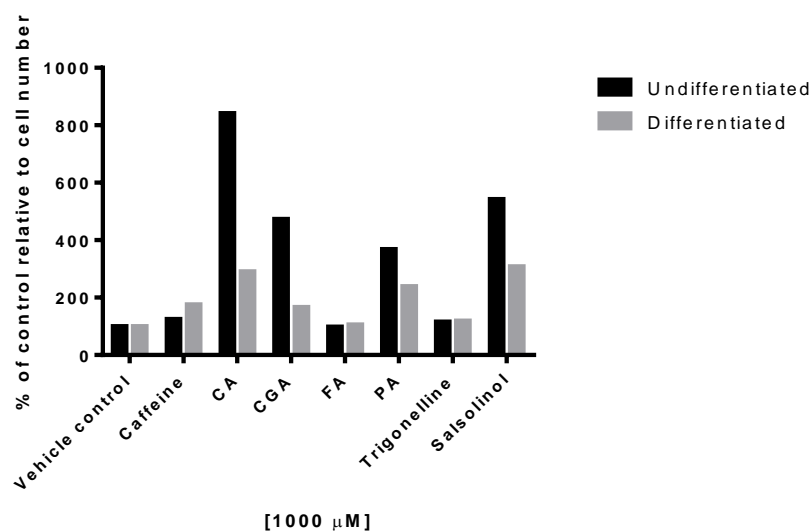


Figure 205 - Measurement of oxidative stress using the MitoSOX assay in undifferentiated and differentiated SH-SY5Y neuroblastoma cells (n=1)

Increased apoptosis in undifferentiated and differentiated SH-SY5Y cells exposed to bioactive coffee constituents for 24 h

Annexin V and caspase-3 activation assays were used to evaluate the effects of bioactive coffee constituents on apoptotic pathways in undifferentiated and differentiated SH-SY5Y neuroblastoma cells.

Annexin V activation

Increased annexin V activation was observed in undifferentiated SH-SY5Y neuroblastoma cells exposed to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M) along with the positive control salsolinol (1000 μ M) ($p < 0.001$), all approximately 5-fold increases, indicating apoptosis. Likewise, increased annexin V activation was observed in differentiated SH-SY5Y neuroblastoma cells with 24 h exposure to CA (1000 μ M), CGA (1000 μ M), PA (1000 μ M) and the positive control salsolinol (1000 μ M) ($p < 0.01$). PA increased annexin V activation by approximately 2-fold and CA and CGA increased it approximately by 1-fold. Trends of increased annexin V activation were seen in differentiated SH-SY5Y neuroblastoma cells exposed to CA and CGA for 24 h however these are not statistically significant as seen in figure 206.

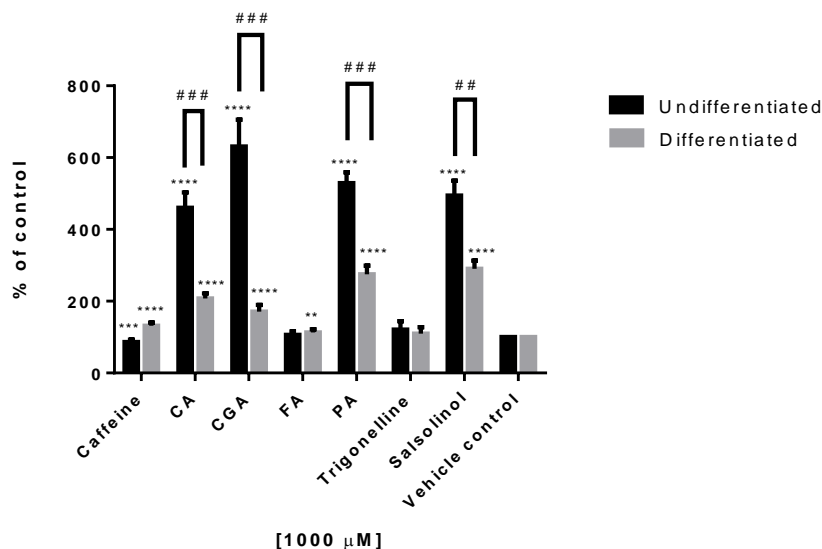


Figure 206 - Annexin V activation in undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to bioactive coffee constituents (n = 3)

Caspase-3 activation

Caspase-3 activation, a marker of apoptosis, was increased in undifferentiated SH-SY5Y neuroblastoma cells after 24 h exposure to CA (1000 μ M), and salsolinol (1000 μ M). CA increased caspase-3 activation by approximately 2-fold. Additionally, although not statistically significant, a trending increase in caspase-3 can be seen after PA (1000 μ M) exposure. Likewise, caspase-3 activation was observed in differentiated SH-SY5Y neuroblastoma cells exposed for 24 h to CA (1000 μ M), PA (1000 μ M) and salsolinol (1000 μ M) as seen in figure 207. CA and PA increased caspase-3 activation by approximately 2-fold and 4-fold, respectively.

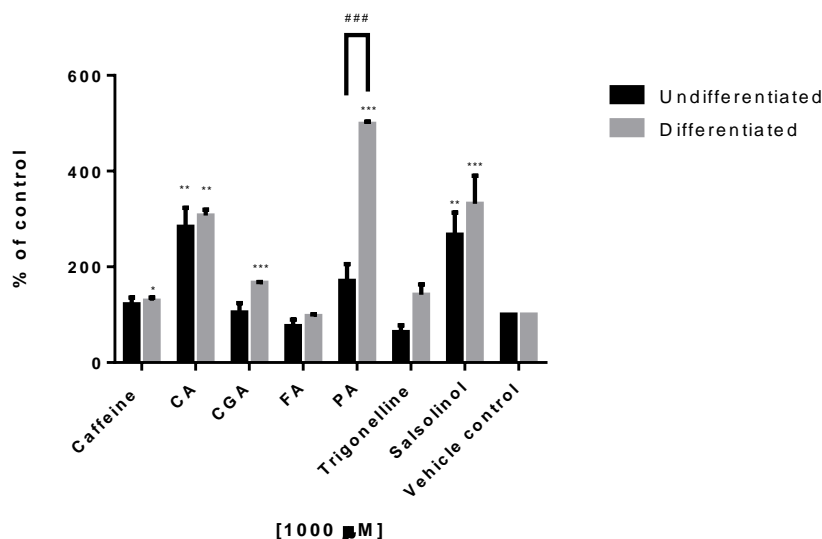


Figure 207 - Caspase-3 activation in undifferentiated and differentiated SH-SY5Y neuroblastoma cells after exposure to bioactive coffee constituents (n = 3)

Necrosis measured using propidium Iodide

Increased cellular necrosis was measured using propidium iodide. Statistically significant increases in necrosis in relation to control were seen in undifferentiated SH-SY5Y with 24 h exposure to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M), all approximately 5-fold increases. Although not statistically significant, a trending increase of necrosis was observed after 24 h exposure of undifferentiated SH-SY5Y neuroblastoma cells to salsolinol (1000 μ M) as seen in figure 208. Two-fold increases in necrosis were observed in differentiated SH-SY5Y neuroblastoma cells treated with PA for 24 h ($p < 0.0001$).

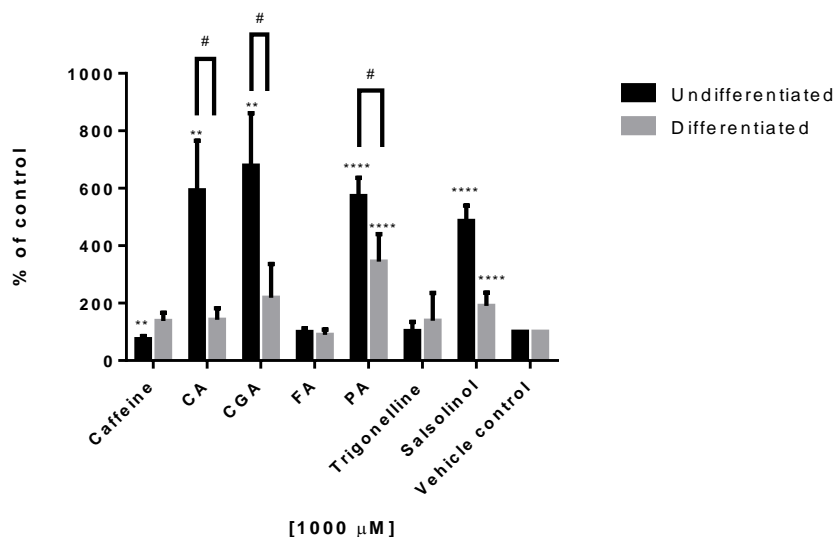


Figure 208 - Percentage necrosis in comparison to the vehicle control using PI assay in undifferentiated and differentiated SH-SY5Y neuroblastoma cells exposed to bioactive coffee constituents (n = 3)

Cyclic AMP

cAMP concentrations were quantified after exposure of SH-SY5Y neuroblastoma cells using an ELISA kit. Statistically significant increases in cAMP levels in undifferentiated SH-SY5Y neuroblastoma cells were observed after exposure to all of the bioactive coffee constituents tested. It was found that caffeine (100 µM and 1000 µM), CA (1000 µM), CGA (100 µM and 1000 µM), FA (1000 µM), PA (100 µM and 1000 µM) and trigonelline (1000 µM) all significantly increased cAMP production. Furthermore, the increases in cAMP levels in caffeine, CA, FA, PA and trigonelline were all shown to be in a concentration-dependent manner as seen in figure 209 below.

Exposure of differentiated SH-SY5Y neuroblastoma cells to key bioactive coffee constituents likewise resulted in similar increases in cAMP concentrations for some but not all coffee constituents. This study showed that caffeine (100 μ M and 1000 μ M), CA (100 μ M and 1000 μ M), CGA (100 μ M and 1000 μ M) to increase cAMP concentrations and trigonelline (100 μ M) to decrease cAMP concentrations. The major difference between these results and those observed for the undifferentiated cells is the lack of concentration-dependence as seen in figure 209 below.

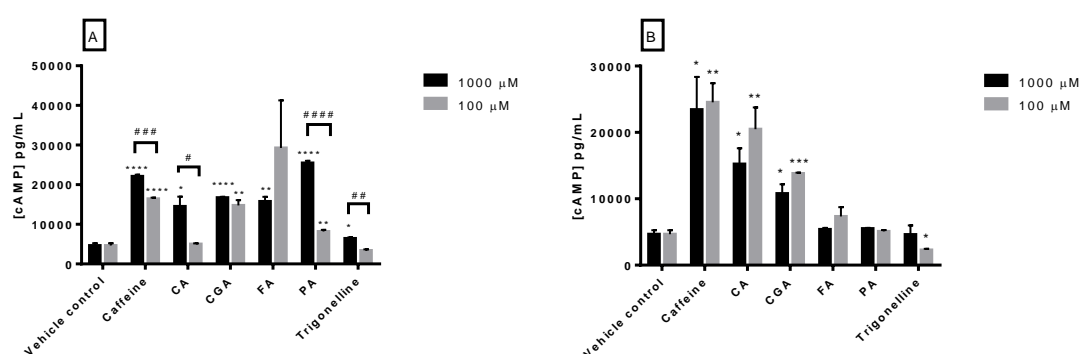


Figure 209 – a) cAMP concentrations produced by undifferentiated SH-SY5Y neuroblastoma cells after exposure to key bioactive coffee constituents; b) cAMP concentrations produced by differentiated SH-SY5Y neuroblastoma cells after exposure to key bioactive coffee constituents.

5.5 Discussion

This study is the first to assess the effects of key bioactive coffee constituents, either alone or in combination with caffeine, on pro-inflammatory mediators in IFN- γ or alpha stimulated differentiated THP-1 human monocytic cells. This model served as a

surrogate microglial cell to assess the effects of these compounds on parameters of neuroinflammation.

The effects of key bioactive coffee constituents were evaluated for their toxicity towards undifferentiated and differentiated THP-1 human monocytic cells, using the model developed in chapter four. Small decreases in viability were observed after undifferentiated THP-1 cells were exposed to CGA (all three concentrations tested) and 100 μ M of PA. Furthermore, PA (1 μ M and 10 μ M) and trigonelline (10 μ M and 100 μ M) increased the viability of undifferentiated THP-1 cells. Similar results were observed in differentiated THP-1 cells. Significant decreases in viability were observed in these cells after exposure to caffeine (1 μ M, 10 μ M and 100 μ M), CGA (100 μ M) and PA (100 μ M). Conversely, increases in the viability of these cells was increased after 24 h treatment with CA (100 μ M) and FA (1 μ M and 10 μ M). Furthermore, the effects of a combination of 100 μ M caffeine combination with various combinations of the other key bioactive coffee constituents, on viability were assessed. Significant changes to the viability in comparison to the effects observed after treatment with the constituents alone only occurred in THP-1 cells treated with a combination of caffeine and FA and caffeine and PA. FA treatment alone (1 μ M) increased the viability of these cells however, when combined with caffeine, this increase was lost. Furthermore, after treatment with PA alone (1 μ M), no change in viability was shown, whereas, when combined with caffeine (100 μ M), the viability of these cells decreased. Given these results, it appears that caffeine decreases the viability of these cells in combination with FA and PA.

The effect of coffee constituents on free radical production using the DCFH-DA assay was evaluated. Decreases in free radical production were observed in these cells exposed to caffeine, FA, PA (100 μ M) and trigonelline (100 μ M). Conversely, CA, CGA and PA (10 μ M and 100 μ M) all increased the free radical production. These results are consistent with previous reported literature. Caffeine, FA and trigonelline are well known to have antioxidant properties [409]. Caffeine and FA have been shown extensively to display antioxidant properties in both *in vitro* cell-based models and *in vivo* animal models [48, 49, 84, 96]. However, to date, the antioxidant effects of trigonelline had only been studied in an *in vivo* study, suggesting the current study is the first to describe the possible antioxidant effects *in vivo* [409]. The other compounds of interest, CA, CGA and PA are all well known to have dose and condition specific effects on free radical formation [64, 88, 97, 103, 106, 108]. All three of these compounds have been shown to have antioxidant effects but can also be pro-oxidant in nature. Given this, it is not surprising to observe concentration-dependent changes in free radical formation.

The effect of caffeine (100 μ M) in combination with various concentrations of coffee constituents was evaluated. Significant decreases in free radical formation were observed in THP-1 cells exposed to caffeine (100 μ M) and CA (100 μ M) and CGA (100 μ M). Furthermore, significant increases in free radical production were observed in THP-1 cells exposed to caffeine (100 μ M) and CGA (10 μ M), FA (1 μ M) and trigonelline (all concentrations tested). These results suggest that caffeine may act as an antioxidant or a pro-oxidant when combined other bioactive coffee constituents.

Key bioactive coffee constituents were then assessed for their effects on markers of inflammation in IFN- γ and IFN- α_{2A} stimulated differentiated THP-1 cells. Four marker of inflammation were assessed including IDO activity and the three cytokines IL-6, IL-1 β and TNF- α . IDO activity was seen to be significantly decreased after 24 h pre-treatment with caffeine, CA, CGA and FA in IFN- γ stimulated differentiated THP-1 cells. Furthermore, treatment with PA further increased IDO activity and trigonelline had no effect on IDO activity. Similar results were observed in IFN- α_{2A} THP-1 cells with exception to PA and trigonelline. Both of these compounds showed a concentration-dependent decrease in IDO activity with a maximal decrease in IDO activity at 100 μ M. Furthermore, when cells were pre-treated with a combination of caffeine (100 μ M) and various concentrations of other key bioactive coffee constituents, no significant changes in IDO activity in IFN- γ stimulated THP-1 cells were observed. In contrast, IDO activity was shown to be decreased in IFN- α_{2A} stimulated THP-1 cells after 24 h pre-treatment with caffeine (100 μ M) in combination with CGA (100 μ M), FA (1 μ M), PA (10 μ M and 100 μ M) and trigonelline (1 μ M, 10 μ M and 100 μ M).

The various key bioactive coffee constituents were shown to have a variety of effects on IL-6, IL-1 β and TNF- α in both IFN- γ and IFN- α_{2A} stimulated THP-1 cells. IL-6 concentrations were found to be decreased in IFN- γ stimulated THP-1 cells pre-treated with caffeine (1 μ M and 10 μ M) and CA (10 μ M). Likewise, IL-6 concentrations were decreased in IFN- α_{2A} stimulated THP-1 cells pre-treated with all six of the coffee constituents. Furthermore, IL-1 β concentrations were shown to be increased in IFN- γ stimulated THP-1 cells pre-treated with FA (10 μ M and 100 μ M) and in IFN- α_{2A} stimulated THP-1 cells pre-treated with all six of the coffee constituents tested. Likewise, TNF- α concentrations were increased in IFN- γ stimulated THP-1 cells

pre-treated with CA, CGA, PA and trigonelline and after pre-treatment with all six of the coffee constituents in IFN- α_{2A} stimulated THP-1 cells.

Combinations of caffeine (100 μ M) and other coffee constituents were then assessed for their effects on pro-inflammatory cytokines in IFN- γ and IFN- α_{2A} stimulated THP-1 cells. IL-6 concentrations were shown to be decreased after pre-treatment with caffeine in combination with PA (1000 μ M) and trigonelline in IFN- γ stimulated THP-1 cells. Additionally, IL-6 concentrations were increased after IFN- α_{2A} stimulated THP-1 cells were pre-treated with all of the coffee constituents in combination with caffeine. Furthermore, changes to IL-1 β concentrations were observed in both IFN- γ and IFN- α_{2A} stimulated THP-1 cells. In IFN- γ stimulated cells, pre-treatment with caffeine in combination with CA and CGA afforded increases in the IL-1 β concentrations whereas pre-treatment with caffeine in combination with FA, PA and trigonelline afforded decreases in IL-1 β concentrations. The changes in concentration of IL-1 β were slightly different in IFN- α_{2A} THP-1 cells. Pre-treatment with caffeine in combination with all five of the remaining constituents of interest afforded significant decreases in IL-1 β concentrations. Changes in TNF- α concentrations were only seen in IFN- γ stimulated THP-1 cells pre-treated with caffeine and other constituents of coffee. Pre-treatment with caffeine in combination with CA, CGA, FA and PA resulted in increases in the concentration of TNF- α in IFN- γ stimulated THP-1 cells.

Countless studies have shown the role of pro-inflammatory cytokines in the upregulation of IDO activity including IFN- γ , IFN- α , TNF- α and IL-1 [15, 59, 60, 122].

In the current study, IFN- γ , the most potent stimulator of IDO activity [410], and IFN- α_{2A} were used as stimuli. As seen in chapter four, both IFN- γ and IFN- α_{2A} significantly increased IDO activity. The effects of coffee constituents alone and in combination with caffeine were assessed in each of the models. Differences were noted in the activities of a number of the coffee constituents between the two models. In the IFN- α_{2A} model, all six coffee constituents studied, caffeine, CA, CGA, FA, PA and trigonelline all significantly decreased the activity of IDO. IFN- γ on the other hand, decreased the activity of IDO after the cells were pre-treated with caffeine, CA, CGA and FA but trigonelline did not alter IDO activity and PA increased IDO activity. As there are no previous studies done to assess the effects of the coffee constituents on IDO activity, with the exception of caffeine, it is difficult to predict why this may have been observed. One possible explanation may be due to the potency of IFN- γ as an inducer of IDO. As outlined above, IFN- γ is the most potent inducer of IDO activity. It is possible that the mechanism by which PA and trigonelline exert their inhibitory effects on IDO in IFN- α_{2A} stimulated THP-1 cells may not be potent enough to exert their effect in IFN- γ stimulated cells. Another possible explanation for the differences observed may be related to the difference in cell signalling between the IFN- γ , a type II IFN, and IFN- α_{2A} , a type I IFN as outlined above. Unfortunately, this study is unable to assess the factors responsible for the differences but future studies are recommended to further elucidate the mechanisms responsible for these differences.

Caffeine's effects on IDO have previously been evaluated in a human study. These results showed IDO activity was increased [37], which is the opposite of what is observed in the current study. One possible explanation for this difference may be due to the concentration of caffeine. In a previous study, a very large dose of caffeine was

given to subjects that may not correlate to the concentrations used in the current study [37]. Furthermore, given that the published study was a human study, it is impossible to control for all confounding factors and stimulus of IDO. In the current study, the only stimulus that cells were receiving was IFN. In a human study, there are many possible inducers of IDO including other circulating cytokines and free radicals [58, 60].

In addition to the comparison between the two types of IFN, comparisons were then also made between the coffee constituents alone and in combination with caffeine for each of the IFN models. Significant differences between the coffee constituents alone and in combination with caffeine were observed in the IFN- γ stimulus model. When constituents were combined with 100 μ M of caffeine, all of the observed decreases and increases in IDO activity were no longer seen. CA in combination with caffeine was the only combination of caffeine and coffee constituent that was different to the results observed in the coffee constituent alone group in IFN- α_{2A} stimulated THP-1 cells. All other combinations were still decreased but to a much lesser degree. These results suggest that coffee constituents alone, especially caffeine, CA, CGA and FA, may provide better protection against IFN- γ induced increases in IDO activity. However, given the limitations of the model used in the current study, further studies confirming these results are recommended.

From the results obtained for the cytokines, a number of compounds significantly decreased IL-6 concentrations. Given the proposed role of IL-6 in depression [58, 60], these compounds may be of interest for further investigation. Decreased IL-6 concentrations were observed in INF- γ stimulated THP-1 cells pre-

treated with caffeine and CA. Furthermore, IFN- α_{2A} stimulated THP-1 cells pre-treated with caffeine, CA, CGA, FA, PA and trigonelline all displayed decreased IL-6 concentrations. Conversely, IFN- α_{2A} stimulated THP-1 cells pre-treated with caffeine in combination with CA, FA, PA and trigonelline all showed increased production of IL-6. Previous studies have not shown IL-6 to influence IDO activity, which is supported by the current results. Although IL-6 concentrations were increased in IFN- α_{2A} stimulated THP-1 cells pre-treated with caffeine in combination with FA, PA and trigonelline, IDO activity still decreased in these cells. Furthermore, CGA [52], PA [106], CA [411] and FA [412] all have been shown previously to decrease IL-6 concentrations. Studies to date have not assessed the effects of combinations of caffeine and other bioactive coffee constituents on the production of IL-6.

The other cytokines of interest in the current study, IL-1 β and TNF- α were both found to be unchanged from the IFN stimulus control in chapter 4. Despite this, significant increases and decreases in the concentrations of these cytokines were observed after pre-treatment with various coffee constituents alone and in combination with caffeine as outlined above. Exposure to PMA, the differentiating agent in the current study, has been shown to increase concentration of IL-1 β and TNF- α in the cells exposed to it [413]. This suggests that a number of coffee constituents, alone and in combination with caffeine, may modulate the effects of PMA on the production of IL-1 β and TNF- α , however further investigations are required.

The cytotoxic effects of the coffee constituents, caffeine, CA, CGA, FA, PA and trigonelline were evaluated in the current study. Furthermore, their effects on the

specific pathways of cell death were also evaluated. Due to financial constraints, some assays were only able to be performed at one concentration. This concentration was chosen based on the cytotoxicity of the compounds on the cells.

The cytotoxic effects of bioactive coffee constituents were measured using the reduction of resazurin to resofurin and LDH activity. Numerous bioactive coffee constituents were shown to have cytotoxic effects towards SH-SY5Y neuroblastoma cells including CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M) using the resazurin proliferation assay. In further support to these observed effects, total LDH activity was raised significantly after 24 h exposure to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M) corresponding to the resazurin results. Similar studies undertaken in other cancer cell lines have shown that both CGA and CA induce apoptotic cell death [414, 415]. CGA was shown to cause apoptosis in a concentration-dependent manner via a mitochondria-dependent pathway [416]. Likewise, CA exposure has been shown to result in apoptosis however the mechanism by which this occurs has yet to be elucidated [414, 415].

This study then further investigated the possible mechanisms by which these constituents caused cytotoxic effects. The first potential mechanism investigated was free radical formation. Free radical formation is central to various cell death pathways [417]. Studies have shown that ROS activation of JNK results in both intrinsic and extrinsic apoptosis signalling and necrotic cell death [417, 418]. This is mediated through prolonged activation of JNK, essential for subsequent cytochrome c and caspase-3 cleavage [417, 418].

The 2',7'-Dichlorodihydrofluorescein diacetate (DCF) assay was used to assess the effects of biologically active coffee constituents on free radical production. Again, the same patterns with regard to the different phenotypes were observed with free radical production as was with viability. Undifferentiated SH-SY5Y neuroblastoma cells were shown to produce greater levels of free radicals when exposed to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M), consistent with the results observed with viability. Likewise, caffeine (1000 μ M) was shown to increase free radical production in differentiated SH-SY5Y cells in greater levels than undifferentiated SH-SY5Y cells however this was not statistically significant. Mitochondrial oxidative stress was measured using DHR 123 and MitoSOX Red in the current study. Both of these assays showed that exposure of undifferentiated SH-SY5Y neuroblastoma cells to CA, CGA and PA resulted in greater increases in mitochondrial oxidative stress in comparison to their differentiated counterpart. The exception to this was after undifferentiated SH-SY5Y neuroblastoma cells were treated with FA, where mitochondrial oxidative stress decreased significantly in comparison to its differentiated counterpart. Significant increases in mitochondrial oxidative stress were observed after undifferentiated SH-SY5Y were exposed to CA, CGA and PA. Furthermore, dbcAMP differentiated SH-SY5Y cells exposed to CA and PA both showed increases in mitochondrial oxidative stress. These results are consistent with the results obtained in the MitoSOX Red assay and complement the already presented results suggesting that the cytotoxicity of the relevant coffee constituents is via, at least in part, a free radical mediated pathway.

Numerous coffee constituents have been shown to have antioxidant properties including CA and CGA [64, 88, 97, 254]. However, these properties are also concentration-dependent [64, 88, 97, 254]. It has been shown at high concentrations

that these compounds can actually act in a pro-oxidant manner. Furthermore, PA, another coffee constituent shown to increase free radical production in both phenotypes of SH-SY5Y cells, also has the ability to act as either a pro-oxidant or an antioxidant [103, 108].

The effects of key biologically active coffee constituents on apoptosis were then evaluated using caspase-3 and annexin V activation as markers of early and mid-apoptosis, respectively. Again, the results observed, particularly for annexin V activation, mirrored the observations of the viability assay. Exposure of undifferentiated cells to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M) resulted in statistically significant increases in the level of annexin V activation. Again, these results were significantly higher than those observed in the differentiated SH-SY5Y cells, with statistically significant increases seen only after exposure to PA (1000 μ M). These results are however not mirrored with early markers of apoptosis, as observed in the caspase-3 activation assay. Statistically significant increases in the activation of caspase-3 were observed after exposure of CA (1000 μ M) in both undifferentiated and differentiated SH-SY5Y cells and PA (1000 μ M) in differentiated SH-SY5Y cells. Several studies have shown CGA and CA to cause apoptosis [414-416], an effect seen with exposure of these compounds to differentiated SH-SY5Y cells. One possible explanation is the high levels of free radical production after exposure of these cells to several of the key bioactive coffee constituents. Free radical production in cells has been shown to mediate apoptosis [419]. The data presented in the current study suggests that this may be occurring.

Propidium iodide solution was used to measure the level of necrosis that exposure of each of the coffee constituents to undifferentiated and differentiated SH-SY5Y resulted in. Again, necrosis was increased in undifferentiated SH-SY5Y cells exposed to CA (1000 μ M), CGA (1000 μ M) and PA (1000 μ M). Necrosis was increased in differentiated SH-SY5Y cells exposed to PA (1000 μ M) however this was not a statistically significant increase.

To determine the possible role of signal transduction pathways in the death of undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells, the effects of the coffee constituents on intracellular cAMP concentrations were assessed. In cells that were pre-treated with coffee constituents that were shown to be cytotoxic above, namely CA, CGA and PA, intracellular cAMP concentrations were significantly raised in undifferentiated SH-SY5Y neuroblastoma cells. Slightly different results were observed in dbcAMP SH-SY5Y cells. CA and CGA both increased intracellular cAMP concentrations however PA did not. Increased intracellular cAMP concentrations are associated with increased activity in signal transduction pathways [420]. This suggests that the cytotoxic effects observed after exposure of undifferentiated SH-SY5Y cells to CA, CGA and PA, and dbcAMP differentiated cells to CA and CGA, are mediated predominantly through cell signalling pathways. Conversely, the cytotoxicity in dbcAMP SH-SY5Y cells exposed to PA is most likely mediated through a receptor level response. Further studies are however required to fully elucidate the specific pathways that these compound mediate their effects through.

Many constituents of coffee have been shown to have beneficial effects in a number of disease conditions including Alzheimer's disease, Parkinson's disease and depression [124]. Many major constituents of coffee have been shown to have protective properties in a number of cells lines along with showing antioxidant activity [124]. However, to date, no studies have investigated the cytotoxic effects of key biologically active coffee constituents on undifferentiated or dibutyryl cyclic AMP differentiated SH-SY5Y neuroblastoma cells, a cell line commonly used in neuronal models of toxicity and inflammation.

SH-SY5Y neuroblastoma cells are routinely used in neuronal toxicity and inflammation studies given their phenotypic resemblance to human foetal sympathetic neurons [278]. Furthermore these cells express many of the receptors expressed by primary human neurons including ionotropic and metabotropic NMDA receptors [279], various subunits of nicotinic acetylcholine receptors (nAChRs), 5-HT receptors, muscarinic receptors, adrenergic receptors and opioid receptors [278]. In addition to the receptors outlined above, these cells express the biosynthetic enzymes for noradrenaline along with numerous neuronal characteristics including the expression of neurofilament proteins, neuron specific enolase, gamma-aminobutyric acid (GABA) and norepinephrine uptake [280].

This study has shown that a number of key bioactive coffee constituents have cytotoxic effects towards both undifferentiated and dbcAMP differentiated SH-SY5Y neuroblastoma cells. These effects were mediated via numerous cell death pathways

including apoptosis and necrosis but appear to be commonly mediated by free radical production.

5.6 Conclusion

The current study has investigated the effects of key bioactive coffee constituents, both alone and in combination with caffeine, on markers of inflammation in IFN stimulated surrogate microglial-like cells. Coffee constituents alone appeared to produce greater reductions in IDO activity after IFN stimulus in comparison to the constituents used in combination with 100 μ M of caffeine. It is difficult to identify a mechanism by which this may have occurred given the limitations of the study with regards to the evaluation of the effects of coffee constituents on the production of cytokines. Furthermore, this study assessed the effects of coffee constituents on the viability of neuronal-like cells. It was found that high concentrations of a number of the constituents including CA, CGA and PA were toxic towards both undifferentiated and dbcAMP differentiated SH-SY5Y, however, undifferentiated cells were more sensitive to these effects. Furthermore, it appears that these effects are mediated through signal transduction pathways given the increases in cAMP. In summary, there is evidence to suggest that coffee constituents may play a beneficial role in the pathophysiology of depression given their ability to modulate IDO and cytokines associated with depression. Furthermore, coffee constituents also appear to have the ability to cause potential harm, especially at high concentrations.

Chapter 6 – Conclusion

This study investigated the effects of coffee and its key bioactive constituents on behaviours of and biomarkers of depression. This, in the first instance, was undertaken using an *in vivo* model of inflammatory depression. This study assessed the effects of caffeine, caffeinated coffee and decaffeinated coffee in a well-accepted LPS model of inflammatory depression. Furthermore, based on the observed results of the *in vivo* study, investigations into the differences in constituents found in caffeinated coffee and decaffeinated coffee were explored using quantitative, analytical HPLC. Subsequently, *in vitro* evaluation of the specific mechanisms by which key bioactive coffee constituents, alone and in combination with caffeine, may elicit their effect were undertaken.

Significant improvements in depressive-like behaviour were observed in this study after pre-treatment with both caffeine and caffeinated coffee in comparison to the LPS alone treatment group. Pre-treatment with caffeine afforded a decrease in immobility time in both the FST and TST comparable to the positive control used in the study, imipramine. Caffeinated coffee, however, afforded a further improvement in the immobility time, that is, a decrease in immobility time, 50% better than caffeine alone in both the TST and FST. Furthermore, specific biomarkers associated with depression including IDO activity, PGE₂ production and neopterin and biopterin concentrations were assessed. IDO activity was decreased in animals pre-treated with caffeinated coffee and exposed to LPS comparable to the decreases shown with pre-treatment with imipramine. The animals pre-treated with caffeine, however, did not show any changes in IDO activity in comparison to animals pre-treated with LPS alone. Furthermore,

PGE₂ production was significantly decreased in animals pre-treated with caffeine, caffeinated coffee and decaffeinated coffee and exposed to LPS in comparison to those animals only receiving LPS treatment alone. Finally, the neopterin to biopterin ratio was evaluated and again, all three treatment groups that is caffeine, caffeinated coffee and decaffeinated coffee, all decreased the rate of conversion of neopterin to biopterin.

These results suggest that the antidepressant-like activity of caffeinated coffee may be due to a reduction in IDO activity. The observed reduction in IDO activity in all of the treatment and control groups correlates with the observations of the behavioural studies, with exception of animals pre-treated with caffeine. Given this, it appears that the antidepressant-like effects may be mediated through a number of different mechanisms. One such mechanism is through anti-inflammatory activities. Caffeine, caffeinated coffee and decaffeinated coffee all decreased PGE₂ concentrations although pre-treatment with caffeinated coffee and decaffeinated coffee provided the greatest reduction in its concentration. Conversely, the concentration of other pro-inflammatory cytokines was unchanged in comparison to both saline and LPS controls. As highlighted earlier, this may be due to the tissue and time specific nature of the release of cytokines [146]. Future studies assessing the effects of caffeine, caffeinated coffee and decaffeinated coffee on the effect of these cytokines at the various time points is warranted in future studies. Another possible mechanism by which caffeinated coffee may provide antidepressant-like activities is through the actions on A_{2A}Rs. Caffeine, an antagonist at A_{2A}Rs, has been shown to display antidepressant activities mediated via this effect [34, 36]. Given the mechanisms, it is possible that multiple mechanisms are working synergistically to result in the enhanced antidepressant-like activities of caffeinated coffee in comparison to caffeine and decaffeinated coffee. One

limitation identified in the *in vivo* study is that an cyclooxygenase inhibitory activity was not evaluated. Given the varying roles of cyclooxygenase-1 and -2 in neuroinflammation and the potential effects of caffeine, caffeinated coffee and decaffeinated coffee have on the inhibition of each of these enzymes, it is worth further investigating in future studies.

Another possibility is that a constituent in caffeinated coffee, in addition to caffeine, is lost in the decaffeination process. Caffeinated coffee is known to have many bioactive constituents with known antidepressant-like, anti-inflammatory and antioxidant activities [124] but to date, no studies have assessed if these constituents are lost through the decaffeination process. Analyses were undertaken assessing if any of the major constituents were missing from decaffeinated coffee. Results showed that one compound, FA, was found to a lesser degree in decaffeinated coffee than caffeinated coffee in addition to caffeine. Further *in vitro* studies were then undertaken to assess if specific mechanisms by which constituents both alone and in combination with caffeine altered inflammatory mediators was undertaken in a surrogate interferon stimulated microglial cell model. A number of bioactive coffee constituents including caffeine, CA, CGA and FA were shown to protect against IFN- γ induced increases in IDO activity. Furthermore, pre-treatment with caffeine, CA, FA, PA and trigonelline protected against IFN- α induced increases in IDO activity. Interestingly, when combined with caffeine, the protective effects of bioactive coffee constituents with regards to IDO activity are diminished. All the protective effects are completely diminished in IFN- γ stimulated THP-1 cells and are significantly less in IFN- α stimulated differentiated THP-1 cells. IDO activity is known to play an important role in the pathophysiology of depression [15, 58-60, 122, 229, 230]. Studies have shown

the correlation between the increase in depressive symptoms and the increase in IDO activity [229]. The current study suggests that key bioactive coffee constituents alone protect against IFN induced increases in IDO activity but due to limitations in the model, it was not possible to determine the mechanism by which this occurs. Furthermore, the effects on IDO activity are diminished when individual constituents are combined with caffeine. One limitation identified in the study was that only one concentration of caffeine was tested which may have influenced the results. Furthermore, coffee constituents were only combined with caffeine and not with other coffee constituents. Future studies investigating the effects of bioactive coffee constituents with various other concentrations of caffeine and other constituents is warranted to determine the possible mechanisms by which caffeinated coffee elucidates its antidepressant-like activity. Furthermore, further studies investigating the effects of coffee constituents, alone and in combination, on the production of PGE₂ would appear to be useful in further investigating these effects. Given the interesting results obtained in the current study, it would be beneficial if the study were repeated in a primary cell model to confirm if these results are comparable between the two models.

Furthermore, there are a number of potential harms identified with the consumption of coffee and caffeine including anxiety and increased risk of suicide with high consumption of coffee [5, 21, 22, 26, 28, 37, 132, 133]. In the current study, possible causes for these harms were evaluated using a surrogate neuronal cell model. The study showed that a number of key bioactive coffee constituents including CA, CGA and PA all significantly decreased the viability of both undifferentiated and differentiated SH-SY5Y neuroblastoma cells, the neuronal-like model used in the current model. Further studies were undertaken to assess specific cell death pathways

and it appears that the cytotoxicity of these compounds may be mediated via a free radical induced apoptotic pathway. These results may provide an explanation as to why caffeine and caffeinated coffee cause symptoms of anxiety and an increased risk of suicide when consumed in high doses. One limitation is that in the current study, the antidepressants investigated in the study amitriptyline, fluoxetine and venlafaxine, were only used in the original state however, *in vivo*, these three drugs undergo extensive metabolism to a number of active metabolites. Further studies are warranted to further assess these effects in a primary cell model.

In conclusion, this study has confirmed the hypotheses of the study, namely that caffeinated coffee along with key bioactive coffee constituents will be beneficial in behaviours and biomarkers associated with depression in both *in vivo* and *in vitro* models of depression and inflammation. This study has shown that there are a number of possible mechanisms by which caffeinated coffee may elucidate its antidepressant action. Furthermore, the results observed in the *in vivo* animal study assessing the effects of caffeine, caffeinated coffee and decaffeinated coffee on parameters of depression provide evidence to support the causal link between caffeinated coffee consumption and a reduced risk of depression. These results correlate with the results observed in the epidemiological studies to provide further evidence to support the causal link. The results observed in the current study support and justify the need for future high quality randomised controlled trials to assess the effects of coffee in humans and assessing its effects on related biomarkers.

References

1. Lara, D.R., *Caffeine, mental health, and psychiatric disorders*. J Alzheimers Dis, 2010. **20 Suppl 1**: p. S239-48.
2. *Australian Health Survey: Nutrition First Results - Foods and Nutrients, 2011-12*. 2015 08/12/2015]; Available from:
<http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/4364.0.55.007main+features12011-12>.
3. Ruusunen, A., et al., *Coffee, tea and caffeine intake and the risk of severe depression in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study*. Public Health Nutr, 2010. **13**(8): p. 1215-20.
4. Lucas, M., et al., *Coffee, caffeine, and risk of depression among women*. Arch Intern Med, 2011. **171**(17): p. 1571-8.
5. Kawachi, I., et al., *A prospective study of coffee drinking and suicide in women*. Arch Intern Med, 1996. **156**(5): p. 521-5.
6. Wang, L., et al., *Coffee and caffeine consumption and depression: A meta-analysis of observational studies*. Aust N Z J Psychiatry, 2016. **50**(3): p. 228-42.
7. Park, R.J. and J.D. Moon, *Coffee and depression in Korea: the fifth Korean National Health and Nutrition Examination Survey*. Eur J Clin Nutr, 2015. **69**(4): p. 501-4.
8. Omagari, K., et al., *Coffee consumption is inversely associated with depressive status in Japanese patients with type 2 diabetes*. J Clin Biochem Nutr, 2014. **55**(2): p. 135-42.
9. Zeni, A.L., et al., *Ferulic acid exerts antidepressant-like effect in the tail suspension test in mice: evidence for the involvement of the serotonergic system*. Eur J Pharmacol, 2012. **679**(1-3): p. 68-74.

10. Huang, Y., et al., *A simple LC-MS/MS method for determination of kynurenine and tryptophan concentrations in human plasma from HIV-infected patients.* Bioanalysis, 2013. **5**(11): p. 1397-407.
11. Pham, N.M., et al., *Green tea and coffee consumption is inversely associated with depressive symptoms in a Japanese working population.* Public Health Nutr, 2014. **17**(3): p. 625-33.
12. Guo, X., et al., *Sweetened beverages, coffee, and tea and depression risk among older US adults.* PloS one, 2014. **9**(4): p. e94715.
13. Chopra, K., B. Kumar, and A. Kuhad, *Pathobiological targets of depression.* Expert Opin Ther Targets, 2011. **15**(4): p. 379-400.
14. Elhwuegi, A.S., *Central monoamines and their role in major depression.* Prog Neuropsychopharmacol Biol Psychiatry, 2004. **28**(3): p. 435-51.
15. Myint, A.M. and Y.K. Kim, *Cytokine-serotonin interaction through IDO: a neurodegeneration hypothesis of depression.* Med Hypotheses, 2003. **61**(5-6): p. 519-25.
16. Nehlig, A., *Are we dependent upon coffee and caffeine? A review on human and animal data.* Neurosci Biobehav Rev, 1999. **23**(4): p. 563-76.
17. Higdon, J.V. and B. Frei, *Coffee and health: a review of recent human research.* Crit Rev Food Sci Nutr, 2006. **46**(2): p. 101-23.
18. Ruiz-Crespo, S., et al., *Coffee component 3-caffeoylquinic acid increases antioxidant capacity but not polyphenol content in experimental cerebral infarction.* Neurochem Res, 2012. **37**(5): p. 1085-90.
19. Butt, M.S. and M.T. Sultan, *Coffee and its consumption: benefits and risks.* Crit Rev Food Sci Nutr, 2011. **51**(4): p. 363-73.

20. Levin, K.A., *Study design III: Cross-sectional studies*. Evid Based Dent, 2006. **7**(1): p. 24-5.
21. Lucas, M., et al., *Coffee, caffeine, and risk of completed suicide: results from three prospective cohorts of American adults*. World J Biol Psychiatry, 2014. **15**(5): p. 377-86.
22. Tanskanen, A., et al., *Heavy coffee drinking and the risk of suicide*. Eur J Epidemiol, 2000. **16**(9): p. 789-91.
23. Gorman, J.M., *Comorbid depression and anxiety spectrum disorders*. Depress Anxiety, 1996. **4**(4): p. 160-8.
24. Charney, D.S., G.R. Heninger, and P.I. Jatlow, *Increased anxiogenic effects of caffeine in panic disorders*. Arch Gen Psychiatry, 1985. **42**(3): p. 233-43.
25. Eaton, W.W. and J. McLeod, *Consumption of coffee or tea and symptoms of anxiety*. Am J Public Health, 1984. **74**(1): p. 66-8.
26. Shanahan, M.P. and R.N. Hughes, *Potentiation of performance-induced anxiety by caffeine in coffee*. Psychol Rep, 1986. **59**(1): p. 83-6.
27. Lee, M.A., O.G. Cameron, and J.F. Greden, *Anxiety and caffeine consumption in people with anxiety disorders*. Psychiatry Res, 1985. **15**(3): p. 211-7.
28. Veleber, D.M. and D.I. Templer, *Effects of caffeine on anxiety and depression*. J Abnorm Psychol, 1984. **93**(1): p. 120-2.
29. Barlow, D.H., et al., *Co-Morbidity and Depression among the Anxiety Disorders: Issues in Diagnosis and Classification*. J Nerv Ment Dis 1986. **174**(2): p. 63-72.
30. Steger, M.F. and T.B. Kashdan, *Depression and Everyday Social Activity, Belonging, and Well-Being*. J Couns Psychol, 2009. **56**(2): p. 289-300.

31. Daly, J.W., *Caffeine analogs: biomedical impact*. Cell Mol Life Sci, 2007. **64**(16): p. 2153-69.
32. Pathak, L., Y. Agrawal, and A. Dhir, *Natural polyphenols in the management of major depression*. Expert Opin Investig Drugs, 2013. **22**(7): p. 863-80.
33. Streit, W.J., R.E. Mrazek, and W.S. Griffin, *Microglia and neuroinflammation: a pathological perspective*. J Neuroinflammation, 2004. **1**(1): p. 14.
34. Kaster, M.P., et al., *Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress*. Proc Natl Acad Sci U S A, 2015. **112**(25): p. 7833-8.
35. Smith, A., *Effects of caffeine on human behavior*. Food Chem Toxicol, 2002. **40**(9): p. 1243-55.
36. El Yacoubi, M., J. Costentin, and J.M. Vaugeois, *Adenosine A2A receptors and depression*. Neurology, 2003. **61**(11 Suppl 6): p. S82-7.
37. Orlikov, A. and I. Ryzov, *Caffeine-induced anxiety and increase of kynurenine concentration in plasma of healthy subjects: a pilot study*. Biol Psychiatry, 1991. **29**(4): p. 391-6.
38. Leonard, B. and M. Maes, *Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression*. Neurosci Biobehav Rev, 2012. **36**(2): p. 764-85.
39. Pechlivanova, D.M., et al., *Effect of long-term caffeine administration on depressive-like behavior in rats exposed to chronic unpredictable stress*. Behav Pharmacol, 2012. **23**(4): p. 339-47.

40. Kale, P.P. and V. Addepalli, *Augmentation of antidepressant effects of duloxetine and bupropion by caffeine in mice*. Pharmacol Biochem Behav, 2014. **124**: p. 238-44.
41. Horrigan, L.A., J.P. Kelly, and T.J. Connor, *Caffeine suppresses TNF-alpha production via activation of the cyclic AMP/protein kinase A pathway*. Int Immunopharmacol, 2004. **4**(10-11): p. 1409-17.
42. Maes, M., et al., *Increased plasma peroxides and serum oxidized low density lipoprotein antibodies in major depression: markers that further explain the higher incidence of neurodegeneration and coronary artery disease*. J Affect Disord, 2010. **125**(1-3): p. 287-94.
43. Piletz, J.E., et al., *Pro-inflammatory biomarkers in depression: treatment with venlafaxine*. World J Biol Psychiatry, 2009. **10**(4): p. 313-23.
44. Bassal, N.K., B.P. Hughes, and M. Costabile, *Arachidonic acid and its COX1/2 metabolites inhibit interferon-gamma mediated induction of indoleamine-2,3 dioxygenase in THP-1 cells and human monocytes*. Prostaglandins Leukot Essent Fatty Acids, 2012. **87**(4-5): p. 119-26.
45. Hughes, M.M., et al., *Tryptophan depletion in depressed patients occurs independent of kynurenine pathway activation*. Brain Behav Immun, 2012. **26**(6): p. 979-87.
46. Abdulla, Y.H. and K. Hamadah, *3',5' cyclic adenosine monophosphate in depression and mania*. Lancet, 1970. **1**(7643): p. 378-81.
47. Fredholm, B.B., *On the mechanism of action of theophylline and caffeine*. Acta Med Scand, 1985. **217**(2): p. 149-53.

48. Devasagayam, T.P.A., et al., *Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species*. Biochim Biophys Acta, 1996. **1282**(1): p. 63-70.
49. Azam, S., et al., *Antioxidant and prooxidant properties of caffeine, theobromine and xanthine*. Med Sci Monit, 2003. **9**(9): p. BR325-30.
50. Vignoli, J.A., D.G. Bassoli, and M.T. Benassi, *Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material*. Food Chem, 2011. **124**(3): p. 863-868.
51. Rammal, H., et al., *Evidence that oxidative stress is linked to anxiety-related behaviour in mice*. Brain Behav Immun, 2008. **22**(8): p. 1156-9.
52. Shen, W., et al., *Chlorogenic acid inhibits LPS-induced microglial activation and improves survival of dopaminergic neurons*. Brain Res Bull, 2012. **88**(5): p. 487-94.
53. Baraldi, S., et al., *Symptomatic treatment of interferon-alpha-induced depression in hepatitis C: a systematic review*. J Clin Psychopharmacol, 2012. **32**(4): p. 531-43.
54. Vismari, L., et al., *A possible role to nitric oxide in the anti-inflammatory effects of amitriptyline*. Immunopharmacol Immunotoxicol, 2012. **34**(4): p. 578-85.
55. Xu, J.G., Q.P. Hu, and Y. Liu, *Antioxidant and DNA-protective activities of chlorogenic acid isomers*. J Agric Food Chem, 2012. **60**(46): p. 11625-30.
56. Qin, L., et al., *Interactive role of the toll-like receptor 4 and reactive oxygen species in LPS-induced microglia activation*. Glia, 2005. **52**(1): p. 78-84.
57. Krakauer, T., *The polyphenol chlorogenic acid inhibits staphylococcal exotoxin-induced inflammatory cytokines and chemokines*. Immunopharmacol Immunotoxicol, 2002. **24**(1): p. 113-9.

58. Maes, M., *Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression.* Prog Neuropsychopharmacol Biol Psychiatry, 2011. **35**(3): p. 664-75.
59. Maes, M., et al., *The new '5-HT' hypothesis of depression: cell-mediated immune activation induces indoleamine 2,3-dioxygenase, which leads to lower plasma tryptophan and an increased synthesis of detrimental tryptophan catabolites (TRYCATs), both of which contribute to the onset of depression.* Prog Neuropsychopharmacol Biol Psychiatry, 2011. **35**(3): p. 702-21.
60. Maes, M., et al., *The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression.* Metab Brain Dis, 2009. **24**(1): p. 27-53.
61. Koo, J.W., et al., *Nuclear factor-kappaB is a critical mediator of stress-impaired neurogenesis and depressive behavior.* Proc Natl Acad Sci U S A, 2010. **107**(6): p. 2669-74.
62. Kreutzberg, G.W., *Microglia: a sensor for pathological events in the CNS.* Trends Neurosci, 1996. **19**(8): p. 312-8.
63. Shi, H., et al., *Effect of chlorogenic acid on LPS-induced proinflammatory signaling in hepatic stellate cells.* Inflamm Res, 2013. **62**(6): p. 581-7.
64. Du, W.Y., et al., *High-dose chlorogenic acid induces inflammation reactions and oxidative stress injury in rats without implication of mast cell degranulation.* J Ethnopharmacol, 2013. **147**(1): p. 74-83.
65. Birks, J., *Cholinesterase inhibitors for Alzheimer's disease.* Cochrane Database Syst Rev, 2006(1): p. CD005593.
66. Ago, Y., et al., *Pharmacological aspects of the acetylcholinesterase inhibitor galantamine.* J Pharmacol Sci, 2011. **116**(1): p. 6-17.

67. Janowsky, D.S., et al., *A cholinergic-adrenergic hypothesis of mania and depression*. Lancet, 1972. **2**(7778): p. 632-5.
68. Oboh, G., et al., *Comparative study on the inhibitory effect of caffeic and chlorogenic acids on key enzymes linked to Alzheimer's disease and some pro-oxidant induced oxidative stress in rats' brain-in vitro*. Neurochem Res, 2013. **38**(2): p. 413-9.
69. Oxenkrug, G.F., *Tryptophan kynurenine metabolism as a common mediator of genetic and environmental impacts in major depressive disorder: the serotonin hypothesis revisited 40 years later*. Isr J Psychiatry Relat Sci, 2010. **47**(1): p. 56-63.
70. Zoheir, N., D.F. Lappin, and C.J. Nile, *Acetylcholine and the alpha 7 nicotinic receptor: a potential therapeutic target for the treatment of periodontal disease?* Inflamm Res, 2012. **61**(9): p. 915-26.
71. Hennings, E.C., J.P. Kiss, and E.S. Vizi, *Nicotinic acetylcholine receptor antagonist effect of fluoxetine in rat hippocampal slices*. Brain Res, 1997. **759**(2): p. 292-4.
72. Rana, B., et al., *Inhibition of neuronal nicotinic acetylcholine receptors by imipramine and desipramine*. Eur J Pharmacol, 1993. **250**(2): p. 247-51.
73. Lopez-Valdes, H.E., J. Garcia-Colunga, and R. Miledi, *Effects of clomipramine on neuronal nicotinic acetylcholine receptors*. Eur J Pharmacol, 2002. **444**(1-2): p. 13-9.
74. Bertrand, D., *The possible contribution of neuronal nicotinic acetylcholine receptors in depression*. Dialogues Clin Neurosci, 2005. **7**(3): p. 207-16.

75. Arase, Y., et al., *Efficacy and safety of combination therapy of natural human interferon beta and ribavirin in chronic hepatitis C patients*. Intern Med, 2011. **50**(19): p. 2083-8.
76. Xu, Y., et al., *Ferulic acid increases pain threshold and ameliorates depression-like behaviors in reserpine-treated mice: behavioral and neurobiological analyses*. Metab Brain Dis, 2013. **28**(4): p. 571-83.
77. Noelker, C., et al., *The flavanoid caffeic acid phenethyl ester blocks 6-hydroxydopamine-induced neurotoxicity*. Neurosci Lett, 2005. **383**(1-2): p. 39-43.
78. Baumeister, A.A., M.F. Hawkins, and S.M. Uzelac, *The myth of reserpine-induced depression: role in the historical development of the monoamine hypothesis*. J Hist Neurosci, 2003. **12**(2): p. 207-20.
79. Yabe, T., et al., *Ferulic acid induces neural progenitor cell proliferation in vitro and in vivo*. Neuroscience, 2010. **165**(2): p. 515-24.
80. Nardini, M., et al., *Absorption of phenolic acids in humans after coffee consumption*. J Agric Food Chem, 2002. **50**(20): p. 5735-41.
81. Mandi, Y. and L. Vecsei, *The kynurenine system and immunoregulation*. J Neural Transm (Vienna), 2012. **119**(2): p. 197-209.
82. Gould, E., et al., *Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation*. J Neurosci, 1997. **17**(7): p. 2492-8.
83. Sharma, O.P., *Antioxidant activity of curcumin and related compounds*. Biochem Pharmacol, 1976. **25**(15): p. 1811-2.

84. Kanski, J., et al., *Ferulic acid antioxidant protection against hydroxyl and peroxy radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies*. J Nutr Biochem, 2002. **13**(5): p. 273-281.
85. Wu, C.H., et al., *The glycation effect of caffeic acid leads to the elevation of oxidative stress and inflammation in monocytes, macrophages and vascular endothelial cells*. J Nutr Biochem, 2011. **22**(6): p. 585-94.
86. Kalonia, H., et al., *Effect of caffeic acid and rofecoxib and their combination against intrastriatal quinolinic acid induced oxidative damage, mitochondrial and histological alterations in rats*. Inflammopharmacology, 2009. **17**(4): p. 211-9.
87. Kang, N.J., et al., *Caffeic acid, a phenolic phytochemical in coffee, directly inhibits Fyn kinase activity and UVB-induced COX-2 expression*. Carcinogenesis, 2009. **30**(2): p. 321-30.
88. Gulcin, I., *Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid)*. Toxicology, 2006. **217**(2-3): p. 213-20.
89. Takeda, H., et al., *Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice*. Eur J Pharmacol, 2002. **449**(3): p. 261-7.
90. Takeda, H., et al., *Rosmarinic acid and caffeic acid reduce the defensive freezing behavior of mice exposed to conditioned fear stress*. Psychopharmacology (Berl), 2002. **164**(2): p. 233-5.
91. Takeda, H., et al., *Caffeic acid produces antidepressive- and/or anxiolytic-like effects through indirect modulation of the $\alpha 1A$ -adrenoceptor system in mice*. Neuroreport, 2003. **14**(7): p. 1067-1070 10.1097/01.wnr.0000073427.02536.b0.

92. Son, Y., et al., *Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways?* J Signal Transduct, 2011. **2011**: p. 792639.
93. Kim, B.C., et al., *Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway.* Biochem Pharmacol, 2005. **70**(9): p. 1398-407.
94. Boudreau, L.H., et al., *Caffeic acid phenethyl ester and its amide analogue are potent inhibitors of leukotriene biosynthesis in human polymorphonuclear leukocytes.* PloS one, 2012. **7**(2): p. e31833.
95. Stone, T.W., C.M. Forrest, and L.G. Darlington, *Kynurenine pathway inhibition as a therapeutic strategy for neuroprotection.* FEBS J, 2012. **279**(8): p. 1386-97.
96. Kikuzaki, H., et al., *Antioxidant properties of ferulic acid and its related compounds.* J Agric Food Chem, 2002. **50**(7): p. 2161-8.
97. Chen, J.H. and C.-T. Ho, *Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds.* J Agric Food Chem, 1997. **45**(7): p. 2374-2378.
98. Sroka, Z. and W. Cisowski, *Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids.* Food Chem Toxicol, 2003. **41**(6): p. 753-8.
99. Yan-Chun, Z. and Z. Rong-Liang, *Phenolic compounds and an analog as superoxide anion scavengers and anti oxidants.* Biochem Pharmacol, 1991. **42**(6): p. 1177-1179.
100. Bilici, M., et al., *Antioxidative enzyme activities and lipid peroxidation in major depression: alterations by antidepressant treatments.* J Affect Disord, 2001. **64**(1): p. 43-51.

101. Herken, H., et al., *Adenosine deaminase, nitric oxide, superoxide dismutase, and xanthine oxidase in patients with major depression: impact of antidepressant treatment*. Arch Med Res, 2007. **38**(2): p. 247-52.
102. Minamisawa, M., S. Yoshida, and N. Takai, *Determination of biologically active substances in roasted coffees using a diode-array HPLC system*. Anal Sci, 2004. **20**(2): p. 325-8.
103. Park, W.H., et al., *A superoxide anion generator, pyrogallol induces apoptosis in As4.1 cells through the depletion of intracellular GSH content*. Mutat Res, 2007. **619**(1-2): p. 81-92.
104. Kattankulathur, K., *Pelagia Research Library*. Eur J Exp Biol, 2011. **1**(3): p. 145-153.
105. *Pyrogallol*. 2015 [cited 2015 18/12/2015]; Available from: <http://www.chemspider.com/Chemical-Structure.13835557.html?rid=8e5bdaab-29a2-4b0f-80b1-776fa3ca313b>.
106. Nicolis, E., et al., *Pyrogallol, an active compound from the medicinal plant Emblica officinalis, regulates expression of pro-inflammatory genes in bronchial epithelial cells*. Int Immunopharmacol, 2008. **8**(12): p. 1672-80.
107. Liao, P.C., et al., *Striatal formation of 6-hydroxydopamine in mice treated with pargyline, pyrogallol and methamphetamine*. J Neural Transm (Vienna), 2003. **110**(5): p. 487-94.
108. Upadhyay, G., et al., *Involvement of multiple molecular events in pyrogallol-induced hepatotoxicity and silymarin-mediated protection: evidence from gene expression profiles*. Food Chem Toxicol, 2010. **48**(6): p. 1660-70.

109. Matic, S., et al., *Methanol extract from the stem of Cotinus coggygia Scop., and its major bioactive phytochemical constituent myricetin modulate pyrogallol-induced DNA damage and liver injury*. Mutat Res, 2013. **755**(2): p. 81-9.
110. van Dijk, A.E., et al., *Acute effects of decaffeinated coffee and the major coffee components chlorogenic acid and trigonelline on glucose tolerance*. Diabetes Care, 2009. **32**(6): p. 1023-5.
111. *Trigonelline*. 2015 [cited 2015 18/12/2015]; Available from:
<http://www.chemspider.com/Chemical-Structure.5369.html?rid=b376bf62-e9c4-4fc5-ae79-9ddb8f3d7782>.
112. Zhou, J., S. Zhou, and S. Zeng, *Experimental diabetes treated with trigonelline: effect on beta cell and pancreatic oxidative parameters*. Fundam Clin Pharmacol, 2013. **27**(3): p. 279-87.
113. Tohda, C., et al., *Trigonelline-induced neurite outgrowth in human neuroblastoma SK-N-SH cells*. Biol Pharm Bull, 1999. **22**(7): p. 679-82.
114. Vaidya, V.A., *Depression - emerging insights from neurobiology*. British Medical Bulletin, 2001. **57**(1): p. 61-79.
115. Pav, M., et al., *Neurobiological aspects of depressive disorder and antidepressant treatment: role of glia*. Physiol Res, 2008. **57**(2): p. 151-64.
116. Oxenkrug, G.F., *Genetic and hormonal regulation of tryptophan kynurenine metabolism: implications for vascular cognitive impairment, major depressive disorder, and aging*. Ann N Y Acad Sci, 2007. **1122**: p. 35-49.
117. Marcus, M., et al. *Depression a global public health concern*. 2012 2012 [cited 2014 7th December 2014]; Available from:
http://www.who.int/mental_health/management/depression/who_paper_depression_wfmh_2012.pdf.

118. Ustun, T.B., et al., *Global burden of depressive disorders in the year 2000*. Br J Psychiatry, 2004. **184**: p. 386-92.
119. Jun, T.Y., et al., *Possible association between -G308A tumour necrosis factor- α gene polymorphism and major depressive disorder in the Korean population*. Psychiatr Genet, 2003. **13**(3): p. 179-81.
120. Heninger, G.R., P.L. Delgado, and D.S. Charney, *The revised monoamine theory of depression: a modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans*. Pharmacopsychiatry, 1996. **29**(1): p. 2-11.
121. Ruhe, H.G., N.S. Mason, and A.H. Schene, *Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies*. Mol Psychiatry, 2007. **12**(4): p. 331-359.
122. Myint, A.M., *Kynurenines: from the perspective of major psychiatric disorders*. FEBS J, 2012. **279**(8): p. 1375-85.
123. Maes, M., R. Smith, and S. Scharpe, *The monocyte-T-lymphocyte hypothesis of major depression*. Psychoneuroendocrinology, 1995. **20**(2): p. 111-6.
124. Hall, S., et al., *A review of the bioactivity of coffee, caffeine and key coffee constituents on inflammatory responses linked to depression*. Food Research International, 2015. **76**: p. 626-636.
125. Messmer, K. and G.P. Reynolds, *An in vitro model of inflammatory neurodegeneration and its neuroprotection*. Neurosci lett, 2005. **388**(1): p. 39-44.
126. Hurley, L.L. and Y. Tizabi, *Neuroinflammation, neurodegeneration, and depression*. Neurotox Res, 2013. **23**(2): p. 131-44.

127. Pravica, V., et al., *A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production.* Hum Immunol, 2000. **61**(9): p. 863-6.
128. Steiner, J., et al., *Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission?* J Neuroinflammation, 2011. **8**: p. 94.
129. Black, C.N., et al., *Is depression associated with increased oxidative stress? A systematic review and meta-analysis.* Psychoneuroendocrinology, 2015. **51**: p. 164-75.
130. Fava, M., et al., *A comparison of mirtazapine and nortriptyline following two consecutive failed medication treatments for depressed outpatients: a STAR*D report.* Am J Psychiatry, 2006. **163**(7): p. 1161-72.
131. Muller, N. and M.J. Schwarz, *A psychoneuroimmunological perspective to Emil Kraepelins dichotomy: schizophrenia and major depression as inflammatory CNS disorders.* Eur Arch Psychiatry Clin Neurosci, 2008. **258 Suppl 2**: p. 97-106.
132. Alsene, K., et al., *Association between A2a receptor gene polymorphisms and caffeine-induced anxiety.* Neuropsychopharmacology, 2003. **28**(9): p. 1694-1702.
133. Childs, E., et al., *Association between ADORA2A and DRD2 polymorphisms and caffeine-induced anxiety.* Neuropsychopharmacology, 2008. **33**(12): p. 2791-2800.

134. Kaplan, G.B., et al., *Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans*. J Clin Pharmacol, 1997. **37**(8): p. 693-703.
135. Nehlig, A. and S. Boyet, *Dose-response study of caffeine effects on cerebral functional activity with a specific focus on dependence*. Brain Res, 2000. **858**(1): p. 71-7.
136. O'Connor, J., et al., *Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2, 3-dioxygenase activation in mice*. Mol Psychiatry, 2009. **14**(5): p. 511-522.
137. Dinel, A.L., et al., *Lipopolysaccharide-induced brain activation of the indoleamine 2,3-dioxygenase and depressive-like behavior are impaired in a mouse model of metabolic syndrome*. Psychoneuroendocrinology, 2014. **40**: p. 48-59.
138. Fujigaki, S., et al., *Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism*. Eur J Immunol, 2001. **31**(8): p. 2313-8.
139. Frenois, F., et al., *Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior*. Psychoneuroendocrinology, 2007. **32**(5): p. 516-31.
140. O'Connor, J.C., et al., *Interferon-gamma and tumor necrosis factor-alpha mediate the upregulation of indoleamine 2,3-dioxygenase and the induction of depressive-like behavior in mice in response to bacillus Calmette-Guerin*. J Neurosci, 2009. **29**(13): p. 4200-9.

141. O'Connor, J.C., et al., *Induction of IDO by bacille Calmette-Guerin is responsible for development of murine depressive-like behavior*. J Immunol, 2009. **182**(5): p. 3202-12.
142. Dunn, A.J., A.H. Swiergiel, and R. de Beaurepaire, *Cytokines as mediators of depression: what can we learn from animal studies?* Neurosci Biobehav Rev, 2005. **29**(4): p. 891-909.
143. Nestler, E.J. and S.E. Hyman, *Animal models of neuropsychiatric disorders*. Nat Neurosci, 2010. **13**(10): p. 1161-9.
144. Dzirasa, K. and H.E. Covington, 3rd, *Increasing the validity of experimental models for depression*. Ann N Y Acad Sci, 2012. **1265**(1): p. 36-45.
145. Walker, A.K., et al., *NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J mice*. Neuropsychopharmacology, 2013. **38**(9): p. 1609-16.
146. Biesmans, S., et al., *Systemic immune activation leads to neuroinflammation and sickness behavior in mice*. Mediators Inflamm, 2013. **2013**: p. 271359.
147. Hwang, S.L., et al., *Indoleamine 2, 3-dioxygenase (IDO) is essential for dendritic cell activation and chemotactic responsiveness to chemokines*. Cell Res, 2005. **15**(3): p. 167-75.
148. Wirthgen, E., et al., *Activation of indoleamine 2,3-dioxygenase by LPS in a porcine model*. Innate Immun, 2014. **20**(1): p. 30-9.
149. Wang, Y., et al., *LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-gamma-independent manner by a JNK signaling pathway in primary murine microglia*. Brain Behav Immun, 2010. **24**(2): p. 201-9.

150. Taylor, M.W. and G.S. Feng, *Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism*. FASEB J, 1991. **5**(11): p. 2516-22.
151. Jurgens, B., et al., *Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells*. Blood, 2009. **114**(15): p. 3235-43.
152. Chen, Y. and G.J. Guillemin, *Kynurenine pathway metabolites in humans: disease and healthy States*. Int J Tryptophan Res, 2009. **2**: p. 1-19.
153. Albuquerque, E.X. and R. Schwarcz, *Kynurenic acid as an antagonist of alpha7 nicotinic acetylcholine receptors in the brain: facts and challenges*. Biochem Pharmacol, 2013. **85**(8): p. 1027-32.
154. Stone, T.W., N. Stoy, and L.G. Darlington, *An expanding range of targets for kynurenine metabolites of tryptophan*. Trends Pharmacol Sci, 2013. **34**(2): p. 136-43.
155. Moroni, F., et al., *Presence of kynurenic acid in the mammalian brain*. J Neurochem, 1988. **51**(1): p. 177-80.
156. Plangar, I., Z. Majlath, and L. Vecsei, *Kynurenines in cognitive functions: their possible role in depression*. Neuropsychopharmacol Hung, 2012. **14**(4): p. 239-44.
157. *Kynurenic acid*. 2015 [cited 2015 18/12/2015]; Available from: <http://www.chemspider.com/Chemical-Structure.3712.html>.
158. Gramsbergen, J.B., et al., *Brain-specific modulation of kynurenic acid synthesis in the rat*. J Neurochem, 1997. **69**(1): p. 290-8.
159. Costantino, G., *New promises for manipulation of kynurenine pathway in cancer and neurological diseases*. Expert Opin Ther Targets, 2009. **13**(2): p. 247-58.

160. Vecsei, L., et al., *Kynurenines in the CNS: recent advances and new questions*. Nat Rev Drug Discov, 2013. **12**(1): p. 64-82.
161. Lugo-Huitron, R., et al., *On the antioxidant properties of kynurenic acid: free radical scavenging activity and inhibition of oxidative stress*. Neurotoxicol Teratol, 2011. **33**(5): p. 538-47.
162. Muller, N., A.M. Myint, and M.J. Schwarz, *The impact of neuroimmune dysregulation on neuroprotection and neurotoxicity in psychiatric disorders--relation to drug treatment*. Dialogues Clin Neurosci, 2009. **11**(3): p. 319-32.
163. Muller, N. and M.J. Schwarz, *The immune-mediated alteration of serotonin and glutamate: towards an integrated view of depression*. Mol Psychiatry, 2007. **12**(11): p. 988-1000.
164. *3-Hydroxykynurenine (CSID:87)*. [cited 2013 31st of October]; Available from: <http://www.chemspider.com/Chemical-Structure.87.html>
165. Chiarugi, A., E. Meli, and F. Moroni, *Similarities and differences in the neuronal death processes activated by 3OH-kynurenine and quinolinic acid*. J Neurochem, 2001. **77**(5): p. 1310-8.
166. Wei, H., et al., *Neuronal apoptosis induced by pharmacological concentrations of 3-hydroxykynurenine: characterization and protection by dantrolene and Bcl-2 overexpression*. J Neurochem, 2000. **75**(1): p. 81-90.
167. *3-Hydroxyanthranilic acid (CSID:84)*. [cited 2013 31st of October]; Available from: <http://www.chemspider.com/Chemical-Structure.84.html>.
168. Morita, T., et al., *3-Hydroxyanthranilic acid, an L-tryptophan metabolite, induces apoptosis in monocyte-derived cells stimulated by interferon-gamma*. Ann Clin Biochem, 2001. **38**(Pt 3): p. 242-51.

169. Stone, T.W. and L.G. Darlington, *Endogenous kynurenines as targets for drug discovery and development*. Nat Rev Drug Discov, 2002. **1**(8): p. 609-20.
170. *Quinolinic acid (CSID:1037)*. [cited 2013 31st of October]; Available from: <http://www.chemspider.com/Chemical-Structure.1037.html>.
171. Braidy, N., et al., *Mechanism for quinolinic acid cytotoxicity in human astrocytes and neurons*. Neurotox Res, 2009. **16**(1): p. 77-86.
172. Cazareth, J., et al., *Molecular and cellular neuroinflammatory status of mouse brain after systemic lipopolysaccharide challenge: importance of CCR2/CCL2 signaling*. J Neuroinflammation, 2014. **11**: p. 132.
173. Aid, S. and F. Bosetti, *Targeting cyclooxygenases-1 and -2 in neuroinflammation: therapeutic implications*. Biochimie, 2011. **93**(1): p. 46-51.
174. Choi, S.-H., S. Aid, and F. Bosetti, *The distinct roles of cyclooxygenase-1 and -2 in neuroinflammation: implications for translational research*. Trends in Pharmacological Sciences, 2009. **30**(4): p. 174-181.
175. Font-Nieves, M., et al., *Induction of COX-2 enzyme and down-regulation of COX-1 expression by lipopolysaccharide (LPS) control prostaglandin E2 production in astrocytes*. J Biol Chem, 2012. **287**(9): p. 6454-68.
176. Aid, S., et al., *Neuronal overexpression of cyclooxygenase-2 does not alter the neuroinflammatory response during brain innate immune activation*. Neuroscience letters, 2010. **478**(3): p. 113-118.
177. Aid, S., et al., *Cyclooxygenase-1 and-2 differentially modulate lipopolysaccharide-induced blood–brain barrier disruption through matrix metalloproteinase activity*. Journal of Cerebral Blood Flow & Metabolism, 2010. **30**(2): p. 370-380.

178. Aid, S., R. Langenbach, and F. Bosetti, *Neuroinflammatory response to lipopolysaccharide is exacerbated in mice genetically deficient in cyclooxygenase-2*. J Neuroinflammation, 2008. **5**(7).
179. Calvello, R., et al., *Novel selective COX-1 inhibitors suppress neuroinflammatory mediators in LPS-stimulated N13 microglial cells*. Pharmacological Research, 2012. **65**(1): p. 137-148.
180. Hwang, J.H., et al., *Caffeine prevents LPS-induced inflammatory responses in RAW264.7 cells and zebrafish*. Chem Biol Interact, 2016. **248**: p. 1-7.
181. Ullah, F., et al., *Caffeine prevents d-galactose-induced cognitive deficits, oxidative stress, neuroinflammation and neurodegeneration in the adult rat brain*. Neurochem Int, 2015. **90**: p. 114-24.
182. Fiebich, B.L., et al., *Effects of caffeine and paracetamol alone or in combination with acetylsalicylic acid on prostaglandin E(2) synthesis in rat microglial cells*. Neuropharmacology, 2000. **39**(11): p. 2205-13.
183. Khan, A.Q., et al., *Caffeic acid attenuates 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced NF-kappaB and COX-2 expression in mouse skin: abrogation of oxidative stress, inflammatory responses and proinflammatory cytokine production*. Food Chem Toxicol, 2012. **50**(2): p. 175-83.
184. Hirata, A., et al., *Ferulic acid dimer inhibits lipopolysaccharide-stimulated cyclooxygenase-2 expression in macrophages*. In vivo, 2005. **19**(5): p. 849-53.
185. Hwang, S.J., et al., *Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells*. Inflamm Res, 2014. **63**(1): p. 81-90.

186. Shan, J., et al., *Chlorogenic acid inhibits lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 cells through suppressing NF-kappaB and JNK/AP-1 activation*. Int Immunopharmacol, 2009. **9**(9): p. 1042-8.
187. Lin, W.C., Y.F. Peng, and C.W. Hou, *Ferulic acid protects PC12 neurons against hypoxia by inhibiting the p-MAPKs and COX-2 pathways*. Iran J Basic Med Sci, 2015. **18**(5): p. 478-84.
188. Duch, D.S., et al., *Urinary excretion of biopterin and neopterin in psychiatric disorders*. Psychiatry Res, 1984. **11**(2): p. 83-9.
189. Coppen, A., et al., *Depression and tetrahydrobiopterin: the folate connection*. J Affect Disord, 1989. **16**(2-3): p. 103-7.
190. Furukawa, Y., et al., *Significance of CSF total neopterin and biopterin in inflammatory neurological diseases*. J Neurol Sci, 1992. **111**(1): p. 65-72.
191. Gostner, J.M., et al., *Regular consumption of black tea increases circulating kynurenine concentrations: A randomized controlled trial*. BBA Clin, 2015. **3**: p. 31-5.
192. Huber, C., et al., *Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma*. J Exp Med, 1984. **160**(1): p. 310-6.
193. Hyland, K., *Inherited disorders affecting dopamine and serotonin: critical neurotransmitters derived from aromatic amino acids*. J Nutr, 2007. **137**(6 Suppl 1): p. 1568S-1572S; discussion 1573S-1575S.
194. Hoekstra, R., et al., *Effect of electroconvulsive therapy on biopterin and large neutral amino acids in severe, medication-resistant depression*. Psychiatry Res, 2001. **103**(2-3): p. 115-23.

195. Knapp, S. and M. Irwin, *Plasma levels of tetrahydrobiopterin and folate in major depression*. Biol Psychiatry, 1989. **26**(2): p. 156-62.
196. Hashimoto, R., et al., *The plasma tetrahydrobiopterin levels in patients with affective disorders*. Biol Psychiatry, 1990. **28**(6): p. 526-8.
197. Kudryavtseva, N.N., I.V. Bakshtanovskaya, and L.A. Koryakina, *Social model of depression in mice of C57BL/6J strain*. Pharmacol Biochem Behav, 1991. **38**(2): p. 315-20.
198. Sparkman, N.L., et al., *Peripheral lipopolysaccharide administration impairs two-way active avoidance conditioning in C57BL/6J mice*. Physiol Behav, 2005. **85**(3): p. 278-88.
199. Shah, H., *How to calculate sample size in animal studies*. Natl J Physiol Pharm Pharmacol, 2011. **1**(1): p. 35-39.
200. Kaur, R., K. Chopra, and D. Singh, *Role of alpha2 receptors in quercetin-induced behavioral despair in mice*. J Med Food, 2007. **10**(1): p. 165-8.
201. Singal, A., N. Tirkey, and K. Chopra, *Reversal of LPS-induced immobility in mice by green tea polyphenols: possible COX-2 mechanism*. Phytother Res, 2004. **18**(9): p. 723-8.
202. Painsipp, E., et al., *Prolonged depression-like behavior caused by immune challenge: influence of mouse strain and social environment*. PloS one, 2011. **6**(6): p. e20719.
203. Jangra, A., et al., *Protective effect of mangiferin against lipopolysaccharide-induced depressive and anxiety-like behaviour in mice*. Eur J Pharmacol, 2014. **740**: p. 337-45.

204. Aguilar-Valles, A., et al., *Role of brain transmigrating neutrophils in depression-like behavior during systemic infection*. Mol Psychiatry, 2014. **19**(5): p. 599-606.
205. Viana, A.F., et al., *Kinin B1 receptors mediate depression-like behavior response in stressed mice treated with systemic E. coli lipopolysaccharide*. J Neuroinflammation, 2010. **7**(1): p. 98.
206. Bailey KR, C.J., *Anxiety-Related Behaviors in Mice.*, in *Methods of Behavior Analysis in Neuroscience. 2nd edition*, B. JJ, Editor 2009, CRC Press/Taylor & Francis; 2009: Boca Raton (FL).
207. Slattery, D.A. and J.F. Cryan, *Using the rat forced swim test to assess antidepressant-like activity in rodents*. Nat Protoc, 2012. **7**(6): p. 1009-14.
208. Porsolt, R.D., et al., *Behavioural despair in rats: a new model sensitive to antidepressant treatments*. Eur J Pharmacol, 1978. **47**(4): p. 379-91.
209. Castagne, V., et al., *Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice*. Curr Protoc Neurosci, 2011. **Chapter 8**(8.10): p. Unit 8 10A.
210. Cryan, J.F., C. Mombereau, and A. Vassout, *The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice*. Neurosci Biobehav Rev, 2005. **29**(4): p. 571-625.
211. Can, A., et al., *The tail suspension test*. J Vis Exp, 2012(59): p. e3769.
212. Włodarczyk, J. and B. Kierdaszuk, *Interpretation of Fluorescence Decays using a Power-like Model*. Biophysical J, 2003. **85**(1): p. 589-598.
213. Group, I.E.W., *ICH Harmonised Tripartite Guideline*, in *Validation of Analytical Procedures: Text and Methodology Q2(R1)*1994.

214. Lin, C.L., et al., *Urinary free cortisol and cortisone determined by high performance liquid chromatography in the diagnosis of Cushing's syndrome*. J Clin Endocrinol Metab, 1997. **82**(1): p. 151-5.
215. Agilli, M., et al., *Comparison of two different HPLC methods and elisa method for measurement of serum neopterin*. J Invest Biochem 2012. **1**(1): p. 43-47.
216. Vrecko, K., et al., *Effect of Interferon- γ and LPS on Tetrahydrobiopterin in Rat PC 12 and Human KNA Cells*. Pteridines, 2001. **12**(4): p. 167-171.
217. Singh, D., et al., *Antioxidants in the prevention of renal disease*. J Med Food, 2006. **9**(4): p. 443-50.
218. Inoue, S., T. Tokuyama, and K. Takai, *Picomole analyses of tryptophan by derivatization to 9-hydroxymethyl-beta-carboline*. Anal Biochem, 1983. **132**(2): p. 468-80.
219. Koch, J.P., et al., *Validation of a high-performance liquid chromatography assay for quantification of caffeine and paraxanthine in human serum in the context of CYP1A2 phenotyping*. Biomed Chromatogr, 1999. **13**(4): p. 309-14.
220. Franklin, A.E., et al., *Lipopolysaccharide-induced hypoactivity and behavioral tolerance development are modulated by the light-dark cycle in male and female rats*. Psychopharmacology (Berl), 2003. **170**(4): p. 399-408.
221. De La Garza, R., 2nd, et al., *Acute diclofenac treatment attenuates lipopolysaccharide-induced alterations to basic reward behavior and HPA axis activation in rats*. Psychopharmacology (Berl), 2005. **179**(2): p. 356-65.
222. Mello, B.S., et al., *Effects of doxycycline on depressive-like behavior in mice after lipopolysaccharide (LPS) administration*. J Psychiatr Res, 2013. **47**(10): p. 1521-9.

223. Walsh, R.N. and R.A. Cummins, *The Open-Field Test: a critical review*. Psychol Bull, 1976. **83**(3): p. 482-504.
224. Prut, L. and C. Belzung, *The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review*. Eur J Pharmacol 2003. **463**(1): p. 3-33.
225. Muller, L.G., et al., *Diene Valepotriates from Valeriana glechomifolia Prevent Lipopolysaccharide-Induced Sickness and Depressive-Like Behavior in Mice*. Evid Based Complement Alternat Med, 2015. **2015**: p. 145914.
226. Finn, I.B. and S.G. Holtzman, *Tolerance to caffeine-induced stimulation of locomotor activity in rats*. J Pharmacol Exp Ther, 1986. **238**(2): p. 542-6.
227. Holtzman, S.G. and I.B. Finn, *Tolerance to behavioral effects of caffeine in rats*. Pharmacol Biochem Behav, 1988. **29**(2): p. 411-8.
228. Teixeira, R.C., H. Zangrossi, and F.G. Graeff, *Behavioral effects of acute and chronic imipramine in the elevated T-maze model of anxiety*. Pharmacol Biochem Behav, 2000. **65**(4): p. 571-6.
229. Wichers, M.C. and M. Maes, *The role of indoleamine 2,3-dioxygenase (IDO) in the pathophysiology of interferon-alpha-induced depression*. J Psychiatry Neurosci, 2004. **29**(1): p. 11-7.
230. Wichers, M.C., et al., *IDO and interferon-alpha-induced depressive symptoms: a shift in hypothesis from tryptophan depletion to neurotoxicity*. Mol Psychiatry, 2005. **10**(6): p. 538-44.
231. Campbell, B.M., et al., *Kynurenines in CNS disease: regulation by inflammatory cytokines*. Front Neurosci, 2014. **8**: p. 12.
232. Henry, C.J., et al., *Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated*

- induction of both pro-inflammatory IL-1 β and anti-inflammatory IL-10 cytokines.* Brain Behav Immun, 2009. **23**(3): p. 309-317.
233. Dobos, N., et al., *The role of indoleamine 2,3-dioxygenase in a mouse model of neuroinflammation-induced depression.* J Alzheimers Dis, 2012. **28**(4): p. 905-15.
 234. Lestage, J., et al., *The enzyme indoleamine 2, 3-dioxygenase is induced in the mouse brain in response to peripheral administration of lipopolysaccharide and superantigen.* Brain Behav Immun, 2002. **16**(5): p. 596-601.
 235. Jung, I.D., et al., *Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells.* FEBS Lett, 2007. **581**(7): p. 1449-56.
 236. Farzi, A., et al., *Synergistic effects of NOD1 or NOD2 and TLR4 activation on mouse sickness behavior in relation to immune and brain activity markers.* Brain Behav Immun, 2015. **44**: p. 106-20.
 237. Uematsu, S., et al., *Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway.* J Immunol, 2002. **168**(11): p. 5811-6.
 238. Wang, T., et al., *Role of reactive oxygen species in LPS-induced production of prostaglandin E2 in microglia.* J Neurochem, 2004. **88**(4): p. 939-47.
 239. Dowlati, Y., et al., *A meta-analysis of cytokines in major depression.* Biol Psychiatry, 2010. **67**(5): p. 446-57.
 240. Weiss, F., et al., *Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration.* Brain Res, 1992. **593**(2): p. 314-8.

241. Fredholm, B.B., et al., *Actions of Caffeine in the Brain with Special Reference to Factors That Contribute to Its Widespread Use*. Pharmacol Rev, 1999. **51**(1): p. 83-133.
242. Lelo, A., et al., *Comparative pharmacokinetics of caffeine and its primary demethylated metabolites paraxanthine, theobromine and theophylline in man*. Br J Clin Pharmacol, 1986. **22**(2): p. 177-82.
243. Rastogi, H. and S. Jana, *Evaluation of inhibitory effects of caffeic acid and quercetin on human liver cytochrome p450 activities*. Phytother Res, 2014. **28**(12): p. 1873-8.
244. Chan, W.-S., P.-C. Wen, and H.-C. Chiang, *Structure-activity relationship of caffeic acid analogues on xanthine oxidase inhibition*. Anticancer Res 1994. **15**(3): p. 703-707.
245. Masuda, T., et al., *Identification of a potent xanthine oxidase inhibitor from oxidation of caffeic acid*. Free Radic Biol Med, 2014. **69**: p. 300-7.
246. Reus, G.Z., et al., *Harmin and imipramine promote antioxidant activities in prefrontal cortex and hippocampus*. Oxid Med Cell Longev, 2010. **3**(5): p. 325-31.
247. Xu, H., J. Steven Richardson, and X.M. Li, *Dose-related effects of chronic antidepressants on neuroprotective proteins BDNF, Bcl-2 and Cu/Zn-SOD in rat hippocampus*. Neuropsychopharmacology, 2003. **28**(1): p. 53-62.
248. Takeda, H., et al., *Caffeic acid produces antidepressive- and/or anxiolytic-like effects through indirect modulation of the alpha 1A-adrenoceptor system in mice*. Neuroreport, 2003. **14**(7): p. 1067-70.
249. Farah, A., et al., *Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees*. J Agric Food Chem, 2006. **54**(2): p. 374-81.

250. Ramalakshmi, K. and B. Raghavan, *Caffeine in coffee: its removal. Why and how?* Crit Rev Food Sci Nutr, 1999. **39**(5): p. 441-56.
251. Australia New Zealand Food Standards Code - Standard 1.1.2 - Supplementary Definitions for Foods 2011 [cited 2015 08/12/2015]; Available from: <https://www.comlaw.gov.au/Details/F2011C00530>.
252. *Manual of Methods of Analysis of Foods: Beverages (Coffee, Tea, Cocoa, Chicory), Sugar and Sugar Products & Confectionary Products*. 2012 [cited 2015 08/12/2015]; Available from: <http://www.fssai.gov.in/Portals/0/Pdf/15Manuals/BEVERAGES,%20SUGARS%20&%20CONFECTIONERY.pdf>.
253. Narita, Y. and K. Inouye, *Degradation kinetics of chlorogenic acid at various pH values and effects of ascorbic acid and epigallocatechin gallate on its stability under alkaline conditions*. J Agric Food Chem, 2013. **61**(4): p. 966-972.
254. Sato, Y., et al., *In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid*. Int J Pharm, 2011. **403**(1-2): p. 136-8.
255. Alonso-Salces, R.M., et al., *Botanical and geographical characterization of green coffee (Coffea arabica and Coffea canephora): chemometric evaluation of phenolic and methylxanthine contents*. J Agric Food Chem, 2009. **57**(10): p. 4224-35.
256. Fujioka, K. and T. Shibamoto, *Chlorogenic acid and caffeine contents in various commercial brewed coffees*. Food Chem 2008. **106**(1): p. 217-221.
257. Ky, C.-L., et al., *Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild Coffea arabica L. and C. canephora P. accessions*. Food Chem, 2001. **75**(2): p. 223-230.

258. Tfouni, S.A.V., et al., *Effect of roasting on chlorogenic acids, caffeine and polycyclic aromatic hydrocarbons levels in two Coffea cultivars: Coffea arabica cv. Catuaí Amarelo IAC-62 and Coffea canephora cv. Apoatã IAC-2258*. Int J Food Sci Technol, 2012. **47**(2): p. 406-415.
259. Moon, J.K., H.S. Yoo, and T. Shibamoto, *Role of roasting conditions in the level of chlorogenic acid content in coffee beans: correlation with coffee acidity*. J Agric Food Chem, 2009. **57**(12): p. 5365-9.
260. Farah, A., et al., *Effect of roasting on the formation of chlorogenic acid lactones in coffee*. J Agric Food Chem, 2005. **53**(5): p. 1505-13.
261. Joët, T., et al., *Influence of environmental factors, wet processing and their interactions on the biochemical composition of green Arabica coffee beans*. Food Chem, 2010. **118**(3): p. 693-701.
262. Decazy, F., et al., *Quality of different Honduran coffees in relation to several environments*. J Food Sci, 2003. **68**(7): p. 2356-2361.
263. Mills, C.E., et al., *The effect of processing on chlorogenic acid content of commercially available coffee*. Food Chem, 2013. **141**(4): p. 3335-40.
264. Streit, W.J., *Microglia as neuroprotective, immunocompetent cells of the CNS*. Glia, 2002. **40**(2): p. 133-139.
265. Rajkowska, G. and J.J. Miguel-Hidalgo, *Gliogenesis and glial pathology in depression*. CNS Neurol Disord Drug Targets, 2007. **6**(3): p. 219-33.
266. Yirmiya, R., N. Rimmerman, and R. Reshef, *Depression as a microglial disease*. Trends Neurosci, 2015. **38**(10): p. 637-58.
267. Chanput, W., J.J. Mes, and H.J. Wichers, *THP-1 cell line: an in vitro cell model for immune modulation approach*. Int Immunopharmacol, 2014. **23**(1): p. 37-45.

268. Schwende, H., et al., *Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3*. J Leukoc Biol, 1996. **59**(4): p. 555-61.
269. Klegeris, A., C.J. Bissonnette, and P.L. McGeer, *Modulation of human microglia and THP-1 cell toxicity by cytokines endogenous to the nervous system*. Neurobiol Aging, 2005. **26**(5): p. 673-82.
270. Klegeris, A. and P.L. McGeer, *Toxicity of human monocytic THP-1 cells and microglia toward SH-SY5Y neuroblastoma cells is reduced by inhibitors of 5-lipoxygenase and its activating protein FLAP*. J Leukoc Biol, 2003. **73**(3): p. 369-78.
271. Daigneault, M., et al., *The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages*. PloS one, 2010. **5**(1): p. e8668.
272. Chen, Q. and A.C. Ross, *Retinoic acid regulates cell cycle progression and cell differentiation in human monocytic THP-1 cells*. Exp Cell Res, 2004. **297**(1): p. 68-81.
273. Vey, E., J.H. Zhang, and J.M. Dayer, *IFN-gamma and 1,25(OH)2D3 induce on THP-1 cells distinct patterns of cell surface antigen expression, cytokine production, and responsiveness to contact with activated T cells*. J Immunol, 1992. **149**(6): p. 2040-6.
274. Taylor, M.W. and G. Feng, *Relationship between interferon-gamma, indoleamine 2, 3-dioxygenase, and tryptophan catabolism*. FASEB J, 1991. **5**(11): p. 2516-2522.

275. Olivieri, G., et al., *Mercury induces cell cytotoxicity and oxidative stress and increases beta-amyloid secretion and tau phosphorylation in SHSY5Y neuroblastoma cells*. J Neurochem, 2000. **74**(1): p. 231-6.
276. Olivieri, G., et al., *N-acetyl-L-cysteine protects SHSY5Y neuroblastoma cells from oxidative stress and cell cytotoxicity: effects on beta-amyloid secretion and tau phosphorylation*. J Neurochem, 2001. **76**(1): p. 224-33.
277. Storch, A., et al., *6-Hydroxydopamine toxicity towards human SH-SY5Y dopaminergic neuroblastoma cells: independent of mitochondrial energy metabolism*. J Neural Transm (Vienna), 2000. **107**(3): p. 281-93.
278. Riganti, L., et al., *Long-term exposure to the new nicotinic antagonist 1,2-bisN-cytisinylethane upregulates nicotinic receptor subtypes of SH-SY5Y human neuroblastoma cells*. Br J Pharmacol, 2005. **146**(8): p. 1096-109.
279. Naarala, J., et al., *Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells*. FEBS Lett, 1993. **330**(2): p. 222-6.
280. Lukas, R.J., S.A. Norman, and L. Lucero, *Characterization of Nicotinic Acetylcholine Receptors Expressed by Cells of the SH-SY5Y Human Neuroblastoma Clonal Line*. Mol Cell Neurosci, 1993. **4**(1): p. 1-12.
281. Guillemin, G.J., et al., *Characterization of the kynurenine pathway in human neurons*. J Neurosci, 2007. **27**(47): p. 12884-92.
282. Jamsa, A., et al., *The retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cell line as a model for Alzheimer's disease-like tau phosphorylation*. Biochem Biophys Res Commun, 2004. **319**(3): p. 993-1000.
283. Encinas, M., et al., *Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic*

- factor-dependent, human neuron-like cells. J Neurochem, 2000. 75(3): p. 991-1003.*
284. Kume, T., et al., *Dibutyl cAMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. Neurosci lett, 2008. 443(3): p. 199-203.*
 285. Thornton, P., et al., *Interleukin-1-induced neurotoxicity is mediated by glia and requires caspase activation and free radical release. J Neurochem, 2006. 98(1): p. 258-66.*
 286. Petty, M.A. and E.H. Lo, *Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. Prog Neurobiol, 2002. 68(5): p. 311-23.*
 287. Xiao, B.-G. and H. Link, *Is there a balance between microglia and astrocytes in regulating Th1/Th2-cell responses and neuropathologies? Immunol Today, 1999. 20(11): p. 477-479.*
 288. Najjar, S., et al., *Neuroinflammation and psychiatric illness. J Neuroinflammation, 2013. 10: p. 43.*
 289. Guillemin, G.J., et al., *IFN-beta1b induces kynurenine pathway metabolism in human macrophages: potential implications for multiple sclerosis treatment. J Interferon Cytokine Res, 2001. 21(12): p. 1097-101.*
 290. Alberati-Giani, D. and A.M. Cesura, *Expression of the kynurenine enzymes in macrophages and microglial cells: regulation by immune modulators. Amino acids, 1998. 14(1-3): p. 251-5.*
 291. Renauld, J.-C., *Class II cytokine receptors and their ligands: Key antiviral and inflammatory modulators. Nat Rev Immunol, 2003. 3(8): p. 667-676.*

292. Runkel, L., *Differences in Activity between alpha and beta Type I Interferons Explored by Mutational Analysis*. Journal of Biological Chemistry, 1998. **273**(14): p. 8003-8008.
293. Kumaran, J., et al., *A structural basis for interferon-alpha-receptor interactions*. FASEB J, 2007. **21**(12): p. 3288-96.
294. Jaitin, D.A., et al., *Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta*. Mol Cell Biol, 2006. **26**(5): p. 1888-97.
295. Walter, M.R. and T.L. Nagabhushan, *Crystal structure of interleukin 10 reveals an interferon gamma-like fold*. Biochemistry, 1995. **34**(38): p. 12118-25.
296. Sano, C., et al., *The modulating effects of proinflammatory cytokines interferon-gamma (IFN-gamma) and tumour necrosis factor-alpha (TNF-alpha), and immunoregulating cytokines IL-10 and transforming growth factor-beta (TGF-beta), on anti-microbial activity of murine peritoneal macrophages against Mycobacterium avium-intracellulare complex*. Clin Exp Immunol, 1999. **115**(3): p. 435-42.
297. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions*. J Leukoc Biol, 2004. **75**(2): p. 163-89.
298. Trinchieri, G., *Type I interferon: friend or foe?* J Exp Med, 2010. **207**(10): p. 2053-63.
299. Haller, O., G. Kochs, and F. Weber, *The interferon response circuit: induction and suppression by pathogenic viruses*. Virology, 2006. **344**(1): p. 119-30.
300. Talpaz, M., J. Mercer, and R. Hehlmann, *The interferon-alpha revival in CML*. Ann Hematol, 2015. **94 Suppl 2**: p. S195-207.

301. Tsaur, I., et al., *Intensified antineoplastic effect by combining an HDAC-inhibitor, an mTOR-inhibitor and low dosed interferon alpha in prostate cancer cells*. J Cell Mol Med, 2015. **19**(8): p. 1795-804.
302. Herold, M., et al., *Long-term follow-up of rituximab plus first-line mitoxantrone, chlorambucil, prednisolone and interferon-alpha as maintenance therapy in follicular lymphoma*. J Cancer Res Clin Oncol, 2015. **141**(9): p. 1689-95.
303. Conversano, C., et al., *Interferon alpha Therapy in Patients with Chronic Hepatitis C Infection: Quality of Life and Depression*. Hematol Rep, 2015. **7**(1): p. 5632.
304. Jiao, Y.M., et al., *Hepatitis C therapy with interferon-alpha and ribavirin reduces the CD4 cell count and the total, 2LTR circular and integrated HIV-1 DNA in HIV/HCV co-infected patients*. Antiviral Res, 2015. **118**: p. 118-22.
305. Manns, M.P., et al., *Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial*. Lancet, 2001. **358**(9286): p. 958-65.
306. Jaeckel, E., et al., *Treatment of acute hepatitis C with interferon alfa-2b*. N Engl J Med, 2001. **345**(20): p. 1452-7.
307. Reuter, K., et al., *Health-related quality of life, fatigue, and depression under low-dose IFN-alpha therapy in melanoma patients*. J Immunother, 2014. **37**(9): p. 461-7.
308. Petersen, T., et al., *Effects of interferon-beta therapy on innate and adaptive immune responses to the human endogenous retroviruses HERV-H and HERV-W, cytokine production, and the lectin complement activation pathway in multiple sclerosis*. J Neuroimmunol, 2009. **215**(1-2): p. 108-16.

309. Heim, M.H. and R. Thimme, *Innate and adaptive immune responses in HCV infections*. J Hepatol, 2014. **61**(1 Suppl): p. S14-25.
310. Vilcek, J., *Novel interferons*. Nat Immunol, 2003. **4**(1): p. 8-9.
311. Leung, S., et al., *Role of STAT2 in the alpha interferon signaling pathway*. Mol Cell Biol, 1995. **15**(3): p. 1312-7.
312. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark, *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins*. Science, 1994. **264**(5164): p. 1415-21.
313. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling*. Nat Rev Immunol, 2005. **5**(5): p. 375-86.
314. Hardy, M.P., et al., *Characterization of the type I interferon locus and identification of novel genes*. Genomics, 2004. **84**(2): p. 331-45.
315. Perry, A.K., et al., *The host type I interferon response to viral and bacterial infections*. Cell Res, 2005. **15**(6): p. 407-22.
316. de Weerd, N.A., S.A. Samarajiwa, and P.J. Hertzog, *Type I interferon receptors: biochemistry and biological functions*. J Biol Chem, 2007. **282**(28): p. 20053-7.
317. Jaks, E., et al., *Differential receptor subunit affinities of type I interferons govern differential signal activation*. J Mol Biol, 2007. **366**(2): p. 525-39.
318. Lavoie, T.B., et al., *Binding and activity of all human alpha interferon subtypes*. Cytokine, 2011. **56**(2): p. 282-9.
319. Cull, V.S., et al., *Type I interferon differential therapy for erythroleukemia: specificity of STAT activation*. Blood, 2003. **101**(7): p. 2727-35.
320. Patten, S.B. and C. Barbui, *Drug-induced depression: a systematic review to inform clinical practice*. Psychother Psychosom, 2004. **73**(4): p. 207-15.

321. Dorr, R.T., *Interferon-alpha in malignant and viral diseases. A review.* Drugs, 1993. **45**(2): p. 177-211.
322. Brassard, D.L., M.J. Grace, and R.W. Borden, *Interferon- α as an immunotherapeutic protein.* J Leukoc Biol, 2002. **71**(4): p. 565-581.
323. Tough, D.F., *Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation.* Leuk Lymphoma, 2004. **45**(2): p. 257-64.
324. Harris, P.E., et al., *Effect of interferon alpha on MHC class II gene expression in ex vivo human islet tissue.* Biochim Biophys Acta, 2006. **1762**(6): p. 627-35.
325. Taylor, J.L. and S.E. Grossberg, *The effects of interferon-alpha on the production and action of other cytokines.* Semin Oncol, 1998. **25**(1 Suppl 1): p. 23-9.
326. Poynard, T., et al., *Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT).* Lancet, 1998. **352**(9138): p. 1426-32.
327. Raison, C., *The effects of hepatitis C and its treatment on mental health.* Focus, 2006. **21**(5): p. 4-6.
328. Hayley, S., J. Scharf, and H. Anisman, *Central administration of murine interferon-alpha induces depressive-like behavioral, brain cytokine and neurochemical alterations in mice: a mini-review and original experiments.* Brain Behav Immun, 2013. **31**: p. 115-27.
329. Kessing, C.F. and W.R. Tyor, *Interferon-alpha induces neurotoxicity through activation of the type I receptor and the GluN2A subunit of the NMDA receptor.* J Interferon Cytokine Res, 2015. **35**(4): p. 317-24.

330. Fritz-French, C. and W. Tyor, *Interferon-alpha (IFNalpha) neurotoxicity*. Cytokine Growth Factor Rev, 2012. **23**(1-2): p. 7-14.
331. Hinkerohe, D., et al., *Effects of cytokines on microglial phenotypes and astroglial coupling in an inflammatory coculture model*. Glia, 2005. **52**(2): p. 85-97.
332. Genin, P., A. Vaccaro, and A. Civas, *The role of differential expression of human interferon- α genes in antiviral immunity*. Cytokine Growth Factor Rev, 2009. **20**(4): p. 283-95.
333. Sellner, J., et al., *Effect of interferon-beta and atorvastatin on Th1/Th2 cytokines in multiple sclerosis*. Neurochem Int, 2008. **53**(1-2): p. 17-21.
334. Tao, Y., et al., *The role of endogenous IFN-beta in the regulation of Th17 responses in patients with relapsing-remitting multiple sclerosis*. J Immunol, 2014. **192**(12): p. 5610-7.
335. Kieseier, B.C. and O. Stuve, *Multiple sclerosis: combination therapy in MS--still a valid strategy*. Nat Rev Neurol, 2011. **7**(12): p. 659-60.
336. Hissong, B.D., et al., *Upregulation of interferon-induced indoleamine 2,3-dioxygenase in human macrophage cultures by lipopolysaccharide, muramyl tripeptide, and interleukin-1*. Cell Immunol, 1995. **160**(2): p. 264-9.
337. Dedoni, S., M.C. Olanas, and P. Onali, *Interferon-beta induces apoptosis in human SH-SY5Y neuroblastoma cells through activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway*. J Neurochem, 2010. **115**(6): p. 1421-33.
338. Huang, S.-i., et al., *Immune response in mice that lack the interferon-gamma receptor*. Science, 1993. **259**(5102): p. 1742-1745.

339. Goodbourn, S., L. Didcock, and R.E. Randall, *Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures*. J Gen Virol, 2000. **81**(Pt 10): p. 2341-64.
340. Dalton, D.K., et al., *Multiple defects of immune cell function in mice with disrupted interferon-gamma genes*. Science, 1993. **259**(5102): p. 1739-42.
341. Wirleitner, B., et al., *Interferon-gamma-induced conversion of tryptophan: immunologic and neuropsychiatric aspects*. Curr Med Chem, 2003. **10**(16): p. 1581-91.
342. Mizuno, T., et al., *Interferon-gamma directly induces neurotoxicity through a neuron specific, calcium-permeable complex of IFN-gamma receptor and AMPA GluR1 receptor*. FASEB J, 2008. **22**(6): p. 1797-806.
343. Fujigaki, H., et al., *The signal transducer and activator of transcription 1a and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide : Involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB pathways, and synergistic effect of several proinflammatory cytokines*. J Biochem, 2006. **139**(4): p. 8.
344. Zhou, Z., et al., *Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases*. J Virol, 2007. **81**(14): p. 7749-58.
345. Ank, N., et al., *Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo*. J Virol, 2006. **80**(9): p. 4501-9.

346. Diegelmann, J., et al., *Comparative analysis of the lambda-interferons IL-28A and IL-29 regarding their transcriptome and their antiviral properties against hepatitis C virus*. PloS one, 2010. **5**(12): p. e15200.
347. Dolganiuc, A., et al., *Type III interferons, IL-28 and IL-29, are increased in chronic HCV infection and induce myeloid dendritic cell-mediated FoxP3+ regulatory T cells*. PloS one, 2012. **7**(10): p. e44915.
348. Kotenko, S.V., *IFN-lambdas*. Curr Opin Immunol, 2011. **23**(5): p. 583-90.
349. Dumoutier, L., et al., *Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling*. J Biol Chem, 2004. **279**(31): p. 32269-74.
350. Dumoutier, L., et al., *Cloning of a new type II cytokine receptor activating signal transducer and activator of transcription (STAT)1, STAT2 and STAT3*. Biochem J, 2003. **370**(Pt 2): p. 391-6.
351. Kupfer, D.J., *The pharmacological management of depression*. Dialogues Clin Neurosci, 2005. **7**(3): p. 191-205.
352. Malhi, G.S., et al., *Pharmacological management of unipolar depression*. Acta Psychiatr Scand Suppl, 2013. **127**(443): p. 6-23.
353. Kenis, G. and M. Maes, *Effects of antidepressants on the production of cytokines*. Int J Neuropsychopharmacol, 2002. **5**(4): p. 401-12.
354. Anderson, I.M., *Selective serotonin reuptake inhibitors versus tricyclic antidepressants: a meta-analysis of efficacy and tolerability*. J Affect Disord, 2000. **58**(1): p. 19-36.
355. Gillman, P.K., *Tricyclic antidepressant pharmacology and therapeutic drug interactions updated*. Br J Pharmacol, 2007. **151**(6): p. 737-48.

356. Obuchowicz, E., et al., *Amitriptyline and nortriptyline inhibit interleukin-1 release by rat mixed glial and microglial cell cultures*. Int J Neuropsychopharmacol, 2006. **9**(1): p. 27-35.
357. Hwang, J., et al., *Inhibition of glial inflammatory activation and neurotoxicity by tricyclic antidepressants*. Neuropharmacology, 2008. **55**(5): p. 826-34.
358. Obuchowicz, E., et al., *Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture*. Pharmacol Rep, 2014. **66**(1): p. 34-43.
359. Hashioka, S., et al., *Antidepressants inhibit interferon-gamma-induced microglial production of IL-6 and nitric oxide*. Exp Neurol, 2007. **206**(1): p. 33-42.
360. Hashioka, S., et al., *Anti-Inflammatory Effects of Antidepressants : Possibilities for Preventives Against Alzheimer ' s Disease*. Cent Nerv Syst Agents Med Chem, 2009. **9**(1): p. 12-19.
361. Kaster, M.P., et al., *Depressive-like behavior induced by tumor necrosis factor- α in mice*. Neuropharmacology, 2012. **62**(1): p. 419-26.
362. Nutt, D.J., et al., *Mechanisms of action of selective serotonin reuptake inhibitors in the treatment of psychiatric disorders*. Eur Neuropsychopharmacol, 1999. **9 Suppl 3**(0): p. S81-6.
363. Trivedi, M.H., et al., *Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice*. Am J Psychiatry, 2006. **163**(1): p. 28-40.
364. Papakostas, G.I., M. Fava, and M.E. Thase, *Treatment of SSRI-resistant depression: a meta-analysis comparing within- versus across-class switches*. Biol Psychiatry, 2008. **63**(7): p. 699-704.

365. Vollmar, P., et al., *Venlafaxine exhibits an anti-inflammatory effect in an inflammatory co-culture model*. Int J Neuropsychopharmacol, 2008. **11**(1): p. 111-7.
366. Schloss, P. and F.A. Henn, *New insights into the mechanisms of antidepressant therapy*. Pharmacol Ther, 2004. **102**(1): p. 47-60.
367. Nair, N.P., S.K. Ahmed, and N.M. Kin, *Biochemistry and pharmacology of reversible inhibitors of MAO-A agents: focus on moclobemide*. J Psychiatry Neurosci, 1993. **18**(5): p. 214-25.
368. Molteni, R., et al., *Modulation of the inflammatory response in rats chronically treated with the antidepressant agomelatine*. Eur Neuropsychopharmacol, 2013. **23**(11): p. 1645-55.
369. Kocki, T., et al., *New insight into the antidepressants action: modulation of kynurenine pathway by increasing the kynurenic acid/3-hydroxykynurenine ratio*. J Neural Transm (Vienna), 2012. **119**(2): p. 235-43.
370. Wood, K., J. Harwood, and A. Coppen, *The effect of antidepressant drugs on plasma kynurenine in depressed patients*. Psychopharmacology (Berl), 1978. **59**(3): p. 263-6.
371. Kolla, N., et al., *Amitriptyline and fluoxetine protect PC12 cells from cell death induced by hydrogen peroxide*. J Psychiatry Neurosci, 2005. **30**(3): p. 196-201.
372. Schmidt, A.J., et al., *Effects of antidepressants on mRNA levels of antioxidant enzymes in human monocytic U-937 cells*. Prog Neuropsychopharmacol Biol Psychiatry, 2008. **32**(6): p. 1567-73.
373. Krass, M., et al., *The antidepressant action of imipramine and venlafaxine involves suppression of nitric oxide synthesis*. Behav Brain Res, 2011. **218**(1): p. 57-63.

374. Abdel-Wahab, B.A. and R.H. Salama, *Venlafaxine protects against stress-induced oxidative DNA damage in hippocampus during antidepressant testing in mice*. Pharmacol Biochem Behav, 2011. **100**(1): p. 59-65.
375. Eren, I., M. Naziroglu, and A. Demirdas, *Protective effects of lamotrigine, aripiprazole and escitalopram on depression-induced oxidative stress in rat brain*. Neurochem Res, 2007. **32**(7): p. 1188-95.
376. Inkielewicz-Stepniak, I., *Impact of fluoxetine on liver damage in rats*. Pharmacol Rep, 2011. **63**(2): p. 441-7.
377. Zafir, A., A. Ara, and N. Banu, *Invivo antioxidant status: a putative target of antidepressant action*. Prog Neuropsychopharmacol Biol Psychiatry, 2009. **33**(2): p. 220-8.
378. Djordjevic, J., et al., *Fluoxetine affects antioxidant system and promotes apoptotic signaling in Wistar rat liver*. Eur J Pharmacol, 2011. **659**(1): p. 61-6.
379. McFarland, A., *Optimization of a PMA differentiation protocol for THP-1 human monocytic cells.*, 2015, Griffith University: Australia.
380. Anoopkumar-Dukie, S., et al., *Resazurin assay of radiation response in cultured cells*. Br J Radiol, 2005. **78**(934): p. 945-7.
381. Anoopkumar-Dukie, S., et al., *Further evidence for biological effects resulting from ionizing radiation doses in the diagnostic X-ray range*. Br J Radiol, 2005. **78**(928): p. 335-7.
382. Anoopkumar-Dukie, S., et al., *Radical mediators and mitogen-activated protein kinase signaling in oxygen-dependent radiosensitivity of human tumor cell lines*. Free Radic Biol Med, 2005. **39**(2): p. 188-94.
383. Uhe, A.M., et al., *Quantitation of tryptophan and other plasma amino acids by automated pre-column o-phthalaldehyde derivatization high-performance*

- liquid chromatography: improved sample preparation. J Chromatogr B Biomed Sci Appl*, 1991. **564**(1): p. 81-91.
384. Funeshima, N., et al., *Inhibition of allogeneic T-cell responses by dendritic cells expressing transduced indoleamine 2,3-dioxygenase. J Gene Med*, 2005. **7**(5): p. 565-75.
 385. Kanai, M., et al., *Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. Mol Brain*, 2009. **2**: p. 8.
 386. Haslam, G., D. Wyatt, and P.A. Kitos, *Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. Cytotechnology*, 2000. **32**(1): p. 63-75.
 387. Wolterbeek, H.T. and A.J. van der Meer, *Optimization, application, and interpretation of lactate dehydrogenase measurements in microwell determination of cell number and toxicity. Assay Drug Dev Technol*, 2005. **3**(6): p. 675-82.
 388. van Engeland, M., et al., *Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry*, 1998. **31**(1): p. 1-9.
 389. Elmore, S., *Apoptosis: a review of programmed cell death. Toxicol Pathol*, 2007. **35**(4): p. 495-516.
 390. Ozaki, Y., M.P. Edelstein, and D.S. Duch, *Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon gamma. Proc Natl Acad Sci U S A*, 1988. **85**(4): p. 1242-6.
 391. Hemmi, S., et al., *Cloning of murine interferon gamma receptor cDNA: expression in human cells mediates high-affinity binding but is not sufficient to*

- confer sensitivity to murine interferon gamma*. Proc Natl Acad Sci U S A, 1989. **86**(24): p. 9901-5.
392. Sanceau, J., et al., *IL-6 and IL-6 receptor modulation by IFN-gamma and tumor necrosis factor-alpha in human monocytic cell line (THP-1). Priming effect of IFN-gamma*. J Immunol, 1991. **147**(8): p. 2630-7.
 393. Heyes, M.P., et al., *Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types*. Biochem J, 1997. **326 (Pt 2)**: p. 351-6.
 394. Wszelaki, N. and M.F. Melzig, *Research on an in vitro cell system for testing the neurotoxicity of kynurenine pathway metabolites*. Pharmazie, 2011. **66**(11): p. 899-903.
 395. Okuda, S., et al., *3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity*. J Neurochem, 1998. **70**(1): p. 299-307.
 396. Morita, T., et al., *L-tryptophan-kynurenine pathway metabolite 3-hydroxyanthranilic acid induces apoptosis in macrophage-derived cells under pathophysiological conditions*. Adv Exp Med Biol, 1999. **467**: p. 559-63.
 397. Kannan, K. and S.K. Jain, *Oxidative stress and apoptosis*. Pathophysiology, 2000. **7**(3): p. 153-163.
 398. Munn, D.H. and A.L. Mellor, *Indoleamine 2,3-dioxygenase and tumor-induced tolerance*. J Clin Invest, 2007. **117**(5): p. 1147-54.
 399. Allan, S.M., P.J. Tyrrell, and N.J. Rothwell, *Interleukin-1 and neuronal injury*. Nat Rev Immunol, 2005. **5**(8): p. 629-40.
 400. Carrasco, E., D. Casper, and P. Werner, *PGE2 receptor EP1 renders dopaminergic neurons selectively vulnerable to low - level oxidative stress and direct PGE2 neurotoxicity*. J Neurosci Res, 2007. **85**(14): p. 3109-3117.

401. Barger, S.W., et al., *Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca²⁺ accumulation*. Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9328-32.
402. Lirk, P., et al., *The neurotoxic effects of amitriptyline are mediated by apoptosis and are effectively blocked by inhibition of caspase activity*. Anesth Analg, 2006. **102**(6): p. 1728-33.
403. Bartholomä, P., et al., *Neuronal cell death induced by antidepressants: lack of correlation with Egr-1, NF-κB and extracellular signal-regulated protein kinase activation*. Biochem Pharmacol, 2002. **63**(8): p. 1507-1516.
404. Abbasi, S.H., et al., *Effect of celecoxib add-on treatment on symptoms and serum IL-6 concentrations in patients with major depressive disorder: randomized double-blind placebo-controlled study*. J Affect Disord, 2012. **141**(2-3): p. 308-14.
405. Jiang, H., et al., *Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis*. Hum Mol Genet, 2004. **13**(16): p. 1745-54.
406. Green, D.R. and J.C. Reed, *Mitochondria and apoptosis*. Science 1998. **281**(5381): p. 1309-12.
407. Budihardjo, I., et al., *Biochemical pathways of caspase activation during apoptosis*. Annu Rev Cell Dev Biol, 1999. **15**: p. 269-90.
408. Wang, Z.Q., J. Liao, and Z. Diwu, *N-DEVD-N'-morpholinecarbonyl-rhodamine 110: novel caspase-3 fluorogenic substrates for cell-based apoptosis assay*. Bioorg Med Chem Lett, 2005. **15**(9): p. 2335-8.

409. Liang, N. and D.D. Kitts, *Antioxidant property of coffee components: assessment of methods that define mechanisms of action*. *Molecules*, 2014. **19**(11): p. 19180-19208.
410. Grohmann, U., et al., *CTLA-4-Ig regulates tryptophan catabolism in vivo*. *Nat Immunol*, 2002. **3**(11): p. 1097-101.
411. Tsai, S.J., C.Y. Chao, and M.C. Yin, *Preventive and therapeutic effects of caffeic acid against inflammatory injury in striatum of MPTP-treated mice*. *Eur J Pharmacol*, 2011. **670**(2-3): p. 441-7.
412. Lampiasi, N. and G. Montana, *The molecular events behind ferulic acid mediated modulation of IL-6 expression in LPS-activated Raw 264.7 cells*. *Immunobiology*, 2016. **221**(3): p. 486-93.
413. Wang, W. and E. Alpert, *Downregulation of phorbol 12 - myristate 13 - acetate -induced tumor necrosis factor -alpha and interleukin - 1 β production and gene expression in human monocytic cells by human alpha - fetoprotein*. *Hepatology*, 1995. **22**(3): p. 921-928.
414. Ho, H.C., et al., *Caffeic acid phenethyl ester inhibits proliferation and migration, and induces apoptosis in platelet-derived growth factor-BB-stimulated human coronary smooth muscle cells*. *J Vasc Res*, 2012. **49**(1): p. 24-32.
415. Chang, W.-C., et al., *Caffeic acid induces apoptosis in human cervical cancer cells through the mitochondrial pathway*. *Taiwan J Obstet Gynecol* 2010. **49**(4): p. 419-424.
416. Yang, J.-S., et al., *Chlorogenic acid induces apoptotic cell death in U937 leukemia cells through caspase-and mitochondria-dependent pathways. In vivo*, 2012. **26**(6): p. 971-978.

417. Marchi, S., et al., *Mitochondria-ros crosstalk in the control of cell death and aging*. J Signal Transduct, 2012. **2012**: p. 329635.
418. Kamata, H., et al., *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases*. Cell, 2005. **120**(5): p. 649-61.
419. Clutton, S., *The importance of oxidative stress in apoptosis*. Br Med Bull, 1997. **53**(3): p. 662-8.
420. Cooper, G., *Pathways of Intracellular Signal Transduction.*, in *The Cell: A Molecular Approach*. 2000, Sunderland (MA): Sinauer Associates; .

Appendix – Publications during candidature

DOI: 10.1016/j.foodres.2015.07.027

Hall, Susan, Ben Desbrow, Shailendra Anoopkumar-Dukie, Andrew K. Davey, Devinder Arora, Catherine McDermott, Matthew M. Schubert, Anthony V. Perkins, Milton J. Kiefel, and Gary D. Grant. "A review of the bioactivity of coffee, caffeine and key coffee constituents on inflammatory responses linked to depression." *Food Research International* 76 (2015): 626-636. (Impact Factor = 2.818)

