Adenosine Receptors And Endothelial Cell Mediated Wound Healing

Zeinab Bonyanian
BSc, MSc

School of Medical Science Griffith Health Griffith University

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STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in 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.

Zeinab Bonyanian (Zina)
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Impaired wound healing is a common and serious complication of diabetes mellitus causing an increased risk of infection and tissue damage. Chronic wounds associated with diabetes may be caused by peripheral neuropathy, vascular complications and alterations in immune function and contributes to non-traumatic amputations. Consequently, therapeutic management of wounds in diabetes is centred on infection control and stimulating revascularization. This research will focus on the latter and investigate the effects of high glucose on endothelial function with a view of finding new strategies to improve wound healing in diabetes. This project identified the adenosine receptor subtypes in EA.hy926 endothelial cell lines that are involved in stimulating wound healing and determined whether their roles are affected in the hyperglycaemic environment associated with diabetes. As caffeine is a non-selective antagonist of the adenosine receptors, this study also investigated the effects of acute and 48 hour chronic caffeine treatments on adenosine receptor populations and on wound closure and cell proliferation. Caffeine has a crucial role in wound healing through various mechanisms such as antioxidant activity or increasing the level of cyclic adenosine monophosphate (cAMP). Therefore, it is hypothesised that a chronic caffeine treatment could speed up wound healing in endothelial cells through up regulation of adenosine receptor subtypes or increased levels of cAMP. Also,
it should be noted that any truly new tests done- showing your research is very
innovation and has not been done before.

The aim of this research was to determine the effect of selective adenosine A1,
A2A and A2B receptor agonists on the rate of wound healing and cell
proliferation in EA.hy926 endothelial cells in a high glucose condition (4500
mg/L or 25 mM) and a low glucose condition (1000 mg/L or 5.5 mM). This
study also investigated the effects of acute caffeine, on the speed of wound
closure, angiogenesis and proliferation of EA.hy926 endothelial cells, using
the wound healing scratch assay, proliferation assay and tube formation assay.
Moreover, the effects of 48 hour caffeine treatment (chronic treatment) on the
speed of wound healing and proliferation of EA.hy926 endothelial cells were
presented using the wound healing scratch assay, proliferation assay expect
tube formation assay. Note that the tube formation assay was not used for
chronic caffeine treatment as the tube formation assay required 18 hours pre-
incubation and the EA.hy926 endothelial cells did not survive a further 48
hour caffeine treatment. Finally this study compared the effect of a 48 hour
caffeine treatment on adenosine receptor subtype protein expression in a high
glucose condition, using the Western blot assay.

The results have demonstrated that under a high glucose condition, the
selective adenosine A1, A2A and A2B receptors agonists, namely 30 nM CPA,
CGS21680 and NECA, significantly increased the rate of wound healing by
42.29%, 49% and 20.57%, respectively in EAhy926 endothelial cells
compared to untreated cells. Also, the results showed that selective agonists of
the adenosine A1 and A2B receptors have high efficacy in increasing the rate of
wound closure under a high glucose condition. EAhy926 endothelial cell
proliferation was significantly stimulated by the selective adenosine A<sub>1</sub> and A<sub>2B</sub> receptor agonists, CPA and NECA. Furthermore, the selective adenosine A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor antagonists, 10 nM DPCPX, ZM241385 and MRS1754, reversed the effects of their agonists and decreased the rate of wound healing. The protein levels of the adenosine A<sub>2A</sub> and A<sub>1</sub> receptors were highly expressed when compared to the adenosine A<sub>2B</sub> receptor in the EAhy926 endothelial cell lines in a high glucose condition.

Acute caffeine did not affect the rate of wound healing or cell proliferation of EA.hy926 endothelial cells. However, caffeine blocked the effects of selective adenosine A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor agonists and decreased the rate of wound healing in EA.hy926 endothelial cells. Nevertheless, caffeine treatment increased angiogenesis by stimulating tube formation in EA.hy926 endothelial cells. As this work has been completed on cells in a high glucose environment, acute caffeine may have a role in stimulating angiogenesis in diabetic patients. Moreover, the 48 hour caffeine treatment significantly elevated the rate of wound healing in EA.hy926 endothelial cells when compared with untreated and acute caffeine treatment groups. The effect of chronic caffeine was attenuated by the adenylyl cyclase inhibitor MDL-12330A. Therefore, chronic caffeine treatment may alter cAMP signalling pathways in EA.hy926 endothelial cell lines. The protein expression of adenosine A<sub>1</sub> and A<sub>2B</sub> receptors did not significantly change with chronic caffeine treatment in EA.hy926 endothelial cell lines but the expression of adenosine A<sub>2A</sub> receptor in the chronic caffeine treatment was considerably lower than the untreated group.
The rate of wound healing in a low glucose condition was significantly accelerated with selective adenosine A₁, A₂A and A₂B receptor agonists in EA.hy926 endothelial cells when compared to a high glucose condition. Furthermore, under a low glucose condition, the efficacy of selective adenosine A₁, A₂A and A₂B receptor agonists and antagonists on EA.hy926 endothelial cell wound healing was greater than their effects under a high glucose condition. However, EA.hy926 endothelial cell proliferation was decreased in the low glucose condition. Moreover, these experiments show that EA.hy926 endothelial cells are not able to remain viable and functional in a low glucose condition after 48 hours. Cell morphology and confluency changes were observed after 48 hour cell growth in EA.hy926 endothelial cells under the low glucose conditions.

Consequently, this hypothesis has been approved that the rates of wound healing in EA.hy926 endothelial cells were increased in chronic caffeine treatment.

In conclusion, there may be a potential therapeutic role for synthetic analogues of adenosine to increase wound healing in the diabetic patient. Furthermore, chronic caffeine also elevated wound healing rates during hyperglycaemic conditions. An effect that appears to be a separate mechanism from its ability to antagonise adenosine receptors.
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LIST OF ABBREVIATIONS

CPA  N°-cyclopentyladenosine
CCPA  2-Chloro-N°-cyclopentyladenosine
(S)-ENBA  (2S)-N°-[2-endo-Norbornyl]adenosine
R-PIA  R-N°-(phenylisopropyl)-adenosine
CHA  N°-cyclohexyladenosine
CGS-21680  2-[(2-carbonyl-ethyl)-phenylethylamino]-5°-N-ethylcarboxamidoadenosine
ATL-146e  4-{[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester
PAPA-APEC  2-(4-[2-[4-(aminophenyl) methylcarbonyl]ethyl]phenyl)ethylamino-5°-N-ethylcarboxamidoadenosine
NECA  5°-N-ethyl-carboxamidoadenosine
BAY-60-6583  2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide
LUF5834  2-Amino-4-(4-hydroxyphenyl)-6-[[1H-imidazol-2-ylmethyl]thio]-3,5-pyridinecarbonitrile
IB-MECA  N°-(3-iodobenzyl)adenosine-5°-N-methyluronamide
C1-IB-MECA  2-chloro-N(6)-(3-iodobenzyl)adenosine-5°-N-methyluronamide
APNEA  N°-2-(4-aminophenyl)ethyladenosine
DPCPX  1,3-dipropyl-8-cyclopentylxanthine
WRC-0571  8-(N-Methylisopropyl)amino-N-(5°-endoxyhydroxy-endonorbornyl)-9-methylenine
N-0840  N°-Cyclopentyl-9-methylenine
ZM-241, 385  4-[2-[(7-Amino-2-(2-furyl)[1,2,4-triazolo[2,3-a] [1,3,5]triazin-5-yl-amino]ethyl]phenol
SCH58261  5-Amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine
CSC  8-(3-chlorostyryl)caffeine
KW6002  (E)-1,3-Diethyl-8-(3,4-dimethoxyphenylethyl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione
MRS1754  8-[4-[[4-(Cyano)phenylcarbamoyl]methyl]oxy]phenyl]-1,3-di-(n-propyl)xanthine
MRE2029-F20  N-Benzoi[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H- purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide
CGS15943  9-Chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine
BW A-1433  1,3-dipropyl-8-(4-acrylate)phenylxanthine
MRS1191  3-Ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate
MRS1220  9-chloro-2-(2-furanyl)-5-[[phenylacetyl]amino][1,2,4]-triazolo[1,5-c]quinazoline
MRS1523  2,3-Diethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate
MRE3008-F20  5N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
<table>
<thead>
<tr>
<th>Term</th>
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<td>IL-1</td>
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<td>IL-12</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>Epidermal growth factor</td>
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<td>TGF-β</td>
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<td>CCL2</td>
<td>(C-C motif) ligand 2</td>
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<td>bFGF</td>
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<td>ECM</td>
<td>Extracellular matrix components</td>
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<td>HbA1c</td>
<td>Glycated haemoglobin A1c levels</td>
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<td>CAM</td>
<td>Chick chorioallantoic membranes</td>
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<td>TSP-1</td>
<td>Thrombospondin-1</td>
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<td>ATP</td>
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<td>Adenosine diphosphate</td>
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<td>5'-AMP</td>
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<tr>
<td>ENT1</td>
<td>Equilibrative nucleoside transporter 1</td>
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<tr>
<td>CD39</td>
<td>Nucleoside triphosphate phosphohydrolase</td>
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<td>CD73</td>
<td>Ecto-5'-nucleotidase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
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<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
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<tr>
<td>EA.hy926</td>
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<tr>
<td>HUVEC</td>
<td>Primary human umbilical vein endothelial cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>DPBS</td>
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<td>Phenylmethanesulfonyl fluoride</td>
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<td>Sodium Pyrophosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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LIST OF PUBLICATIONS AND CONFERENCES


Bonyanian, Z., Slevin, M., Rose'Meyer, R and Ahmed, N. (2013) Glycation of monomeric C-reactive protein is associated with impaired wound healing in diabetes, The Gold Coast Health and Medical Research Conference

Bonyanian, Z and Rose’Meyer, R. (2015) Effect of acute and chronic caffeine on cell proliferation and wound healing assays, The Gold Coast Health and Medical Research Conference, Chronic Diseases – Preventing and Improving Health Outcomes
CHAPTER 1

LITERATURE REVIEW
CHAPTER 1: LITERATURE REVIEW

1.1. SIGNIFICANCE

Many people experience some type of injury or wound during their life. In the majority of cases, wounds heal in a simple and rapid process, though this can leave noticeable scars on the skin. In some cases wound healing may be delayed or impaired due to health factors, leading to complications that adversely affect the patient’s quality of life. For instance, impaired wound healing is a common and serious complication of diabetes mellitus. It causes dysregulation of the process at multiple levels, increasing the risk of infection and tissue damage. Chronic wounds associated with diabetes may be caused by peripheral neuropathy, vascular complications and alterations in immune function and increases the risk of non-traumatic amputations. Some stages of the normal wound healing processes are disturbed in diabetes such as changes in the bactericidal activity of granulocytes, the excessive accumulation of extracellular matrix basement membrane and decreased tissue perfusion. Consequently, therapeutic management of wounds in diabetes is essentially focused on infection control and stimulating revascularization. Despite this knowledge, amputation rates still remain unacceptably high in diabetic patients with impaired wound healing. Hence, recent studies have focussed on finding new strategies to improve wound healing in diabetes.

Adenosine is a purine nucleoside that can bind four receptor subtypes; A1, A2A, A2B and A3. Adenosine mediates most of its intracellular effects through the regulation of the second messenger system adenyl cyclase and cyclic adenosine monophosphate (cAMP) production. Adenosine can induce wound healing through increased angiogenesis and deposition of the collagen
together with stimulation of extracellular matrix production in the wound site \(^4\text{--}^{11}\). Several studies have reported that adenosine and adenosine receptor agonists can increase proliferation and cell migration, which promotes wound healing \(^3\text{,}^{12}\text{,}^{13}\). Animal model studies have shown that adenosine A\(_{2A}\) receptor agonists increase dermal wound closure in healthy rats and in streptozotocin-induced diabetic rats with wound healing complications \(^14\text{,}^{15}\). Therefore, the adenosine A\(_{2A}\) receptor, by accelerating wound healing, could be important for diabetic patients with impaired wound healing and microvascular complications \(^16\). While adenosine may appear to be beneficial with the process of wound healing, there is controversy regarding the protective role of adenosine in tissue repair. Alternatively, another study reported that the deposition of collagen as a main function of wound healing, may lead to pathological remodelling of organs such as the kidney and heart, which causes significant morbidity and mortality \(^17\). This study found that adenosine signalling, mainly via adenosine A\(_{2A}\) and A\(_{2B}\) receptors, contributed to the pathogenesis of fibrosis \(^17\). Although, adenosine receptors may decrease inflammation and accelerate wound healing, loss of these receptors can lead to disruption of the organ structure and the loss of its functional integrity in different type of fibrosing disorders \(^17\). For instance, following hypoxia, stimulation of the adenosine A\(_{2B}\) receptors causes the expression of renal endothelin-1 (ET-1), which increases the proliferation of mesangial cells and collagen production in the kidney \(^17\text{--}^{19}\). Furthermore, activation of adenosine A\(_{2B}\) receptors may modulate the release of ET-1 and Interleukin-6 (IL-6) in interstitial lung disease to cause deposition of collagen, vascular remodeling
and eventually pulmonary hypertension. Consequently, the adenosine A$_{2B}$ receptor is a common feature for inducing fibrosis of these two organs$^{17,20}$. Common sources of caffeine include tea, coffee, cocoa beans and cola nuts. Caffeine is now also being added to beverages such as soft drinks and energy drinks, bottled water, chocolates, chewing gum and medications$^{21-25}$. Several studies have been reported that caffeine consumption has beneficial and protective effects on various diseases such as chronic liver disease, Type 2 diabetes and Parkinson’s disease$^{26-29}$. A study observed that the consumption of caffeinated beverages such as coffee and tea decreased the risk of type 2 diabetes in humans$^{30}$. Furthermore, another study showed that coffee intake of more than two cups daily was associated with lower alanine aminotransferase levels, which decreases the risk of chronic liver disease and reduce liver fibrosis$^{29,31,32}$. Topical coffee powder has been used as a wound covering in humans to treat acute and chronic wounds that occur in diabetes mellitus, sharp cuts and burns$^{33}$. As a result, this study determined that coffee improved the wound healing in the patients with all type of wounds. It had many advantages such as its bactericidal activity, antioxidant properties, deodorizing effects, allowed longer times to change the wound dressing, maintained a moistened wound, increased absorptive capacity, enhanced autolytic debridement, was cost effective and caused no adverse reactions$^{33}$. Furthermore, caffeine consumption can decrease the risk of Type 2 diabetes$^{34,35}$. Caffeine has been reported to have many different effects in cells that may account for its wound healing properties. Studies have confirmed that antioxidants may accelerate wound closure$^{36-38}$. Caffeine stimulates a variety of cellular and pharmacologic reactions in the central nervous system such as
stimulation of motor activity and antioxidant activity. According to reports, caffeine can enhance the release of Ca$^{2+}$ from internal Ca$^{2+}$ stores via activation of ryanodine receptor.

Caffeine is a non-selective antagonist of adenosine receptors. Through blocking adenosine receptors, caffeine decreases fibrosis and collagen deposition in the lung and liver. Caffeine also has low affinity for the adenosine receptor subtypes, being more effective in a high dose caffeine treatment (25 mg/kg per day) compared to a lower dose of caffeine (2.5 mg/kg per day) in the rat ventral prostate model. Moreover, an in vivo study using mice, showed that caffeine not only blocked the adenosine A$_{2A}$ receptor but also acted as a cAMP phosphodiesterase inhibitor. As a result, caffeine inhibits intracellular cAMP stimulation through antagonising adenosine A$_{2A}$ receptors, however, it increases immunosuppressive cAMP accumulation by preventing its degradation when it acts as a phosphodiesterase inhibitor to reduce inflammatory responses. Studies have reported that chronic caffeine treatment up-regulates adenosine A$_{2A}$ receptors to increase the anti-inflammatory property of adenosine and decrease the liver damage in mice. Also, the brain damage associated with ischaemic episodes can be attenuated by chronic caffeine intake. Marangos and colleagues (1984) reported that chronic caffeine treatment may have different effects on brain adenosine receptor subtypes according to the duration of caffeine treatment. Their animal study, with 11, 16 and 23 days of chronic caffeine treatment, found that after 11 days of treatment, the number of adenosine receptors were significantly changed compared to the control conditions; at 16 days chronic caffeine treatment the number of adenosine receptors significantly increased.
only in the cerebellum and brain stem regions. Subsequently, after 23 days treatment, the numbers of adenosine receptors increased significantly in all regions of the brain. Overall, this study found that the level of adenosine receptor subtypes in adult animals following chronic caffeine treatment was increased 20-30% in comparison with control models. Overall, the effects of caffeine on wound healing could be altered if given acutely or chronically. This thesis will consider the different effects of chronic and acute caffeine on the wound healing and the potential role for this compound’s use in diabetes.

Please note that sections of this review have been published in “Caffeine and its potential role in attenuating impaired wound healing in diabetes” Bonyanian Zeinab and Rose'Meyer Roselyn B. Journal of Caffeine Research. November 2015, 5(4): 141-148. doi:10.1089/jcr.2015.0011. (Appendix 1 for paper)

1.2. DEFINITION OF DIABETES AND CLASSIFICATION

Diabetes mellitus is a chronic disease that is characterized by hyperglycaemia and abnormalities in the metabolism of carbohydrates, proteins and lipids. It is caused by a defect in insulin function or pancreatic secretions or both. According to the World Health Organisation (WHO), 346 million people worldwide have diabetes and it estimates that the number of deaths from diabetes will double between the years 2005 and 2030. Diabetes mellitus is diagnosed using the classic symptoms of hyperglycemia (deficiency of the insulin hormone that produced by the pancreas), glycosuria (secretion of glucose into the urine) and polydipsia (excessive thirst or drinking excessive amounts of liquids). In addition, blood tests that are used to diagnose diabetes mellitus include fasting plasma glucose levels >=7 mmol/L or
>=11.1mmol/L 2 hours after a 75g glucose drink. Glycated haemoglobin A1c levels (HbA1c), provides information regarding blood glucose levels over the previous 120 days with a normal measure of HbA1c being less than 6%\textsuperscript{53}. Diabetes mellitus is classified into four different subtypes including Type 1, Type 2, gestational diabetes and impaired glucose tolerance (pre-diabetes)\textsuperscript{10}. Type 1 diabetes occurs as the result of the destruction of the pancreatic $\beta$-cells which prevents insulin secretion whereas Type 2 diabetes is a common form of diabetes mellitus that is the outcome of impaired insulin activity or secretion\textsuperscript{10}. Gestational diabetes is defined as glucose intolerance associated with pregnancy and is associated with alterations in reproductive and stress hormone levels that rise significantly during gestation and its symptoms are similar to Type 2 diabetes\textsuperscript{15,36,37}. Gestational diabetes is regularly diagnosed via prenatal screening of glucose tolerance rather than following the occurrence of its symptoms\textsuperscript{16}. Prolonged hyperglycaemia caused by diabetes mellitus may lead to several complications such as neuropathy, a high risk of foot ulceration and amputation, impaired wound healing, cataract development, retinopathy and associated blindness and nephropathy\textsuperscript{3,52}. The development of complications of the vascular system occur in diabetic patients more often than in non-diabetic people and this is a main contributor to morbidity and mortality\textsuperscript{12,13}. In addition, vascular complications may contribute to some diabetic pathogenesis such as retinopathy, nephropathy, neuropathy and impaired wound healing\textsuperscript{13}. 

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1.3. COMPLICATIONS OF DIABETES

1.3.1. Diabetic Atherosclerosis

Atherosclerosis is one of the more serious complications of vascular disease that could occur through long-term diabetes mellitus \(^3,^{54}\). The risk of vascular disorders such as coronary, cerebrovascular and peripheral arterial disease could be increased by diabetes mellitus; also, atherosclerosis is a main cause of mortality or disability in the patient \(^3,^{54}\). Atherosclerosis may occur via deposition of lipid and cholesterol in the vascular walls leading to the formation of stiff structures called plaques that block the blood flow \(^3\).

1.3.2. Diabetic Nephropathy

Diabetic nephropathy is another common diabetes complication, which is characterized by deposition of extracellular matrix protein in the mesangium, thickness of the basement membrane, albuminuria, decrease of filtration and renal failure \(^3,^{14}\). The lack of balance between the synthesis and destruction of extracellular matrix components causes the pathologic deposition of laminins and collagen together with fibronectins \(^{14}\).

1.3.3. Diabetic Retinopathy

Diabetic retinopathy is a common microvascular disorder that is a major cause of blindness in the diabetic population of many developed countries \(^3,^{15},^{55}\). Diabetes was originally considered a disease of the affluent and treatment options for diabetics has allowed individuals to live long enough to develop
retinopathy. However, as the prevalence of this condition rises in both
developed and developing countries it would be expected that the occurrence
of retinopathy would increase across the world. Diabetic retinopathy could be
classified as non-proliferative or proliferative. Also, retinopathy is
characterized by vascular blocking, angiogenesis, developing of blood vessels
proliferation, microaneurysms and infarction influence on the retina of the eye
3. The pathological features of retinopathy are: thickening of the retinal
capillary basement membrane; loss of tight junctions in the retinal
endothelium; enhanced permeability of capillaries; and loss of pericytes so
that the capillaries can be coated by the contractile cells which will control the
vessels’ content and perfusion.

1.3.4. Diabetes-Impaired Wound healing

Cardiovascular complications associated with diabetes occur due to
endothelial damage, which impairs angiogenesis and then delays wound
healing, contributing to atherosclerosis, thrombosis and hypertension. Impaired wound healing is a serious diabetic complication that leads to
approximately 50% of non-traumatic amputations in diabetic patients.
Furthermore, a reduced capacity for angiogenesis, decreased
lymphangiogenesis and the destruction of endothelial cells increases the risk
of cardiovascular disease and impaired diabetic wound healing. It is
noteworthy that both angiogenesis and lymphangiogenesis are essential factors
in the wound healing process. Diabetics develop vascular complications
such as arteritis stiffness and poor blood circulation particularly to the lower
limbs that lead to hypoxia and reduced blood flow to areas of tissue damage, which may impair wound healing \(^3, 38\). Peripheral neuropathy is another component associated with delayed wound healing through reduced sensorial nerve function and nerve transmission. Lack of pain perception increases the risk of wound development and delayed wound healing in diabetic patients as they do not perceive the onset and development of ulcers or infections in their extremities \(^34, 38\). In diabetes mellitus, wound healing is also compromised due to a delay of inflammatory cell influx into the damaged area and therefore the inflammatory response \(^38\). However, once the inflammatory cells penetrate the wound they contribute to chronic inflammatory processes that inhibit the extracellular matrix components (ECM) deposition, tissue remodelling and wound healing \(^3, 34\).

### 1.4. Skin Wound Healing Process

Wounds in the skin heal through three essential phases, namely the inflammatory, proliferative and maturation stages \(^35, 39\). In addition, growth factors and cytokines together with chemokines have crucial roles in the different phases of wound healing process \(^39, 61\).

#### 1.4.1. Inflammatory Phase

The destruction of blood vessels during tissue injury instigates platelet accumulation and activation and the coagulation process that initiates the inflammatory reaction \(^39\). As the inflammatory process proceeds keratinocytes release pre-stored interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) \(^39\).
In addition, other growth factors are secreted from the platelet α-granules. These include platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) \(^{39}\). Furthermore, TGF-β stimulates the conversion of monocytes to macrophages to increase the inflammatory reaction and tissue debridement \(^{39}\).

### 1.4.2. Proliferative Phase

This phase includes epithelialisation, the formation of granulation tissues and revascularization \(^{39, 61}\). Migration of the epidermal cell has a key role in reepithelialisation of wounds and these cells advance from the surrounding wound margins towards the centre of the wound \(^{62}\). Furthermore, epidermal growth factor, TGF-α and keratinocyte growth factor have a major role in stimulating the reepithelialisation process \(^{63}\). Macrophages release different types of pro-inflammatory cytokines such as IL-1 and IL-6 and growth factors, which initiate the formation of granulation tissue \(^{39}\). Granulation tissue is created through the proliferation of fibroblast cells and vascular endothelial cells, which initiate the repair process \(^{35}\). In addition, macrophages release TGF-β and PDGF to promote the proliferation of fibroblasts to generate new ECM \(^{63-65}\). Macrophages also release vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to stimulate the formation of new blood vessels through angiogenesis. This process depends on other factors such as ECM formation and the proliferation and migration of endothelial cells \(^{63}\).
1.4.3. Maturation Phase

The maturation phase consists of wound contraction, the removal of excess blood vessels via apoptosis and the conversion of collagen with its cross linking and rearrangement along stress lines to strengthen the tissue. Granulation tissues are removed during revascularization to generate a matrix and collagen is replaced with elastin fibers. In tissue remodelling and wound contraction, the TGF-β family which includes TGF-β1, TGF-β2, TGF-β3 and TGF-β8, stimulates the synthesis of fresh collagen type III to replace old collagen type I that is broken down by PDGF following up-regulation of matrix metalloproteinases. Newly formed collagen then binds to fibroblasts through integrin receptors to lead to scar tissue formation and healing of the wound.

1.5. Angiogenesis

Angiogenesis is a process by which new capillary blood vessels are formed from primary vessels. This process is controlled through interactions between growth factors that either induce or inhibit the growth of blood vessels. Angiogenesis has an essential role in normal processes such as fetal development, tissue repair, and the female reproductive cycle and in abnormal processes including arthritis, stroke, ulcer development, tumour growth and diabetic retinopathy. Several studies have shown that adenosine and adenosine receptor agonists up-regulate the expression of angiogenic factors and anti-angiogenic factor thrombospondin-1 (TSP-1) to stimulate angiogenesis. Furthermore, several in vitro and in vivo studies reported that adenosine and its receptor agonists stimulate blood vessel growth.
in pulmonary endothelial cells, the corneas of amphibian, mammalian embryos, rat skeletal muscle and chick chorioallantoic membranes (CAM) \(^68, \, 73, \, 77-82\). Studies have reported that adenosine plays an essential role in angiogenesis and the wound healing process \(^{41, \, 69, \, 83, \, 84}\) through elevating the release of angiogenic factors \(^{69, \, 85}\). Subsequently, a reported in vivo study found that caffeine can induce defects in the angiogenesis process in zebrafish embryos through blocking the adenosine receptors, an effect which was reversed after stopping caffeine treatment \(^{69}\).

### 1.6. ADENOSINE

Adenosine is a purine nucleoside which comprises an adenine molecule bound to a ribose sugar and it has a short half-life of 0.6-10 seconds \(^{73, \, 86, \, 87}\) (Figure 1.6.1). Adenosine is key local modulator of tissue function, particularly when there is loss of oxygen delivery, leading to a severe reduction in cellular energy production \(^{88}\). Adenosine induces vasodilation, which increases substrate and oxygen delivery to damaged tissue \(^{89}\). Research has found that adenosine generated during inflamed, ischaemic and hypoxic conditions decreases damage to tissues and stimulates recovery through a number of receptor-mediated processes \(^{89}\). During low oxygen conditions, ATP is degraded to adenosine \(^{90}\). As intracellular levels of adenosine rise the nucleotide then follows a concentration gradient out of the cell via carrier mechanisms \(^{90}\). Adenosine regulates organ and cellular activity through binding of cell surface receptors, which are all members of the 7-transmembrane spanning family, heterotrimeric G protein related receptors \(^{9, \, 91}\). The concentration of adenosine in the extracellular environment is usually
kept at minimal levels due to reuptake processes using transporters that are selective for adenosine. Following its movement back to the cell, adenosine is phosphorylated with adenosine kinase to generate AMP (adenosine monophosphate) and ultimately ATP, or it may be broken down by the enzyme adenosine deaminase to produce inosine.

Figure 1.6.1. The chemical structure of adenosine
1.6.1. Adenosine Metabolism

Adenosine is formed through the metabolism of the bioactive nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP) and cyclic adenosine monophosphate (cAMP) together with S-adenosylhomocysteine (SAH) \(^{92}\). There are several metabolic pathways that utilize adenosine. This includes the intracellular and extracellular phosphorylation of 5'-adenosine monophosphate (5'-AMP) to 5'-nucleotides and the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine via S-adenosylhomocysteine hydrolase in intracellular space \(^{92}\). The extracellular adenosine concentration is maintained through carrying adenosine from the intracellular space to the extracellular space by the bi-directional equilibrative nucleoside transporter 1 (ENT1). There are two enzymes located on the cell surface, CD39 (nucleoside triphosphate phosphohydrolase) and CD73 (ecto-5'-nucleotidase) that catalyze break down of ATP to form AMP, which is then converted to adenosine in the extracellular space (Figure 1.6.2) \(^{4,93}\). Moreover, adenosine can be further broken down by moving back into the cell to form inosine through the enzyme adenosine deaminase, or adenosine may be converted to 5'-AMP by adenosine kinase \(^{89}\).
Figure 1.6.2. Adenosine metabolism in the intracellular and extracellular environment of the cell\textsuperscript{4}
1.6.2. Adenosine Receptors

Adenosine receptors consist of four subtypes A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B} and A\textsubscript{3}, which are members of the G protein-coupled receptors and as such are recognised as seven-transmembrane domain receptors\textsuperscript{86, 88, 94, 96}. The adenosine receptors are widely expressed on various cell types such as endothelial cells, fibroblasts, neutrophils and macrophages. Therefore, adenosine as an effective endogenous mediator, regulates physiological effects such as stimulation of angiogenesis and wound healing through interaction its receptors\textsuperscript{5, 73, 97, 98}. Originally the pharmacological properties of the adenosine receptors were used to characterize and differentiate the four subtypes of receptor\textsuperscript{88, 99}. More recently the genes that encode the adenosine receptor subtypes have been identified and published, as well as their primary amino acid sequence\textsuperscript{88, 99}. These four subtypes of adenosine receptors have different functions and molecular weights\textsuperscript{88, 99}. The adenosine receptors are coupled to their particular G proteins and are well recognized to inhibit and/or stimulate adenylyl cyclase\textsuperscript{88, 91}. The adenosine A\textsubscript{1} and A\textsubscript{3} receptors comprise a molecular mass of approximately 38 kDa and 52 kDa respectively and are coupled to G\textsubscript{i/o} to mediate adenylyl cyclase inhibition, attenuating the cyclic adenosine monophosphate (cAMP) dependent pathway and decreasing cAMP production. They also activate K\textsuperscript{+} channels, inhibit Ca\textsuperscript{2+} channels and mediate catabolism of phospholipids (Table 1.6.1)\textsuperscript{88, 91, 100-102}.

A study investigating the avian heart has reported that activation of both adenosine A\textsubscript{1} receptors may stimulate an accumulation of diacylglycerol (DAG)\textsuperscript{103}. Furthermore, this study showed that adenosine A\textsubscript{1} receptors stimulate phospholipase C (PLC) to cause a concomitant accumulation of
phosphoinositides\textsuperscript{103}. U-73122, a PLC inhibitor, reversed DAG production after the activation of adenosine A\textsubscript{1} receptors and suppressed adenosine A\textsubscript{1} receptor mediated cardioprotection\textsuperscript{88,103}.

The adenosine A\textsubscript{2A} receptor subtype has a molecular mass of around 45 kDa and is coupled to the G\textsubscript{s} protein. The adenosine A\textsubscript{2B} receptor has a molecular mass of around 53 kDa and can be coupled to both G\textsubscript{s} and G\textsubscript{q} proteins to stimulate adenylyl cyclase, increased stimulation of intracellular cAMP production, intracellular calcium mobilization, activation of mitogen-activated protein kinase (MAPK) and phospholipase C (PLC) (Table 1.6.1)\textsuperscript{9,88,91,102,104,105}. There are several potent and selective agonists and antagonists for adenosine receptors that have a high affinity to its related subtype; some of these agonists and antagonist were utilised in this research study and are listed in Table 1.6.1.
<table>
<thead>
<tr>
<th>Adenosine Receptor subtype</th>
<th>Number of amino acids</th>
<th>G protein Coupling</th>
<th>Mechanism</th>
<th>Agonists</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>326</td>
<td>$G_{i/o}$</td>
<td>AC inhibition, Decrease cAMP productions, Activation $K^+$ channels, inhibition of $Ca^{2+}$ channels</td>
<td>❖ Selective CPA CCPA (S)-ENBA R-PIA CHA</td>
<td>❖ Selective DPCPX WRC-0571 N-0840 ❖ Non-Selective Caffeine</td>
</tr>
<tr>
<td>A&lt;sub&gt;2A&lt;/sub&gt;</td>
<td>410</td>
<td>$G_s$</td>
<td>AC stimulation, Increase cAMP productions</td>
<td>❖ Selective CGS-21680 ATL-146e PAPA- APEC</td>
<td>❖ Selective ZM-241, 385 SCH58261 CSC KW6002 ❖ Non-Selective Caffeine Theophylline</td>
</tr>
<tr>
<td>A&lt;sub&gt;2B&lt;/sub&gt;</td>
<td>320</td>
<td>$G_s$ &amp; $G_q$</td>
<td>AC stimulation Increase cAMP productions, Activation MAPK and PLC, $Ca^{2+}$ mobilization</td>
<td>❖ Selective None due to low agonist affinity ❖ Non-Selective NECA BAY-60-6583 LUF5834</td>
<td>❖ Non-Selective MRS1754 Caffeine Theophylline Alloxazine MRE2029-F20 CGS15943</td>
</tr>
<tr>
<td>A&lt;sub&gt;3&lt;/sub&gt; **</td>
<td>320</td>
<td>$G_{i/o}$</td>
<td>AC inhibition, Decrease cAMP productions, Activation $K^+$ channels, Deactivation $Ca^{2+}$ channels</td>
<td>❖ Selective** IB-MECA C1-IB-MECA APNEA ❖ Non-Selective NECA</td>
<td>❖ Selective** BWA-1433 MRS1191 MRS1220 MRS1523 MRE 3008-F20 ❖ Non-Selective** Theophylline</td>
</tr>
</tbody>
</table>

Table 1.6.1. Adenosine receptors classification and functions. Adenylate cyclase (AC), mitogen-activated protein kinase (MAPK), phospholipase C (PLC), ** Adenosine A<sub>3</sub> receptor agonist and antagonist were not utilised in this research study. 7, 86, 88, 92.
1.6.3. Signalling pathways of the adenosine receptors

Activation of each adenosine receptor by its agonist causes different signalling events. Adenosine A₁ and A₃ receptors, through binding to Gᵢ/₀ proteins, inhibit the activity of adenylate cyclase thus decreasing the activation and production of cyclic AMP (cAMP). One study using Chinese hamster ovary cells (CHO) demonstrated that inhibition of adenylyl cyclase through functional binding of adenosine A₃ receptors could be observed in CHO cells transfected with the receptor cDNA. The adenosine A₂A and A₂B receptors bind to Gₐ to stimulate adenylyl cyclase and increase cAMP accumulation to activate protein kinase A (PKA) and eventually stimulate cell proliferation, and is the primary signalling pathway associated with these receptors. Also, activation of protein kinase C (PKC) and increasing intracellular calcium levels occur following elevation of cAMP levels and activation of phospholipase C (PLC) through the Gₐ protein. The Gₐ protein mediated signalling is the second main signalling pathway of adenosine A₂A and A₂B receptors. A study has shown that adenosine at low concentrations can stimulate the high affinity adenosine A₁, A₂A and A₃ receptors whereas high concentrations of adenosine activates the low affinity A₂B receptors.

The adenosine A₁ receptors can bind to the Gᵢ/₀ protein, which leads to decreased intracellular cAMP levels. The interaction of adenosine with the adenosine A₁ receptor can elevate intracellular Ca²⁺ levels through the stimulation of phospholipase C (PLC). PLC breaks down phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). In addition, increased levels of intracellular Ca²⁺ activate enzymes such as protein kinase C (PKC) and
phospholipase A$_2$ (PLA2) together with phospholipase D (PLD) $^{84, 95}$. A study using selective adenosine receptor agonists and antagonists showed that adenosine A$_1$ receptors can have pro-inflammatory effects in different tissues $^{110}$. For example, in a study on treatment of experimental lung injury in mice, it was demonstrated that DPCPX (1,3-dipropyl-8-cyclopentylxanthine), a selective adenosine A$_1$ receptor antagonist, could prevent endothelial damage, migration of neutrophils and damage of alveolus in reperfusion in the lungs $^{111}$. Endothelial cells have a large capacity to store and release adenosine $^{108}$. In addition, many of inflammatory processes of endothelial cells can be modulated by adenosine A$_{2B}$ receptors, which are expressed on endothelial cell surfaces $^{71, 108}$. Interactions of adenosine with adenosine A$_{2A}$ and A$_{2B}$ receptors leads to increased cell proliferation, chemotaxis and capillary tube formation, therefore confirming a role for adenosine A$_{2A}$ and A$_{2B}$ receptors in stimulating angiogenesis $^{108, 112, 113}$. Furthermore, activated adenosine A$_{2B}$ receptors in human microvascular endothelial cells stimulate the secretion of VEGF and the insulin-like growth factor-I together with the basic fibroblast growth factor (bFGF) $^{71}$. 

The binding of adenosine to the adenosine A$_3$ receptors can initiate the classical second messenger pathway through adenylyl cyclase inhibition, increasing Ca$^{2+}$ levels and the stimulation of PLC $^{84, 114-116}$. Adenosine A$_3$ receptors are coupled to mitogen-activated protein kinase (MAPK) to stimulate extracellular signal-regulated kinases (ERK1/2). Hence adenosine A$_3$ receptors have a role in cell proliferation, differentiation, survival and death $^{84, 104}$. A study in melanoma cells demonstrated that activation of adenosine A$_3$ receptors stimulates PI3K-dependent phosphorylation of protein
kinase B (PKB/Akt) to decrease levels of ERK1/2 phosphorylation and inhibit cell proliferation \(^{117}\). Angiogenesis and cell preservation can also be mediated through adenosine A\(_3\) receptors, which enhance VEGF and HIF-1 \(\alpha\) production \(^{118}\). Furthermore, through activation of Protein kinase C (PKC) or phosphoinositol 3-kinase (PI3K), the adenosine A\(_3\) receptor can activate degranulation and cell migration of rodent mast cells via increased intracellular Ca\(^{2+}\) levels \(^{84},^{114-116}\).

Studies have noted that in comparison to other adenosine receptor subtypes, the adenosine A\(_3\) receptor has the lowest peptide homology between species \(^{110},^{119}\).

\section*{1.6.4. Association of adenosine receptors with wound healing}

Stimulation of the adenosine A\(_{2A}\) and A\(_{2B}\) receptors enhances migration of fibroblasts and endothelial cells through cAMP- and PKA mediated signalling pathways \(^{5}\). Prior studies have also illustrated that adenosine and its analogues, through binding adenosine A\(_{2A}\) receptors, can enhance proliferation and migration of endothelial cells as well as the release of vascular endothelial growth factors (VEGF) \(^{5},^{120}\). This demonstrates that adenosine receptors enhance wound healing through increased growth factor secretion into the target area \(^{5}\). On the other hand, occupation of adenosine receptors can impair wound healing through inhibition of inflammatory cytokines secretion such as TNF-\(\alpha\), IL-6, IL-8, IL-12 and vascular endothelial growth factor (VEGF) \(^{121},^{123}\). Adenosine A\(_1\), A\(_{2A}\) and A\(_3\) receptors can downregulate TNF-\(\alpha\) and IL-12 production in monocytes and macrophages \(^{46},^{124}\). Also, the stimulation of
adenosine receptors, especially the adenosine A$_2$ receptor in U-937 human macrophages and human umbilical vein endothelial cells, inhibits the secretion of vascular endothelial growth factor (VEGF) and IL-6, IL-8 respectively. VEGF is a main factor that stimulates angiogenesis and vascular permeability. Therefore adenosine may accelerate angiogenesis by increasing VEGF production. According to Hashimoto et al., 1994, adenosine A$_2$ receptor stimulation in U-937 human macrophages, leads to the accumulation of VEGF mRNA. Other studies have demonstrated that adenosine can stimulate monocytes and macrophages to increase IL-10 production. As adenosine receptor agonists have been demonstrated to enhance wound repair in diabetic animals, it has been proposed as a new innovation for the advancement of agents that enhance wound healing in healthy people as well as those with impaired wound healing.

1.7. CHEMICAL STRUCTURE OF CAFFEINE

Caffeine is a 1,3,7-trimethylxanthine and is synthesised from the purine nucleotide xanthosine (Figure 1.7.1).
Figure 1.7.1. Chemical structure of caffeine
1.7.1. Caffeine Consumption

Caffeine is present in several of dietary sources such as food and beverages, mostly coffee and tea, that are widely consumed around the world. The caffeine content in some popular dietary sources includes: coffee 40 to 180 mg/150 ml; tea 24 to 50 mg/150 ml; cola 15 to 29 mg/180 ml; cocoa 2 to 7 mg/150 ml; and 1 to 36 mg/28 g for chocolate.

1.7.2. Caffeine Absorption

Caffeine is rapidly absorbed through the gastrointestinal tract in humans and absorption reaches 99% within 45 minutes after ingestion. Caffeine is metabolized in the liver by the microsomal enzyme CYP1A2. One study observed that the absorption of caffeine from coffee is faster than from cold drinks and suggested several reasons for this. The low temperature of the cold beverage may reduce the blood flow rates within the intestine and the rate of gastric emptying may be decreased by phosphoric acid and sugar in the cold beverage. The hydrophobic properties of caffeine allow it to diffuse through the body and penetrate all cell membranes, the blood-brain barrier and the placenta, however, caffeine does not accumulate in the tissues or organs. The peak plasma concentration of caffeine is reached between 15 and 20 minutes after oral ingestion in humans and this is the equivalent to 8 to 10 mg/l for 5 to 8 mg/kg doses. Furthermore, smoking accelerates the metabolism of caffeine thus the half-life of caffeine in a smoker adult male may decrease by 30% to 50%. The half-life of caffeine doubles in women who are taking oral contraceptives (OCPs) and can be increased in the last three months of pregnancy. Also caffeine levels will be elevated in the patients.
with chronic liver disorder. Moreover, one study has shown that ingestion of high doses of caffeine (doses more than 300 mg) can induce anxiety, panic attacks and hallucinations in human. Acute caffeine poisoning in humans occurs following ingestion of caffeine at the levels of a few hundred micromoles per litre or 40–400 mg/L in plasma.

### 1.7.3. Caffeine Metabolism

Caffeine is metabolized via the liver to produce dimethylxanthines and monomethylxanthines, dimethyl uric acids and monomethyl uric acids, trimethylallantoin and dimethylallantoin, and derivatives of uracil. Also, several substances are formed by caffeine metabolism such as 1,3-dimethylxanthine (theophylline) and 1,7-dimethylxanthine (paraxanthine) which also have marked pharmacological activity. Millimolar concentrations of caffeine cause the release of intracellular calcium through binding to ryanodine receptors on the sarcoplasmic reticulum. Moreover, the inhibition of phosphodiesterases may occur at higher concentrations of caffeine in humans than those usually reached during the consumption of beverages. According to Fredholm et al., 1999, caffeine is able to block adenosine A2A and A1 receptors at low concentrations, which can be reached after a single cup of coffee. However, higher concentrations of 20 times this are required to inhibit the cyclic nucleotide breakdown by phosphodiesterases; 40 times higher concentrations will block GABA receptor and 100 times higher concentrations will mobilize Ca2+ from intracellular calcium depots. The highest two levels of caffeine referred to above are improbable in humans through any form of normal usage of...
caffeinated beverages. Furthermore, an *in vivo* study using rats demonstrated that the effects of low doses of caffeine were similar to the selective adenosine A$_{2A}$ receptor antagonist SCH 58261 but not the selective adenosine A$_1$ receptor antagonist DPCPX.

### 1.7.4. Caffeine and Ryanodine Receptors

Ryanodine receptors mediate rapid calcium (Ca$^{2+}$) release from the sarco/endoplasmic reticulum (SR/ER) and are responsible for activating several Ca$^{2+}$-activated physiological processes. Caffeine is used commonly as a ryanodine receptor agonist and stimulates Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. Caffeine stimulates Ca$^{2+}$ release via sensitizing the channel to cytosolic Ca$^{2+}$ activation. The ryanodine receptors channels can be activated by the resting cytosolic Ca$^{2+}$ upon the addition of caffeine. Caffeine at submaximal concentrations has the ability to stimulate a partial and temporary Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores in cells expressing ryanodine receptors or from sarcoplasmic reticulum membrane vesicles in a phenomena recognized as “quantal” Ca$^{2+}$ release.

### 1.7.5. Various Effects of Caffeine

Caffeine exerts “positive” subjective effects on mood in humans at low doses of caffeine (20–200 mg). Caffeine is however well recognized as increasing anxious behaviour in human and animal models and can increase risk of panic attacks. It has been reported that patients who suffer from panic disorder, a serious form of anxiety disorder, were particularly sensitive to low doses of
caffeine (i.e. instant coffee at 65 mg caffeine per cup). Adenosine \( A_1 \) receptors regulate the release of various neurotransmitters such as glutamate. Therefore, excitatory transmission is increased when the adenosine \( A_1 \) receptors are blocked by caffeine directly or indirectly when caffeine affects GABAergic transmission.

Many studies using rats have shown that caffeine stimulates electrical activity in the cortex. Also, cats treated with caffeine exhibited activation of the cortical electroencephalography (EEG) similar to activity recorded at physiological awakening times and comparable to the EEGs that are generated via direct stimulation of the reticular formation, a part of the brain that plays an essential role in vigilance and awakening. Methylxanthines increase the excitability of rat hippocampal sections through antagonizing the actions of adenosine and also activate the EEG theta rhythm in the hippocampus of rabbit. Adenosine decreases the development of long-term potentiation while xanthines have the opposite effect through blocking adenosine receptors in rat hippocampal slices.

Caffeine can promote different acute cardiovascular effects including the up-regulation of circulating catecholamines in the cardiovascular system. Arterial stiffness and endothelium-dependent vasodilation also occur, leading to elevated systolic and diastolic blood pressure. In addition, an increase in the respiration rate is an effect that is dependent on the plasma caffeine levels.

Adenosine prevents catecholamine and renin release, and also inhibits lipolysis. Furthermore, caffeine as a nonselective adenosine receptor subtype antagonist, blocks the actions of adenosine. Consequently, caffeine
increases circulating catecholamine levels and via this mechanism elevates the basal metabolic rate. Hence, lipolysis occurs, causing the release of free fatty acids. This increases the levels of serum free fatty acids to 50% -100% above normal (the reference range is 0.00–0.72 mmol/L)\(^{133}\). Caffeine stimulates the small intestine, causing secretion of water and sodium\(^{22,133}\). According to a medical report, caffeine may stimulate apoptosis in UVB-damaged cells as well as block adenosine receptors to regulate blood vessels contraction\(^{22,162}\). Furthermore, caffeine may be utilized as a psychoactive drug to treat the patients with Parkinson’s disease\(^{22,162}\).

### 1.7.6. Caffeine and Wound Healing

Caffeine is a methylxanthine and a member of an alkaloid group that is a main component in coffee and other beverages such as cocoa and tea\(^{6,42,163,164}\). Caffeine has the potential to alter wound healing through a variety of mechanisms. It has been shown that caffeine can act as antioxidant in the body and components including xanthine and theobromine may have antioxidant roles\(^{6,163,164}\). Following tissue injury, damaged cells in the wound generate numerous amounts of reactive oxygen species (ROS), which are generated in the early stage of wound healing, and antioxidants are needed to scavenge free radical species and accelerate wound closure\(^{33,165}\).

In addition, some studies have demonstrated that antioxidant intake can accelerate wound healing\(^{6,165,166}\). For instance, acceleration of wound healing has been demonstrated using different components containing antioxidant compounds such as the methanolic and ethanolic extract of *Boesenbergia rotunda*, green tea extract and coffee powder\(^{33,167,168}\). Sol Gel has been
developed as an application method to apply antioxidants including a mixture of vitamin C in Pluronic F127 \textsuperscript{165}. The application of sol-gel on skin wounds has been shown to promote wound closure and enhanced cell proliferation and collagen synthesis as well as reducing apoptosis in streptozotocin induced diabetic rats \textsuperscript{165}. Coffee powder has likewise been shown to improve and support wound healing through its antibacterial and antioxidant properties in human and animal models \textsuperscript{33}. Therefore, coffee powder is utilised directly, to cover different types of wounds associated with diabetes mellitus, sharp cuts and burns \textsuperscript{169}. On the other hand, an \textit{in vitro} study in human keratinocytes and epidermal cells has shown that caffeine limited cell proliferation and cell migration in a dose- and time-dependent manner, thereby attenuating two main functions for wound healing \textsuperscript{6}.

\section*{1.8. ASSOCIATION OF CAFFEINE WITH DIABETES AND EFFECT ON ADENOSINE RECEPTORS}

Caffeine can alter the activity of adenosine and its effects either through modifying glucose levels, which are a fundamental cause of impaired wound healing, or through acute inhibition of adenosine receptors or chronic regulation of the adenosine receptor subtypes. The non-selective adenosine A\textsubscript{2B} receptor agonist NECA has been shown to increase glucose production in hepatocytes \textsuperscript{170}. Similarly, the application of NECA through oral gavage caused an increase in fasting glucose levels in mice \textsuperscript{171}. The implementation of adenosine A\textsubscript{2B} receptor knockout in mice and blockade of the adenosine A\textsubscript{2B} receptors with the selective antagonist ATL-801 in this study has indicated that the adenosine A\textsubscript{2B} receptor may act as an initial mediator of this effect \textsuperscript{171}.
Caffeine can act as a non-selective blocker of the adenosine A₁, A₂A and A₂B adenosine receptors. However, the ATL-801 is around 5000 times more potent than caffeine. A study of diabetic KKA¹ mice model has shown that the intake of large amount of coffee decreased hyperglycaemia, fat mass, and the expression of TNF-α and IL-6 in white adipose tissue. It also reduced the synthesis of fatty acids through decreased hepatic expression of the SREBP-1 and FAS genes. By blocking the adenosine A₂B receptor in diabetic mice, caffeine increased the sensitivity to insulin and the metabolism of glucose in the liver and skeletal muscle and brown adipose tissue.

Chronic caffeine treatment has the capacity to increase adenosine receptor populations. A study investigating chronic caffeine treatment over one week reported significant increases in the number of adenosine receptors in rat cerebral cortical membranes. A mouse model of acute lung injury demonstrated that an acute low dose caffeine treatment (5 or 15 mg/kg) increased lung injury by acting as an adenosine A₂A receptor antagonist to inhibit the anti-inflammatory effect of adenosine A₂A receptors, whereas an acute high dose of caffeine (50 mg/ kg) and chronic caffeine treatment decreased the lung damage and inhibited inflammation in an adenosine A₂A receptor-independent manner. In addition, this study showed that acute low dose caffeine decreased the level cAMP in wild type (WT) mice but it did not have any effect on cAMP levels in adenosine A₂A receptor knockout (KO) mice. Another in vivo study has shown that caffeine inhibits cAMP phosphodiesterase and can suppress immune activity by reducing cAMP levels in immune cells to decrease tissue injury during acute inflammation. These results demonstrated that neutrophil infiltration and expression of
inflammatory cytokines were inhibited through chronic caffeine and acute high dose caffeine (50 mg/kg) treatments in both WT and KO mice. Therefore the reduction in lung damage was not related to presence of adenosine A<sub>2A</sub> receptors<sup>175</sup>. Furthermore, other studies have shown that chronic caffeine decreases tissue injury in the kidney and liver<sup>175,178,179</sup>. Additionally, chronic caffeine–induced up-regulation of adenosine A<sub>2A</sub> receptors may increase the anti-inflammatory role of adenosine to decrease tissue injury<sup>180,181</sup>. Similarly up-regulation of adenosine A<sub>2B</sub> receptors with chronic caffeine was shown to be protective against acute lung injury in mice. However, there was no significant change in the expression of adenosine A<sub>2A</sub>, A<sub>1</sub>, and A<sub>3</sub> receptors in pre- and post-acute lung injury groups with chronic and acute caffeine treatment in comparison with a control group. Activation of the adenosine A<sub>2B</sub> receptor, which leads to increased cAMP levels as previously described, can decrease acute inflammation, which protects tissues from further injury or mitigates tissue damage<sup>182-185</sup>. Overall, chronic caffeine treatment upregulates adenosine A<sub>2B</sub> receptor numbers, which may enhance this receptor's anti-inflammatory role when activated via extracellular adenosine<sup>175</sup>.

1.9. CYTOKINES

As discussed previously, depending on the cell type inflammatory, endothelial, or structural adenosine and caffeine can have different effects on the cytokines released during the stages of wound healing (Figure 1.9.1). Pro-inflammatory cytokines consist of IL-1, IL-6 and TNF-α which are the main elements involved in the inflammatory phase of wound healing (Figure 1.9.1)<sup>63</sup>. IL-1 is
generated immediately following tissue injury from macrophages, neutrophils, keratinocytes and monocytes \(^{63}\). IL-1 and TNF-\(\alpha\) stimulate fibroblast production and the release of FGF\(_7\) \(^{39,186}\). The release of IL-6 and TNF-\(\alpha\) from activated neutrophils and macrophages that infiltrate the site of damage are essential in initiating the wound healing process and improving reepithelialisation \(^{39}\). IL-6 persists through the inflammatory process and it stimulates mitogenic and proliferative activity in keratinocytes and attracts neutrophils to the wound \(^{39,187}\). TNF-\(\alpha\) indirectly improves reepithelialisation through increased stimulation of FGF\(_7\) production, however it has been shown that TNF-\(\alpha\) itself can reduce re-epithelialization of wound \(^{39}\). In addition, TNF-\(\alpha\) attenuates the synthesis of ECM proteins and tissue inhibitor of metalloproteinase (TIMP-1) and stimulates the synthesis of metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, and MT1-MMP) \(^{39,188-190}\).

A study using the mouse macrophage cell line RAW 264.7, reported that adenosine A\(_1\), A\(_2\) and A\(_3\) receptors have an essential role in the regulation of cytokines such as IL-10, TNF-\(\alpha\) \(^{128}\). Furthermore, this study showed that drugs such as methotrexate, sulfasalazine and GP-1-515, can elevate extracellular adenosine concentrations and are potent anti-inflammatory agents \(^{128}\). Moreover, there are several mechanisms that might contribute to the anti-inflammatory properties of these drugs including: stimulation of adenosine A\(_2\) and A\(_3\) receptors to increase the production of IL-10; and stimulation of adenosine A\(_1\), A\(_2\) and A\(_3\) receptors to reduce the release of TNF-\(\alpha\) \(^{128}\). Another study demonstrated that adenosine can potently inhibit the expression of pro-inflammatory cytokines such as TNF-\(\alpha\) from mouse peritoneal macrophages in response to Toll-like receptors (TLR) activation \(^{191,192}\). An effect mediated
through the adenosine $A_{2A}$ receptor acting by different mechanisms included inhibition of nuclear factor-κB (NF-κB) \(^{192-195}\). Adenosine $A_3$ receptors are also able to suppress pro-inflammatory cytokine expression \(^{126, 191, 192}\). The adenosine $A_{2A}$ receptor is more effective at inhibiting TNF-α production in murine peritoneal macrophages than the adenosine $A_{2B}$ receptor \(^{192}\). Moreover, the adenosine $A_{2B}$ receptor has been commonly considered to be a pro-inflammatory receptor \(^{192}\). It is reported that activation of the adenosine $A_{2B}$ receptors can stimulate the production of IL-8 in a human mast cell line HMC-1 and also stimulate production of IL-6 and monocyte chemotactic protein-1 from human primary bronchial smooth muscle cells \(^{192, 196, 197}\).

With respect to the effects of caffeine on cytokine release an in vivo study reported that the effect of caffeine on inflammatory responses is dose dependent in mice \(^{45}\). For example, caffeine at low doses (10 and 20 mg/kg) increased pro-inflammatory cytokine release including TNF-α, IL-4 and IL-12 to enhance tissue damage, while caffeine at higher doses (100 mg/kg) reduced tissue destruction via the inhibition of pro-inflammatory cytokine responses and the release of an anti-inflammatory cytokine IL-10 \(^{45}\). Also, this study showed that lower doses of caffeine intensely exacerbated acute liver damage and elevated pro-inflammatory cytokines levels in mice \(^{45}\). In contrast, caffeine at higher doses entirely inhibited liver damage and pro-inflammatory cytokines responses via the adenosine $A_{2A}$ receptor-independent mechanism \(^{45}\).

Another study reported that plasma caffeine levels were strongly associated with reduced inflammatory cytokine levels of Aβ 1-40, IL-12 (p70), IFN-γ and TNF-α in the hippocampus \(^{198}\). Also, a significant reduction in hippocampal
inflammatory cytokines was observed in caffeine treated mice with higher plasma caffeine levels in comparison with lower caffeine levels.
Figure 1.9.1. Figure showing the role of pro-inflammatory cytokine responses in wound healing. Interleukin-8 (CXCL8), interleukin-1α (IL-1α), interleukin-1β (IL-1β), (C-C motif) ligand 2 (CCL2), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), tumour necrosis factor (TNF) and platelet-derived growth factor (PDGF)
1.10. **CONCLUSION**

Impaired wound healing and cardiovascular complications are significant disorders in diabetes mellitus that could be caused by attenuated angiogenesis together with endothelial cell dysfunction. Adenosine is a purine nucleoside that could activate cell proliferation through the interaction with adenosine receptor subtypes (A₁, A₂A, A₂B, and A₃). Adenosine A₁ and A₂ receptor agonists improve wound healing, and adenosine can stimulate angiogenesis as well. In addition, occupation of the adenosine receptor could reduce the elements that impair wound healing through inhibition of inflammatory cytokine secretion such as TNF-α, IL-6, and IL-8. While caffeine is a nonselective antagonist of the adenosine receptor subtypes, it has also been found to increase cell proliferation and wound healing. Caffeine may inhibit the role of adenosine in the development of glucose intolerance and insulin resistance, and alter the ability of adenosine to moderate the inflammatory processes. In addition, caffeine acts as an antioxidant and phosphodiesterase inhibitor, and may improve wound healing through these mechanisms.

1.11. **HYPOTHESES AND AIMS**

This project will identify the adenosine receptor subtypes involved in stimulating wound healing and determine whether their roles are affected in the hyperglycaemic environment associated with diabetes. The project will also investigate the effects of acute and chronic caffeine treatments on adenosine receptor populations and whether they subsequently alter the wound closure and adenosine functions within the cellular and vascular systems.
Caffeine may have a crucial role in wound healing through various pathways such as: antioxidant pathways and increasing the level of cyclic adenosine monophosphate (cAMP). Therefore, it is hypothesised that a chronic caffeine treatment could speed up wound healing in endothelial cells through up-regulation of adenosine receptor subtypes and increased levels of cAMP.

This study will investigate several hypotheses as follows;

HA; Cell proliferation and wound healing in EAh926 endothelial cells will be stimulated by selective adenosine receptor agonists

H0; Cell proliferation and wound healing in EAh926 endothelial cells will not be stimulated by selective adenosine receptor agonists

HA; Acute caffeine has an inhibitory effect on the wound healing rate and cell proliferation in EAh926 endothelial cells

H0; Acute caffeine does not have an inhibitory effect on the wound healing rate and cell proliferation in EAh926 endothelial cells

HA; Chronic caffeine has a stimulatory effect on the wound healing rate and cell proliferation in EAh926 endothelial cells

H0; Chronic caffeine will not have stimulatory effects on the wound healing rate and cell proliferation in EAh926 endothelial cells

HA; There will be differences in rates of wound healing and cell proliferation in EAh926 endothelial cells during high and low glucose conditions.

H0; There will be no differences in rates of wound healing and cell proliferation in EAh926 endothelial cells during high and low glucose conditions.
The aims of this research project are to:

I. Identify the effect of adenosine receptor subtypes on signalling pathways associated with EAhy926 endothelial cell proliferation and wound healing process.

II. Determine the effect of chronic and acute caffeine treatment on the speed of wound closure and angiogenesis in EAhy926 endothelial cells.

III. Compare the effect of chronic caffeine treatments on adenosine receptor subtype population protein expression.

IV. To determine the effect of high and low glucose levels on EAhy926 endothelial cell proliferation and wound healing process.
CHAPTER 2

METHODOLOGY
CHAPTER 2: METHODOLOGY

This chapter describes the various methods and materials used in this study, ranging from cell culture techniques to tube formation assays and Western blotting techniques. These methods have been implemented to determine the presence of adenosine A_1, A_{2A} and A_{2B} receptors in the endothelial cell population used for these studies and to investigate the effect of acute and chronic caffeine treatment on endothelial cell function such as wound healing, angiogenesis and calcium signalling. The results of these studies are presented in chapters 3, 4 and 5.

2.1. HUMAN ENDOTHELIAL HYBRID CELL LINE (EA.HY926)

The permanent human endothelial hybrid cell line (EA.hy926) was used for the studies presented in this thesis. This cell line was created through the hybridisation of primary human umbilical vein endothelial cells (HUVEC) and a human lung carcinoma cell line (A549/8) in 1983\textsuperscript{200, 201}. Following the establishment of these cells they have been used for a number of studies related to vascular function including the biochemical pathways of coagulation and fibrinolysis\textsuperscript{202}. Also, these endothelial cell have been reported to contain tissue-type plasminogen activator (t-PA), which when released into the bloodstream is the main enzyme that facilitates the process of fibrinolysis and thrombolysis\textsuperscript{202-208}. In addition, t-PA and a plasminogen activator inhibitor (PAI) were synthesised and secreted by human endothelial cells\textsuperscript{202, 209-212}. Many studies using the EA.hy926 cell line have reported that these cells maintain the expression of cell surface thrombomodulin proteins, the synthesis
of von Willebrand factor and prostacyclins and the release of platelet activation factor. Moreover, another study demonstrated the fibrinolytic characteristics of the EAhy926 cell line which secretes both tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1). Several studies have investigated the properties of EA.hy926 endothelial cells with respect to angiogenesis, cell proliferation, cell migration and apoptosis. A study reported that down-regulation of dihydrofolatereductase prevents the stimulation of G1 phase thus inhibiting cell proliferation. Another study observed that cordycepin (3′-deoxyadenosine) a derivative of adenosine, stimulated apoptosis and inhibited EA.hy926 cell migration in a dose- and time-dependent manner. Furthermore, homocysteine and adenosine impaired angiogenesis in EA.hy926 endothelial cells through activation of the MEK/ERK signalling pathway. This study also reported that dipyridamole, a nucleoside transport inhibitor prevented impaired angiogenesis in the EA.hy926 cell line through the suppression of intracellular accumulation of S-adenosylhomocysteine.

2.1.1. Cell Culture Technique

The EA.hy926 endothelial cell line used for these studies was generously donated to our research group by the Apoptosis Research Group, Griffith University, Gold Coast campus. The EA.hy926 endothelial cells were seeded into a 75 cm² cell culture flask containing 13 µL of complete media. Complete media comprised high glucose (4500 mg/L, 25 mM) Dulbecco’s Modified Eagle’s Medium (DMEM) (ThermoFisher Scientific, VIC, Australia) supplemented with a mixture of Hypoxanthine-Aminopterin-Thymidine (HAT)(Sigma Aldrich, NSW, Australia), 10% Foetal Bovine Serum (FBS),
and 1% Antibiotic-Antimycotic (penicillin/streptomycin/amphotericin B) (Invitrogen, VIC, Australia). Please note that the high glucose media was used to grow the EA.hy926 endothelial cell line in culture for Chapters 3 to 5 to mimic a diabetic condition. The low glucose media (5.5mM) was used for experiments in Chapter 6 to create a normal low glucose condition (non diabetes). The high glucose media is the recommended media for this cell line as the cells do not grow well at the lower glucose concentration. Cells were incubated in a humidified incubator at 37 ºC and 5 % carbon dioxide (CO₂). The cells were maintained under these conditions until they adhered to the surface of the flask. They were grown for 4-5 days until they reached the optimal confluency to commence experiments.

2.1.2. Cell Passaging

The cells were passaged after 2-3 days, once they had reached 90% confluence. The remaining media was discarded from the 75 cm² cell culture flask and replaced with 8-10mL pre-warmed Dulbecco’s Phosphate-Buffered Saline (DPBS) (ThermoFisher Scientific, VIC, Australia) to wash the cells, then the DPBS was discarded from the culture flask. Afterwards, the cells were incubated with 2 mL pre-warmed trypsin (ThermoFisher Scientific, VIC, Australia) to detach the cells from the surface of the culture flask and placed for 5 minutes in a humidified incubator at 37 ºC, 5% CO₂. Concurrently, a culture flask containing 10mL of complete media was placed into the incubator for 15 minutes to allow the solution to reach a temperature of 37 ºC and pH of 7 to 7.6. Then 4 mL of complete media was added to the detached cells,
denature the trypsin and the solution aspirated a few times to prevent clumping of cells. The total 6 mL cell solution was then equally divided into 3 pre-incubated 75 cm² culture flasks containing 10 mL high glucose media, giving 12 mL total solution in each flask. The cells and high glucose media were gently mixed with a 10 mL pipette to obtain a uniform solution. The cells were then incubated at 37 °C, 5% CO₂, for 2 days to reach 90% confluence. Cells were tested for mycoplasma infections using the MycoAlert® Myco Plasma Detection kit (Lonza, Basel, Switzerland). Mycoplasma-positive cell lines underwent antimycoplasma treatment with antibiotics including plasmocin, BM-Cyclin and baytril for periods of 7–21 days.

For the duration of these experiments, the cells would have undergone 20 passages for the wound healing experiments and another 20 passages for the cell proliferation assays.

2.1.3. **Cryopreservation (Cell Freezing)**

When the cells reached 80% confluence, the existing media was discarded from the 75 cm² cell culture flask and the cells washed with pre-warmed DPBS. Then 2 mL pre-warmed trypsin was added to the 75 cm² flask and the flask incubated for 5 minutes at 37 °C, 5% CO₂. After the incubation period, 8 mL of high glucose media was added to the cells and mixed, then the 10 mL suspension was transferred to a 15 mL falcon tube, and centrifuged for 5 minutes at 1200 RPM and 4 °C. In the meantime, the media used to freeze the cells was prepared by adding 50% filtered FBS to 40% high glucose media and 10% dimethyl sulfoxide (DMSO). After 5 minutes of centrifuging, the
supernatant was discarded and the pellet or cell residue was retained. Then 4.5 mL of freshly prepared media was gradually added to the cells and mixed. Subsequently, 1.5 mL of the cell solution was added to each of the three 2 mL cryotubes. The cryotubes were stored in a -20 °C freezer for 1 hour then placed into a -80°C freezer.

2.1.4. Cell Counting

The harvested cells were centrifuged for 5 minutes at 1200 RPM and 4 °C then the pellet was collected and resuspended in 5-10 mL pre-warmed high glucose media. An aliquot of 10 µL cell suspension was mixed with 10 µL of trypan blue (0.4%)(Sigma Aldrich, NSW, Australia). Then using a haemocytometer chamber in a Nikon ECLIPSE TS100 light microscope (Nikon, USA)under 40 x magnification, cells were counted in the grids. Then after calculations, the required number of cells were seeded into the designated plates. To calculate the viable number of the cells per mL, the cells were counted from each set of 16 corner squares and divided by 4 as there are 4 sets of 16 corner squares (Figure 2.1.1). The result was multiplied by $10^4$ (10,000), and by dilution factor of 2 as the cell suspension was diluted 1:1 with trypan blue.

\[
\frac{\text{Total cells counted}}{\text{Number of squares}} \times \text{Dilution factor} \times 10^4 = \text{Total number of the viable cells /mL}
\]

i.e. \[\frac{125 \times 2}{4} \times 10^4 = 625,000 \text{ cells /mL}\]
Figure 2.1.1. The figure is showing grid lines of the haemocytometer chamber. Each red square indicates a corner square (a set of 16 squares)
2.2. THE SCRATCH WOUND HEALING ASSAY

The wound-healing assay used throughout this thesis is a straightforward and low-cost method and is one of the advanced techniques used to examine cell migration *in vitro* \(^{218,219}\). This simple method imitates the migration of cells in the wound healing process observed during *in vivo* conditions \(^{218,219}\). The primary stages of an *in vitro* wound healing assay includes creating a wound or scratch on a confluent cell monolayer, collecting the images at the starting point and at certain time intervals of cell migration until wound closure occurs. The images are then viewed to measure and determine the rate of cell migration \(^{218,219}\). Furthermore, this method may determine the efficacy of different type of treatments or drugs on the rates of cell migration and wound healing.

The EA.hy926 endothelial cells (≈2x10\(^5\)) were seeded in 12-well plates, in high glucose media, and once they had reached 80-85% confluence, the scratch wound healing assay was performed. The EA.hy926 cell monolayer was then scratched with a 200 µL pipette tip (yellow tip) using a ruler as a guide, to create a straight line through the centre of the well. Then the media was removed and the cells rinsed once gently with pre-warmed DPBS, to remove any cellular debris or remaining media \(^6\). The cells were subsequently treated with fresh media combined with drugs in different concentrations and timeframes in accordance with the designed experiments. The preparation of the drugs used for these experiments is described in Chapter 3.
Experimental timeline

2.2.1. Data Collection from the Wound Healing Scratch Assay

Cell migration was monitored using the Nikon ECLIPSE TS100 light microscope (Nikon, USA) with attached camera. The wells were photographed at the starting point of the experiment (0 hours) then at 2, 4 and 6 hours. Rates of cell migration were then evaluated by measuring the width of the wound at each 2 hour time period until the end of the experiment, using Image J software. Data values (μm/hour) were collected for each sample at 0, 2, 4 and 6 hours then the data was transferred to Microsoft Excel to determine the slope of the line, for example see figure 2.2.1. The slope of the line has been used to measure the speed of wound healing (μm/hour). The data from single doses of agonists or antagonists was analysed to calculate the mean and SEM which was then represented in histograms.

Statistical analysis was completed using GraphPad Prism software (version 6.0) (CA, USA to examine the rates of wound healing in a dose and time dependent manner. The significance of differences between groups was evaluated using one-way analysis of variance (ANOVA). Values are presented
(Chapter 3) as mean± SEM and differences were considered statistically significant at $P < 0.05$. 
Figure 2.2.1. Graph showing the effect of adenosine receptor agonist (30nM) or antagonist (10nM) on the rate of wound closure (µM/hour) over time (hour). The control group had no drug treatment.
2.3. **CELL PROLIFERATION ASSAYS**

Cells are basic structural and functional parts of an organism. Cell proliferation and differentiation plays an essential role in the development and maintenance of organisms from embryogenesis to mature tissue homeostasis by replacing the old, dead or damaged cells with new ones. Cell proliferation assays may include metabolic activity analysis, determination of DNA replication rates or the detection of antigens on cell surfaces. The Cell Counting Kit-8 (CCK-8) from Sigma Aldrich (NSW, Australia) was utilized to measure the cell viability and proliferation in this study. CCK-8 contains a highly water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] which generates a yellow colour due to the formation of water-soluble formazan upon cellular reduction (Figure 2.3.1). In live cells, a dehydrogenase enzyme in the cytosol of cells reduces WST-8 to produce a formazan dye that is soluble in the tissue culture media and is directly related to the number of viable cells.
Figure 2.3.1. The chemical structure of WST-8 in CCK-8 and formation of its formazan dye.\textsuperscript{221}
The CCK-8 assay has several advantages when compared to other colorimetric assays such as the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) and WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) methods. CCK-8 is a convenient and simple assay and does not require any further steps regarding premixing. The water-soluble formazan dyes are non-radioactive and also have no toxic effects on cells. Detection of CCK-8 formazan dye from absorbance measurements is also more sensitive than for other conventional tetrazolium salts. Unlike CCK-8 or WST-8, MTT produces a formazan dye, which is extremely insoluble in water. Its residue damages the cells and an accurate absorbance measurement cannot be obtained. Therefore, to prevent this problem the MTT assay requires an additional step to dissolve the formazan.

The ATP detection assay is a reliable and sensitive luminescence method that can be utilized as an alternative to the tetrazolium reduction or colorimetric assays such as MTT, MTS, XTT, WST-1, and WST-8. The ATP assay detects viable cells by measuring ATP as a marker of live cells. Performing the luminescence assay has advantages in that the protocol is simple, highly sensitive and fast.

For most of the experiments, the EA.hy926 endothelial cells were seeded in triplicate at 5000 cells/well in 96 well plates. For the purpose of creating a calibration curve other wells were prepared with duplicate known numbers of viable cells (including 4000, 5000, 6000, 7000, 8000 and 9000 cells per well). Hence, cell numbers were compared to the average absorbance of each well to
create a standard curve (to see the calibration curve refer to Chapter 3). Once cells were seeded, the plate was incubated for 24 hours at 37 °C, 5% CO₂. CCK-8 was either thawed at room temperature for 30 mins, or defrosted in the water bath at 37 °C for 5 mins. After 24 hours incubation, the high glucose complete media was discarded from each well and subsequently replaced with freshly prepared high glucose complete media (200 µL) containing 10 µL of various drugs. Then 10 µL of the CCK-8 solution was carefully loaded into each well of the plate to avoid producing any air bubbles (air bubbles in the wells could interfere with the optical density O.D. reading). The 96 well culture plate was then incubated at 37 °C, 5% CO₂, for 4 hours. Then the plate was placed into the Infinite M200 PRO-TECAN microplate reader (Tecan group, Switzerland) to measure absorbance at 450 nm. The standard curve can be seen in Figure 2.3.2. The standard curve was used to determine cell numbers, to allow an accurate quantification of cell proliferation.

Statistical analysis for the cell proliferation was performed using a one-way analysis of variance (ANOVA) with version 6.0 of GraphPad Prism software. Statistical differences in mean values of cell proliferation with different drug treatments and also compare means with the control group. A P-value of < 0.05 considered statistically significant.

Timeline for experimental protocol
Cultured cells in 96 well plate

Add 10 μL CCK-8 solution

Incubate for 24hrs at 37 °C

Incubate for 4hrs at 37 °C

Then measure absorbance at 450 nm
Figure 2.3.2. A standard curve for the CCK8 cell proliferation assay at 450 nm absorbance.
2.4. WESTERN BLOTTING TECHNIQUE

2.4.1. Sample Preparation

The EA.hy926 cells were seeded in a 75 cm² cell culture flask. Once the cells reached 80% to 85% confluency, the cells were detached from the surface of the flask using a rotatable Techno Plastic Products (TTP) cell scraper (Zellkultur Lab, Switzerland). It should be noted that the cells were not trypsinized to separate them from the surface of the flask, as trypsin breaks down the proteins and cell membrane and the whole cell (with its membrane) was required for this experiment. The harvested cells were transferred to a 15 mL falcon tube, then counted as previously described and prepared for cell lysis.

2.4.2. Cell Lysis

The cells were lysed using lysis buffer (1mM Phenylmethanesulfonyl fluoride (PMSF), 10µM Leupeptin, 3mM Benzamidine, 5µM Pepstatin A, 1mM Sodium orthovanadate, 0.1% Triton-X and Kinexus Buffer*), which was freshly prepared using the recommended formula from the Heart Foundation Research centre at Griffith University. Kinexus Buffer was prepared from mixture of Mops Buffer, 2mM EGTA, 5mM EDTA, 30mM NaF, 40mM B-Glycerophosphate and 20mM Sodium Pyrophosphate (NaPP). The cell suspension was centrifuged at 1200 RPM, for 5 minutes at 4 °C and then the supernatant was removed, then the cells were washed with ice-cold DPBS. The cells were then re-centrifuged at 1200 RPM, for 5 minutes at 4 °C. The supernatant was subsequently removed, leaving only the cell pellet. The cells
were agitated with the lysis buffer using the pipette tip, then transferred into an eppendorf tube and maintained on ice, with constant agitation for 30 minutes. Afterwards, the cell and lysis buffer mixture was centrifuged at 13200 RPM, for 10 minutes at 4 °C. The supernatant was then aspirated and preserved in a fresh eppendorf tube and kept on ice. To determine the protein concentration, the bicinchoninic acid assay (BCA) (Pierce BCA protein assay kit, ThermoFisher Scientific, VIC, Australia) was applied.

Experimental timeline

1. Cultured cells reaching 80-85% confluence
   - Incubate for 3 days at 37 °C
2. Cells centrifuged at 1200 RPM
   - 5 minutes
3. Mixture of cells and lysis buffer were agitated and kept in ice
   - 30 minutes
4. Mixture centrifuged at 13200 RPM
   - 10 minutes
5. Protein sample mixed with BCA reagent
   - Incubate for 30 minutes at 37 °C
6. Then measure the absorbance at 540 nm
2.4.3. **BCA Assay**

The BCA protocol was used to measure protein concentrations of cell lysates and compare these to known standard sample concentrations. Protein samples and protein standards (Table 2.4.1) were combined with the BCA reagents using the published company protocol for this kit and the final product absorbance was measured using a spectrophotometer.

A clear 96 well plate (Sarstedt, Germany) was used to load the samples. The BCA standard samples were prepared using bovine serum albumin (BSA) in Kinexus buffer, standard samples (10 µL) were loaded in duplicate into each allocated well. The protein samples were prepared in 1:5 dilution (5 µL samples in 20 µL Kinexus buffer) then 10 µL of the protein samples loaded into the empty wells. Afterward, 200 µL of BCA mixture of reagent part A (BCA) and reagent part B (cupric sulphate) were added to standard and protein samples. The 96 well plate was covered with aluminium foil to avoid light exposure and incubated for 30 minutes at 37 °C. After the incubation period, the plate was placed into the Infinite M200 PRO-TECAN microplate reader (Tecan group, Switzerland) to measure the absorbance at 540 nm. Once the sample protein concentration was determined using the standard curve, the samples were diluted and aliquoted. The aliquots were frozen at -80 °C to avoid protein degradation.
<table>
<thead>
<tr>
<th>PCR tubes</th>
<th>BSA (µg/µL)</th>
<th>Diluent Volume (µL)</th>
<th>BSA Volume (µL)</th>
<th>From Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.5</td>
<td>0</td>
<td>21</td>
<td>2.0 Stock</td>
</tr>
<tr>
<td>B</td>
<td>1.5</td>
<td>13</td>
<td>39</td>
<td>2.0 Stock</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>14</td>
<td>28.0</td>
<td>From sample B</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>19.5</td>
<td>19.5</td>
<td>From sample C</td>
</tr>
<tr>
<td>E</td>
<td>0.25</td>
<td>16.5</td>
<td>16.5</td>
<td>From sample D</td>
</tr>
<tr>
<td>F</td>
<td>0.125</td>
<td>11</td>
<td>11.0</td>
<td>From sample E</td>
</tr>
<tr>
<td>G</td>
<td>Blank</td>
<td>21</td>
<td>0.0</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 2.4.1. Standard sample preparation for the BCA standard curve.
2.4.4. *Western Blot Assay*

The Western blot assay is a common technique used to separate and detect specific proteins from a complex mixture. This technique separates proteins by molecular weight and type using gel electrophoresis. Proteins are then transferred or blotted onto a membrane and the target protein detected using specific antibodies \(^{226-228}\). Western blot techniques allow the semi-quantification of proteins \(^{228}\).

A sample of the total protein (50 µg) was mixed in 6:1 loading buffer (a mixture of β-mercaptoethanol and 2x trypan blue loading dye) and the samples placed in a dry block heater (Ratek Instruments, Boronia, Australia) and denatured at 97 °C for 5 minutes. Samples were then immediately transferred onto ice in a cooler box (esky) and maintained on ice for another 5 minutes.

In addition, a loading control in the Western blot assay is essential to normalize protein expression levels across all wells \(^{229}\). In selecting a proper loading control for each experiment, there are a few factors that need to be considered including the protein molecular weight and sample type (tissue) \(^{229,230}\). For instance, a loading control should have a different molecular weight to the target protein either small or large enough to be detected from the target protein. Additionally, a selected loading control should be highly expressed in the sample \(^{229,230}\). Beta (β)-Actin (42 kDa) and GAPDH (36 kDa) are the most common loading controls due to their stable expression across all eukaryotic cell types \(^{229}\). Moreover, GAPDH is essential for glycolysis and has numerous roles in nuclear function including apoptosis and regulation of transcription \(^{229}\).
2.4.5. Preparation of Gel Electrophoresis

Proteins were separated according to their molecular weight using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which is suitable for medium molecular weight proteins up to 100 kDa. The SDS-PAGE gel consists of two parts: resolving gel and stacking gel. The resolving gel was prepared (Table 2.4.2) and loaded into 2/3 of the gel cassette (BioRad, NSW, Australia) and allowed to solidify for 45 minutes. Meanwhile, the stacking gel was prepared (Table 2.4.2) and added on top of the resolving gel, then the 15 well combs were inserted into the gels and left to solidify for another 35 minutes. The gels were then placed into the electrophoresis tank, which had been filled with 1X running buffer (3g/L Trizma Base, 14.4g/L Glysine, 1g/L SDS). The combs were subsequently removed and pre-stained protein ladder or molecular weight marker (ThermoFisher Scientific, VIC, Australia) was loaded in the first well and 28 µL of samples into the remaining wells. The gel electrophoresis was run at 150 volts for approximately 50 minutes until the samples reached the bottom of the gel.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Resolving Gel Volume (µL)</th>
<th>Stacking Gel Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>10000</td>
<td>1250</td>
</tr>
<tr>
<td>0.5M Tris-HCl pH 6.8</td>
<td>-------</td>
<td>3150</td>
</tr>
<tr>
<td>1.5M Tris-HCl pH 8.8</td>
<td>10000</td>
<td>-------</td>
</tr>
<tr>
<td>10% SDS</td>
<td>400</td>
<td>125</td>
</tr>
<tr>
<td>N,N,N',N'Tetramethylethylenediamine (TEMED)</td>
<td>20</td>
<td>12.5</td>
</tr>
<tr>
<td>10% Ammonium Persulfate (APS)</td>
<td>200</td>
<td>62.5</td>
</tr>
<tr>
<td>Distilled deionised water</td>
<td>19400</td>
<td>7.950</td>
</tr>
</tbody>
</table>

Table 2.4.2. Materials required for the SDS-PAGE electrophoresis gels used in the Western blot studies
2.4.6. *Electrotransfer Of Proteins Onto Membrane*

Blotting paper and sponge pads were immersed in the transfer buffer (Trizma Base 3g/L, Glysine 20g/L, Methanol 25%) for 10 minutes. The SDS-PAGE gels were cut from the top and bottom where there were no protein bands. In the meantime, the Immobilon-FL polyvinylidinedifluoride (PVDF) transfer membranes (Merck Millipore, VIC, Australia) were activated by soaking in methanol for 1 minute then rinsing in milli-Q water for a further 1 minute. Afterward, the cassettes were placed into the Western blot tray filled with transfer buffer and the cassette was prepared including a sponge pad, blotting paper, SDS-PAGE gels, PVDF transfer membranes, blotting paper and sponge pad. Once the components were assembled, the cassette was firmly closed. The tank was filled with transfer buffer and a block of ice was placed into the tank to keep it cool. The cassette was placed into the tank and the transfer gel was run at 75 volts for 1 hour and 30 minutes.
2.4.7. Membrane Blocking And Antibody Incubation

Following protein transfer, the PVDF transfer membranes were soaked in 1X TBST (Trizma Base 2.43g/L, NaCl 9g/L, HCl (for adjusting 7.6 pH) and 0.1% Tween 20) for 5 minutes, and then membranes were blocked with Odyssey Blocking Buffer (ThermoFisher Scientific, VIC, Australia) for one hour on a rocking platform at room temperature. The PVDF transfer membranes were washed with 1X TBS (Trizma Base 2.43g/L, NaCl 9g/L and HCl (for adjusting 7.6 pH)) for 5 minutes to remove the residue of the blocking buffer and then the PVDF transfer membranes incubated with the primary antibody (5 mL), on a rocking platform at 4 °C overnight. Tween 20 (0.1-0.2%) was added to the Odyssey blocking buffer, which was used to dilute antibodies.

The same protocol was applied in preparing the secondary antibody, however, 0.02% SDS was also added to the mixture. More information regarding primary and secondary antibody suppliers and concentrations is presented in Chapter 3.

To remove the residual antibody, the PVDF transfer membranes were washed 3 times with 1X TBST for 5 minutes using a rocking platform at room temperature. The PVDF transfer membranes were incubated in 5 mL of secondary antibody for 1 hour on a rocking platform away from light sources at room temperature. After the incubation period, the PVDF transfer membrane was washed 3 times with 1X TBST for 5 minutes on a rocking platform at room temperature in order to remove the remainder of the secondary antibody. The rinsed membranes were then washed with 1X TBS for 2 minutes, and the membranes were subsequently ready to scan on the Li-Cor Odyssey Western blotting system (LI-COR Biotechnology, USA).
2.4.8. Data Collection From Western Blot Assay

Images of the membranes were analysed using Image Studio application (Version 5.2.5 by Li-Cor Odyssey, USA). Data was collected by measuring the density and size of each protein band (Figure 2.4.1). The loading control β-Actin was used to normalize all the data. As a result, the signals from each band of the protein samples were divided by normalization factor of the same band in the normalization channel (Table 2.4.3).

Statistical analyses were performed for Western blot assay using an unpaired, two-tailed Student’s t-test for a single comparison and a one way-ANOVA for more than two or multiple comparisons. Data analysis was performed using version 6.0 of GraphPad Prism software. Values are presented as mean± SEM. A P-value of < 0.05 was considered to be statistically significant.
Figure 2.4.1. Image showing an example of measuring density of the protein band with the yellow numbers in lanes 1 and 2 indicating the signal strength of each band.
<table>
<thead>
<tr>
<th>Normalization Channel (β-Actin)</th>
<th>Signal</th>
<th>Normalization Factor</th>
<th>Target Protein</th>
<th>Signal</th>
<th>Normalized Signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>17100</td>
<td>17100 ÷ 26400*</td>
<td>Control 1</td>
<td>3170</td>
<td>3170 ÷ 0.741</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 0.741</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 2</td>
<td>19600</td>
<td>19600 ÷ 26400*</td>
<td>Control 2</td>
<td>2740</td>
<td>2740 ÷ 0.841</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 0.841</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 3</td>
<td>26400*</td>
<td>26400* ÷ 26400*</td>
<td>Control 3</td>
<td>2730</td>
<td>2730 ÷ 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>= 1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4.3. Showing example calculations of normalized data using the normalization channel (which is β-Actin). The red font (*) indicates the highest signal value and normalization factor values are indicated in blue font.
2.5. **TUBE FORMATION ASSAY (ANGIOGENESIS)**

Angiogenesis is an essential process required to develop new blood vessels from the existing vasculature to support wound healing, tissue and embryonic development. However, angiogenesis may play a pathological role in conditions such as metastasis and tumour growth and impaired wound healing as observed in diabetics. The tube formation assay is a quick, quantitative and highly reliable method to demonstrate *in vitro* angiogenic activity of endothelial cells. Also, the tube formation technique can examine the effect of various compounds on angiogenic and anti-angiogenic activity.

For more than 20 years, studies have demonstrated that the implementation of a basement membrane extracellular matrix is an appropriate environment to stimulate angiogenesis in endothelial cells. Endothelial cells rapidly attach to the reconstructed basement membrane extracellular matrix after seeding and form capillary-like structures *in vitro*. Basement membrane extracellular matrices have been prepared from extracts of the engelbreth-holm-swarm tumours (EHS), three-dimensional fibrin gel matrices, collagen gels from the collagen of rat tail tendons or alternatively are available from laboratory suppliers such as BD Biosciences (USA), Invitrogen (Australia) and others commercial sources. Commercially produced Matrigel is the most commonly reported basement membrane extracellular matrix over past 15 years. It is made from an extracellular gelatine form mixture and basement membrane proteins isolated from the murine Engelbreth Holm Swarm (EHS) tumour. Furthermore, growth factor reduced Matrigel (GFRM) is another form of the Matrigel.
matrix, in which the level of stimulatory cytokines and growth factors have
been significantly reduced to prevent overstimulation of endothelial cells that
may occur when using standard Matrigel.\textsuperscript{237, 240}

It is important to note that the formation of capillary-like structures are
specific for the various types of endothelial cells that are derived from
different tissues. Other cell types can form different structures\textsuperscript{235, 237, 241}. For
example, metastatic tumour cells will migrate and invade the basement
membrane matrix while salivary gland cells and mammary epithelial cells
form spheres with a central lumen on the matrix\textsuperscript{235, 237}. Furthermore, tube
formation assays using different types of endothelial cells such as human
umbilical vein endothelial cells (HUVEC), EA hy926 cells, calf pulmonary
artery endothelial cells (CPAECs) and bovine aortic endothelial cells (BAEC)
have been reported\textsuperscript{58, 216, 239, 242}.

The tube formation protocol was performed to examine the angiogenic activity
of the EA.hy926 cell line. The cell line was grown in a 75 cm\textsuperscript{2} tissue culture
flask until it reached 80% confluence. Geltrex (Reduced Growth Factor
Basement Membrane Matrix) (Invitrogen, VIC, Australia) was utilized to coat
a 96 well, flat clear bottom plate. The pipette tips and the 96 well plate were
maintained in the -20°C freezer for a day prior to the assay, to prevent any
solidification of the Geltrex during the experiment and when loading into the
wells\textsuperscript{216}.

Geltrex (50 µL) was loaded into the each well of the plate and incubated at
37 °C, 5% CO\textsubscript{2}, for 30-40 minutes to allow the gel to solidify. Afterwards, the
cells were harvested using 2 mL trypsin incubated in the flask for 5 minutes at
37 °C, 5% CO\textsubscript{2}. After the incubation period, 8 mL high glucose complete
media were added to the cells and mixed, then the cell suspension centrifuged for 5 minutes at 300 RCF at 4 °C. The supernatant was discarded and a sufficient amount of DMEM added to the cells to resuspend the cells. Cells (50,000) were loaded gently into the each well of a 96 well plate and the high glucose compete media together with the drug treatment were then added to give a final volume of 100 µL (more information about the treatment and its concentration in presented in subsequent chapters). The plate was incubated for a maximum of 18 hours at 37 °C, 5% CO₂.

2.5.1. Data Collection Of The Tube Formation Assay

The angiogenesis process was monitored through the Nikon ECLIPSE TS100 light microscope (Nikon, USA) with attached camera. The tube networks were photographed in six random selected microscopic fields after 4 hours incubation at the starting point of the tube formation, and after 18 hours at the end of incubation. The quantity of total number of junctions, nodes and branches in each individual image was determined using Angiogenesis Analyzer of Image J software (Figure 2.5.1)²¹⁶. Statistical analysis was completed using one-way ANOVA to determine significance of differences between groups and means of each group were compared to the control group. Values are presented as mean± SEM. Differences were considered statistically significant at P < 0.05.
Figure 2.5.1. Image showing an example of monitoring the tube formation after 18 hours and analysing the quantity of total number of junctions, nodes and branches.
CHAPTER 3

Role of Adenosine $A_1$, $A_{2A}$ and $A_{2B}$

Receptor in Wound Healing
CHAPTER 3: ROLE OF ADENOSINE $A_1$, $A_{2A}$ AND $A_{2B}$ RECEPTOR IN WOUND HEALING

3.1. INTRODUCTION

3.1.1. Adenosine

Adenosine is a ubiquitous purine nucleoside that is produced following the dephosphorylation of ATP and adenine nucleotides and it protects the tissues and the cells during various conditions such as tissue damage or hypoxic-ischaemic situations\textsuperscript{4,88}. Its role becomes important during times of metabolic stress and cellular energy imbalance caused by the difference in the amount of energy required and energy supplied to cells\textsuperscript{4,88}. Research has shown that the generation of adenosine can induce wound healing and angiogenesis in endothelial cells. Adenosine can also contribute to the development of fibrosis including pulmonary fibrosis, hepatic fibrosis, peritoneal fibrosis and cardiac fibrosis\textsuperscript{4}. Adenosine levels vary between 20-300 nM under physiological conditions and these levels may rise during conditions including intense exercise or in a low oxygen environment such as being at high altitude\textsuperscript{243}. Under severe pathological conditions such as ischaemia, the concentration of adenosine can rise to 30 \textmu M\textsuperscript{243,244}. 
3.1.2. **Signalling pathways of adenosine receptors in wound healing**

Repair of damaged tissues is a vital homeostatic mechanism. It comprises several phases including inflammation, neovascularization, tissue regeneration, and tissue reorganization \(^{41}\) (Chapter 1).

Adenosine and its receptors have essential roles in the production of matrix and stimulation of neovascularization, which both crucial process for tissue repair and wound healing \(^4,^{41}\). Conversely, adenosine by binding to its receptors, promotes the development of fibrosis in the skin, lungs and liver \(^{41}\).

3.1.3. **The Role of Adenosine A\(_1\) Receptor Agonists in Wound Healing**

Cyclopentyladenosine (CPA) has a similar structure to adenosine and is classified as a highly selective agonist for the adenosine A\(_1\) receptor as noted in Table 1.6.1 \(^{245}\). CPA, through binding to the adenosine A\(_1\) receptor located on many cell types, can reduce damage associated with ischaemia, attenuate calcium levels in the cytosol or reduce coronary vasodilation \(^{245-250}\). In addition, CPA through the activation of adenosine A\(_1\) receptors in macrophage cell lines mediates nitric oxide (NO) release as well as the production of inflammatory cytokines including IL-10 and TNF-\(\alpha\), which are essential for tissue repair \(^{128,189,245,251,252}\). *In vivo* studies using mice have shown that CPA stimulates the proliferation of endothelial cells, keratinocytes and fibroblasts \(^{245,250}\). Also CPA acts as a mitogen and has strong vasodilator properties, which support cell proliferation and wound healing \(^{245,250}\). Also, a dose–response relationship for the efficacy of CPA on human dermal microvascular endothelial cell proliferation has been reported \(^{245}\).
3.1.4. The Role of Adenosine $A_{2A}$ and $A_{2B}$ Receptor Agonists in Wound Healing

Adenosine $A_{2A}$ and $A_{2B}$ receptor agonists both cooperate to promote angiogenesis and migration of endothelial cells and fibroblasts through cAMP- and PKA (protein kinase A) - dependent pathways $^5,97$. A study using human coronary endothelial cells reported the presence of the adenosine $A_{2A}$ receptors on the cell surface that stimulates an increase in cAMP production $^89$. Also, another study using human aortic endothelial cells in culture, observed messenger RNA encoding adenosine $A_{2A}$ and $A_{2B}$ receptors $^{253,254}$. Activation of the adenosine $A_{2B}$ and $A_3$ receptors in human mast cells stimulates angiogenesis through the release of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) while in microvascular endothelial cells they elevate the expression of angiopoietin 2 $^{255}$. Adenosine $A_{2B}$ receptors through $G_q$ protein signaling activate the expression of angiogenic factors IL-8, VEGF and basic fibroblast growth (bFGF) in the human microvascular endothelial cell lines (HMEC-1) $^{71}$.

Moreover, the rate of cell proliferation has been reported to be enhanced by different concentrations of adenosine when applied to human umbilical vein endothelial cell lines (HUVEC) through the stimulation of VEGF production $^{256}$. Endothelial cells in the vasculature generate adenosine from adenine nucleotides and respond to adenosine through increased proliferation and migration $^4,120,257-260$. Adenosine also causes vascular leakage through coronary vasodilation $^4,120,257-260$. In addition to the promotion of wound healing following activation of adenosine receptors, stimulation of the adenosine $A_{2A}$ receptors may also enhance extracellular matrix production in
healing wounds. Human foreskin dermal fibroblasts were recognized to express adenosine A₁ and A₂ receptors that produce matrix through regulating intracellular levels of cAMP. Also, the stimulation of adenosine A₂A receptors in these cells directly increases collagen production. A study using a bleomycin-induced dermal fibrosis model reported that stimulation of adenosine A₂A receptors is responsible for increased collagen production in the skin. Adenosine A₂A receptor knockout mice or mice treated with the adenosine A₂A receptor antagonist, such as ZM241385, were protected from increased diffuse dermal fibrosis. While the receptor populations on the endothelial cells have been identified there is limited published research investigating selective adenosine A₂A and A₂B receptor agonists or antagonists.

### 3.1.5. The Role of Adenosine A₃ Receptor Agonists in Wound Healing

The adenosine A₃ receptor couples to several G proteins such as G₁ α₂, G₁ α₃ and G₉ α. The adenosine A₃ receptor has been reported to induce selective RhoA-dependent activation of phospholipase D (PLD). Also adenosine A₃ receptor activation modulates mitogen-activated protein kinase (MAPK) signalling and stimulates protective extracellular signal-regulated kinases (ERK1/2) in cardiomyocytes and contributes to a protective function in cardiovascular diseases. The adenosine A₃ receptor signalling plays a principally anti-inflammatory role in bleomycin-treated mice, however it also acts to elevate the levels of matrix metalloproteinase-9 (MMP-9) that can stimulate the activation of transforming growth factor (TGF)-β1 and downstream fibrotic pathways. Moreover, this study
reported that stimulation of the adenosine A\textsubscript{3} receptors can contribute to the intensification of pulmonary fibrosis through regulation of MMP-9, and/or mediating eosinophil activation\textsuperscript{268}. Therefore, the adenosine A\textsubscript{3} receptors can contribute to fibrotic and inflammatory processes and can adjust the balance between normal wound closure and the development of tissue fibrosis\textsuperscript{268}. Furthermore, adenosine A\textsubscript{3} receptor agonists have the capacity to provide protective responses as well as adverse inflammatory responses in the heart and vasculature\textsuperscript{265}. Several studies using \textit{in vivo} mice models or \textit{in vitro} (neuronal cells, macrophages and bone marrow cells) protocols have demonstrated that the exogenous activation of adenosine A\textsubscript{3} receptors leads to considerable cardioprotection and tissue injury prevention during and following ischaemia reperfusion\textsuperscript{265,269-272}. Adenosine A\textsubscript{3} receptors can induce a vasoprotective response and have a significant role in vascular injury and modifying the post-ischaemic myocardial phenotype\textsuperscript{265,273}. During tissue injury, tumor growth, ischaemia and inflammation, adenosine is released into the extracellular space and stimulates the secretion of vascular endothelial growth factor (VEGF) and interleukin 8 (IL8) through adenosine A\textsubscript{2B} receptors and it also stimulates angiopoietin-2 secretion through adenosine A\textsubscript{3} receptors\textsuperscript{255}. As a result, the adenosine A\textsubscript{3} receptor on mast cells and human umbilical vein endothelial cells (HUVEC) acts as a promoter of angiogenic signals\textsuperscript{255}.

While all the adenosine receptor subtypes have been implicated in cell proliferation and angiogenesis, this study focuses on the adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptors as mRNA of the adenosine A\textsubscript{3} receptor has not been found in previous studies using the EA.hy926 endothelial cells.
3.2. AIMS

The aims of this research are as follows:

1. To measure the effect of selective adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor agonists and antagonists on the rate of wound closure in EA.hy926 endothelial cells, using the wound healing scratch assay \textit{in vitro}.

2. To determine the effect of selective adenosine receptor agonists and antagonists in stimulating EA.hy926 endothelial cell proliferation.

3. To establish the protein expression level of adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor subtypes in EA.hy926 endothelial cells, using Western-blotting assays.

3.3. METHODS AND MATERIALS

3.3.1. Preparation of Adenosine Receptor Agonists And Antagonists

The selective adenosine receptor agonists and antagonists used in this study included:

- \text{N}\textsuperscript{6}-cyclopentyladenosine (CPA, agonist) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, antagonist) for the adenosine A\textsubscript{1} receptor;

- \text{2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680, agonist)} and \text{4-(-2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385, antagonist)} for the adenosine A\textsubscript{2A} receptor; and

- \text{5’-N-ethylcarboxamidoadenosine (NECA, agonist)} and \text{N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1754, antagonist)} for the adenosine A\textsubscript{2B} receptor.
The adenosine A₃ receptor agonist and antagonist were not utilised for these studies as previous work in our laboratory (unpublished data), have shown that there is no mRNA expression for the adenosine A₃ receptors in the EA.hy926 endothelial cell line. Furthermore, this work showed that the addition of the non-selective adenosine receptor antagonist 8-sulphylphenyltheophylline did not affect the rate of wound closure in the scratch assay, indicating that residual adenosine (if it is released) in cell culture conditions does not affect cell function in this assay.

It should also be noted that NECA is a non-selective agonist for the adenosine A₂ receptors, hence ZM241385 (10 nM) was added to experiments exploring adenosine A₂B receptor function to exclude the adenosine A₂A receptor. Different concentrations of agonists were utilised (1-300 nM) in the initial scratch assay experiments to obtain concentration response curves, determine the EC₅₀ value and establish an appropriate concentration of agonist required for rest of the experiments. The Kᵢ values for adenosine receptor antagonists are as follows, DPCPX (adenosine A₁ receptor, 3.8 nM), ZM241385 (adenosine A₂A receptor, 1.4 nM) and MRS1754 (adenosine A₂B receptor, 1.97 nM). A final concentration of 10 nM was used for all the adenosine receptor antagonists in this study.

Stock solutions of the adenosine agonists and antagonists (1 mM) were dissolved in DMSO and stored as aliquots in the -20 °C freezer until further use. All drugs were freshly prepared on the day of the experiments from thawed stock solution by diluting with high glucose complete media to reach the concentration required for experiments. The reagents used in this experiment are DMSO, CPA, DPCPX, NECA, MRS1754 and CGS21680.
from Sigma Aldrich, NSW, Australia and ZM241385 from Tocris Bioscience, Bristol, UK.

3.3.2. **Wound Healing Scratch Assay**

EA.hy926 endothelial cells (2x10⁵ cells) in high glucose complete media were seeded into the 12-well plates in triplicate, then incubated until the cells reached 80-85% confluence. The cell layer was then scratched with a 200 µL pipette tip and prepared agonists and antagonists were loaded into the wells. The wounds were photographed at the starting point of the treatment (0 hours) and at 2, 4 and 6 hours (end of the treatment). For more detail on this assay refer to Chapter 2. For the vehicle control, diluted DMSO was added to the scratch assay. In these experiments DMSO (0.03%) did reduce wound closure rates when compared to control conditions. Analysis of the concentration response curves to the selective adenosine receptors agonists were performed using GraphPad Prism software (CA, USA) (version 6.0).

3.3.3. **Cell Proliferation Assay**

The EA.hy926 endothelial cells were seeded in triplicate at 5000 cells/well in 96 well plates and also, 4000, 5000, 6000, 7000, 8000 and 9000 cells were seeded in duplicate per well, respectively, to form the standard curve. They were all incubated for 24 h at 37 ºC, 5% CO₂ (Chapter 2). The adenosine receptor agonists and antagonists were prepared using high glucose complete media at 30 nM and 10 nM concentrations respectively. In addition, for control experiments cells were treated with normal high glucose complete
media and vehicle controls using DMSO at two different dilutions of 0.004% and 0.03% v/v (Table 3.3.1). The plate was placed into the micro-plate reader and the absorbance measured at 450 nm (Chapter 2).
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>CPA</td>
<td>CPA 30 nM + high glucose Complete Media</td>
</tr>
<tr>
<td>DPCPX</td>
<td>DPCPX 10 nM + high glucose Complete Media</td>
</tr>
<tr>
<td>CPA+ DPCPX</td>
<td>CPA 30 nM + DPCPX 10 nM + high glucose Complete Media</td>
</tr>
<tr>
<td>CGS21680</td>
<td>CGS21680 30 nM + high glucose Complete Media</td>
</tr>
<tr>
<td>ZM 241385</td>
<td>ZM241385 10 nM + high glucose Complete Media</td>
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<td>CGS21680 + ZM 241385</td>
<td>CGS21680 30 nM + ZM241385 10 nM + high glucose Complete Media</td>
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<tr>
<td>NECA</td>
<td>NECA 30 nM + ZM241385 10 nM + high glucose Complete Media</td>
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<td>MRS1754</td>
<td>MRS1754 10 nM + high glucose Complete Media</td>
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<td>Complete Media</td>
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<tr>
<td>Control</td>
<td>Cells + high glucose Complete Media</td>
</tr>
<tr>
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<td>0.004% DMSO + high glucose Complete Media</td>
</tr>
<tr>
<td>Vehicle Control 2</td>
<td>0.03% DMSO + high glucose Complete Media</td>
</tr>
<tr>
<td>Negative Control</td>
<td>No cells –only high glucose Complete Media</td>
</tr>
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</table>

Table 3.3.1. Treatment conditions and the concentrations
3.3.4. Western Blotting Technique

The EA.hy926 endothelial cells were harvested, lysed and prepared for the Western blotting technique as explained in Chapter 2. Protein samples (50 µg) were loaded into the gels and run at 150 volts for approximately 50 minutes until the samples reached the bottom of the gel. The protein samples were then transferred to PVDF membranes using 75 volts for 1 hour and 30 minutes. The membranes were treated with blocking buffer for 1 hour then they were incubated overnight at 4°C with rabbit polyclonal primary antibody for adenosine A<sub>1</sub> receptor (1:500) (Abcam, Cambridge, UK), adenosine A<sub>2A</sub> receptor (1:500) (Santa Cruz Biotechnology, CA, USA) or adenosine A<sub>2B</sub> receptor (1:200) (Merck Millipore, VIC, Australia). Afterward, the membranes were incubated in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000) for adenosine A<sub>1</sub> and A<sub>2A</sub> receptor and (1:500) for adenosine A<sub>2B</sub> receptor (Santa Cruz Biotechnology, CA, USA) for 1 hour at room temperature. Furthermore, beta (β)-Actin antibody was using as a loading control (1:1000) (Thermo Fisher, VIC, Australia) for all three adenosine receptor subtypes and a horseradish peroxidase-conjugated goat anti-rabbit antibody was used as the secondary antibody (1:3000).

The expression of the proteins was examined by comparing their size with protein ladder or molecular weight markers.
3.4. RESULTS

3.4.1. The Effect Of Selective Adenosine Receptor Agonists On EA.hy926 Endothelial Cells Using The Wound Healing Scratch Assay

The data for the wound healing scratch assay was obtained from measuring the width of the scratch at 2 hourly intervals, using Image J software. The average speed of wound healing was examined by comparing the effect of different concentrations of adenosine receptor agonists. The results were analysed using a one way ANOVA. The effective concentration producing 50% maximal effective concentration response (EC$_{50}$) to agonist (log concentration) was calculated based on curve fitting using GraphPad Prism (Figure 3.4.1).
Figure 3.4.1. Concentration response curves (CRC) showing the effect of selective adenosine receptor agonists on EA.hy926 endothelial cells and the rate of wound healing, n= 6 per group.
3.4.2. Wound Healing Scratch Assay Using The Selective Adenosine A$_1$ Receptor Agonist

The adenosine A$_1$ receptor agonist CPA caused a significant concentration dependant increase in the rate of wound healing at all concentrations of CPA when compared to the control group (P< 0.0001) (Figures 3.4.2 and 3.4.3). The EC$_{50}$ value for CPA in the wound healing scratch assay of EA.hy926 endothelial cell was 7.16 x 10$^{-9}$ M (confidence intervals 4.75 x 10$^{-9}$ – 1.08 x 10$^{-8}$ M). The data also shows that the efficacy of CPA and its ability to stimulate wound healing was greater than the two other selective adenosine receptor agonists, CGS21680 and NECA (adenosine A$_{2A}$ receptor and A$_{2B}$ receptor agonists respectively) (Figure 3.4.1). However, CPA was not the most potent agonist in stimulating the wound healing scratch assay with the order of potency being CGS21680 < CPA < NECA (Figure 3.4.1).
Figure 3.4.2. Wound healing scratch assay using different concentrations of CPA on EA.hy926 endothelial cells. CPA concentrations from left to right are control (0 nM), 1 nM, 3 nM, 10 nM, 30 nM, 100 nM and 300 nM CPA. Magnification x40.
Figure 3.4.3. Graph showing the rate of the wound healing with increasing CPA concentrations compared to the control.
Data is represented as mean ± SE, n= 6, **P < 0.01, ***P < 0.001 and **** P < 0.0001 versus control (0).
3.4.3. Wound Healing Scratch Assay Using The Selective Adenosine $A_{2A}$ Receptor Agonist

CGS21680 was used as a selective agonist for the adenosine $A_{2A}$ receptors. The rates of wound healing at all concentrations of CGS21680 were significantly increased when compared to the control group (P< 0.0001) (Figures 3.4.4 and 3.4.5). The EC$_{50}$ value for CGS21680 in the wound healing scratch assay of EA.hy926 endothelial cells was 3.25 x 10$^{-9}$ M (confidence intervals 2.83 x 10$^{-10}$ – 3.74 x 10$^{-8}$ M). The CGS21680 was more potent than the other two adenosine $A_1$ and $A_{2B}$ receptor agonists (CPA and NECA) used in this assay (Figure 3.4.1). While CGS21680 was the most potent of the selective agonists used in this assay, its efficacy was low when compared to the other two agonists with respect to rates of wound healing.
Figure 3.4.4. Wound healing scratch assay using different concentrations of CGS21680 (CGS) on EA.hy926 endothelial cells. CGS concentrations from left to right are: control group (0 nM), 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM. Magnification x40.
Figure 3.4.5. Graph illustrating the rate of the wound healing with increasing CGS21680 concentrations compared to the control. Data is represented as mean ± SE, n=6, *P < 0.05, **P < 0.01 and **** P < 0.0001 versus control (0).
3.4.4. Wound Healing Scratch Assay Using The Selective Adenosine $A_{2B}$ Receptor Agonist

NECA was used as an agonist for adenosine $A_{2B}$ receptor in this study, but as previously explained, NECA is a non-selective agonist for the adenosine $A_{2}$ receptor. Therefore, NECA was combined with the adenosine $A_{2A}$ receptor antagonist ZM241385 (10 nM) to block the adenosine $A_{2A}$ subtype. The rates of wound healing at all concentrations of NECA were significantly increased when compared to the control group ($P < 0.05$) to ($P < 0.0001$). (Figures 3.4.6 and 3.4.7). The EC$_{50}$ value for NECA in the wound healing scratch assay of EA.hy926 endothelial cells was $1.48 \times 10^{-8}$ M (confidence intervals $2.83 \times 10^{-10} – 3.74 \times 10^{-8}$ M). Of the three agonists tested, NECA was the least potent on EA.hy926 endothelial cells in stimulating wound healing in the scratch assay (Figure 3.4.1).
Figure 3.4.6. Wound healing scratch assay using different concentrations of NECA combined with ZM241385 (10 nM) on EA.hy926 endothelial cells. NECA concentrations from left to right are: control group (0 nM), 1 nM, 3 nM, 10 nM, 30 nM, 100 nM and 300 nM. Magnification x40
Figure 3.4.7. Graph showing the rate of the wound healing with increasing NECA concentrations compared to the control. Data is represented as mean ± SE, n= 6, *P < 0.05, **P < 0.01, *** P < 0.001 and **** P < 0.0001 versus control (0)
3.4.5. The Effect Of Selective Adenosine Receptor Antagonists On Agonist Induced Increases In Rates Of Wound Closure

Selective adenosine receptor antagonists were used in the EA.hy926 endothelial cell wound healing scratch assay. Single concentrations of selective adenosine receptor agonists (30 nM) were used in the wound healing scratch assay and tested against selective adenosine receptor antagonists at 10 nM. The rates of wound healing with all the adenosine receptor agonists increased as follows; adenosine A\textsubscript{1} receptor agonist (CPA 30 nM) increased by 42.29%, adenosine A\textsubscript{2A} receptor agonist (CGS21680 30 nM) rose by 49% and adenosine A\textsubscript{2B} receptor agonist (NECA 30 nM) was elevated by 20.57% when compared with their respective control group (P < 0.0001) (Figure 3.4.8).

The selective adenosine A\textsubscript{1} and A\textsubscript{2B} receptor antagonists (DPCPX 10 nM) and (MRS1754 10 nM) considerably decreased the rate of wound healing in comparison with the control group respectively (P < 0.0001) (Figure 3.4.8). Furthermore, the wound closure rate was significantly reduced by the selective adenosine A\textsubscript{2A} receptor antagonist (ZM241385 10 nM) when compared to its control group (P < 0.05) (Figure 3.4.8). The combined group included a mixture of the selective adenosine receptor agonist (30 nM) with its relevant selective adenosine receptor antagonist (10 nM) such as (CPA+DPCPX), (CGS21680+ ZM241385) and (NECA+MRS1754). These results show the rate of wound healing has been significantly decreased with the combined group in comparison with its relevant selective adenosine agonist alone as CPA, CGS21680 and NECA, respectively (P<0.0001) (Figure 3.4.8).
Figure 3.4.8. Graph showing the effect of adenosine receptor agonist, antagonist and their combination on rate of wound healing. One-Way ANOVA was applied to analyse data. Data is expressed as mean ± SEM, n= 6, **P < 0.01, *** P < 0.001 and **** P < 0.0001 versus the control. CGS21680 (CGS), ZM241385 (ZM) and MRS1754 (MRS)
3.4.6. Effect Of Selective Adenosine Receptor Agonists And Antagonists On Cell Proliferation

A standard curve was generated to convert sample absorbance values into cell numbers using 4000, 5000, 6000, 7000, 8000 and 9000 cells per well. Analysis of the standard curve showed that absorbance values were proportional to cell numbers, as indicated by figure 2.3.2 in Chapter 2.

The results show that cell proliferation was significantly decreased by the vehicle control treatment (0.004% and 0.03% DMSO) when compared with the control group (P < 0.0001) (Figure 3.4.9). Also, increased cell proliferation was observed in cells treated with the adenosine A₁ and A₂B receptor agonists (CPA 30 nM and NECA 30 nM) when compared with the control groups (P < 0.01) and (P < 0.05) respectively (Figure 3.4.9). However, in comparison with control groups, the other treatment groups did not show any changes (P > 0.05). Figure 3.4.9 shows that all the adenosine receptor subtype agonists (CPA, CGS21680 and NECA 30 nM) increased cell proliferation but they did not have any significant differences when compared with each other (P > 0.05). Moreover, the results showed that agonist-induced cell proliferation was reversed by antagonist treatment; (CPA+DPCPX), (CGS21680+ ZM241385) and (NECA+MRS1754). As a result, the effects of the adenosine receptor agonists were blocked by their selective adenosine receptor antagonists.
Figure 3.4.9. Graph showing the effect of different treatments on cell proliferation. Control group or untreated (Ctrl), vehicle control group (0.004% and 0.03% DMSO), Adenosine receptor agonists 30 nM (CPA, CGS21680 and NECA), Adenosine receptor antagonists 10nM (DPCPX, ZM241385 and MRS1754) and combination group (mixture of adenosine receptor agonist 30 nM with antagonist 10 nM). The data was obtained through one way ANOVA. Data is expressed as mean ± SEM n= 6, *P < 0.05, **P < 0.01, *** P < 0.001 and **** P < 0.0001 versus control.
3.4.7. Protein Expression Of Adenosine Receptor Subtypes On EA.hy926 Cells

The results of Western blotting techniques confirmed the presence of adenosine A₁, A₂A and A₂B receptors of expected molecular size A₁ (∼37 kDa), A₂A (∼45 kDa) and A₂B (∼50-52 kDa) in EA.hy926 endothelial cells. Furthermore, β-actin with a molecular size of (∼42 kDa) was used as a loading control (Figure 3.4.10). As figure 3.4.10 shows, three different blots were utilized for each adenosine A₁, A₂A and A₂B receptor antibody and they were individually analysed against internal controls.

Data analysis shows that the protein expression of adenosine A₂A receptor was considerably higher in EA.hy926 endothelial cells when compared with adenosine A₁ and A₂B receptors (P < 0.05) and (P < 0.001) respectively (Figure 3.4.11). Moreover, the adenosine A₁ receptor had higher expression when compared with adenosine A₂B receptors (P < 0.01) (Figure 3.4.11).
Figure 3.4.10. Western blot images showing protein bands of (A) A₁ ≈ 37 kDa, (B) A₂A ≈ 45 kDa, (C) A₂B ≈ 50-52 kDa and (D) β-actin ≈ 42 kDa. In all images the first lane contains the molecular weight marker. Each protein sample is loaded in the subsequent wells in triplicate.
Figure 3.4.11. Graph demonstrating the relative expression of adenosine A₁, A₂A and A₂B receptor subtypes on E.Ahy926 endothelial cells. The data was analysed using one way ANOVA. Data is expressed as mean ± SEM (n=6), *P < 0.05, **P < 0.01 and *** P < 0.001
3.5. DISCUSSION

This study measured the effect of selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists and antagonists on the rate of wound closure in EA.hy926 endothelial cells. This project also determined the effect of selective adenosine receptor agonists on cell proliferation and tube formation and determined the protein expression level of the adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor subtypes in EA.hy926 endothelial cells. Many studies have used cultured endothelial cells to model different physiological and pathological conditions, particularly in the study of angiogenesis. Primary endothelial cells have a restricted lifespan and they can demonstrate characteristics that vary due to their multi-donor origin. Immortalized cell lines have been reported to be generally better characterized and more constant in their endothelial properties than primary endothelial cells that were given an extended life span. This study reported that the best characterized human macro vascular endothelial cell lines and human micro vascular endothelial cell lines are EA.hy926 and HMEC-1, respectively. The permanent human endothelial hybrid cell line (EA.hy926) was generated by hybridisation of primary human umbilical vein endothelial cells (HUVEC) and the human lung carcinoma cell line (A549/8). Many studies have used the EA.hy926 endothelial cell line. For example, one study using these cells reported that the compound cordycepin (3'-deoxyadenosine) significantly decreased cell proliferation and angiogenesis and inhibited cell migration.

The results of this study illustrate that the rates of wound healing in EAhy926 endothelial cells were significantly increased by the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists (CPA, CGS21680 and NECA respectively).
when compared with their control group. Also, the concentration response curves to the selective adenosine A₁, A₂ₐ and A₂ₐ receptors show that the rate of wound healing was concentration dependent. Adenosine exerts its effects via G-protein coupled cell surface receptors that can be divided into four subtypes: adenosine A₁ and A₃ receptors that inhibit adenylyl cyclase and adenosine A₂ₐ and A₂ₐ receptors that stimulate adenylyl cyclase ²⁴⁵. Other research has determined that stimulation of adenosine receptors increased endothelial cell migration via cAMP- and PKA-dependent pathways ⁵. Previous studies investigating cells in the wound area observed that the cells were distributed into two cell cycle phases; the log phase (G₁) and the quiescent phase (G₀) ²⁴⁵. A study using human fibroblasts and endothelial cells reported that both cell types in G₁ and G₀ phases responded in a dose-dependent manner to the adenosine A₁ receptor agonist (CPA). Also, studies using human endothelial cells confirmed that adenosine is a mitogen for endothelial cells ²⁴⁵,²⁵⁶,²⁷⁶. Another study observed that the topical application of the selective adenosine A₂ₐ receptor agonist (CGS21680) in mice, significantly increased the wound closure rates when compared with mice treated with vehicle 1.5% methylcellulose (control group) ⁵.

The efficacy and potency of the three selective adenosine receptor agonists (CPA, CGS21680 and NECA) on the rate of wound healing varies in the EAHy926 endothelial cell line. For instance, the adenosine A₂ₐ receptor agonist (CGS21680) was more potent than CPA and NECA (adenosine A₁ and A₂ₐ receptors agonist respectively) on wound closure. On the other hand, the efficacy of CPA in rate of wound healing in EAHy926 endothelial cells was greater than CGS21680 and NECA. Similar to previous literature, the
adenosine A\textsubscript{2B} receptors agonist (NECA) were the least potent and had reduced efficacy in stimulating wound healing when compared to the other two adenosine receptor agonists (CPA and CGS21680). The adenosine A\textsubscript{2B} receptor has the lowest affinity for selective agonists, that is, the agonists for the other receptor subtypes have potencies in the low nanomolar range while the most potent of adenosine A\textsubscript{2B} receptor agonists have affinities around the 1 \( \mu \text{M} \) range\textsuperscript{91}. Similarly, a study using human umbilical vein endothelial cells reported that the selective adenosine A\textsubscript{2A} receptor agonist CGS 21680 was more potent in activating adenylyl cyclase production than the non-selective adenosine A\textsubscript{2B} receptor agonist (NECA). Although NECA was less potent than CGS21680, it exhibited higher efficacy in the activation of adenylyl cyclase\textsuperscript{71}. The rates of wound healing in EAhy926 endothelia cells were significantly increased by all three adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor agonists (CPA, CGS21680 and NECA) when compared to their control groups and relevant selective adenosine receptor antagonists (DPCPX, ZM241385 and MRS1754 respectively). Also, when the selective agonists and antagonists were used together, the antagonists decreased wound closure rates indicating that all the adenosine receptor antagonists were able to reverse the effect of adenosine receptor agonists on the rate of wound healing in EAhy926 endothelial cells. This indicates that all three adenosine receptor subtypes studied in these cells induce wound healing. Moreover, an \textit{in vivo} study using mice showed that the topical application of the selective adenosine A\textsubscript{2} receptor antagonist DMPX (3,7-dimethyl-1-propargyl xanthine) did not affect the rate of wound healing itself, however it reversed the effect of the selective adenosine A\textsubscript{2} receptor agonist (CGS21680) on the rate of wound closure\textsuperscript{5}. It should be noted that
DMPX and ZM 241385 are highly selective adenosine A\textsubscript{2A} receptor antagonists. Thus both of them are able to reverse the effect of the selective adenosine A\textsubscript{2A} receptor agonist (CGS-21680)\textsuperscript{277}. A decrease in rates of cell proliferation and wound healing was also caused by the addition of the vehicle control DMSO, which was used to make the stock solutions of the adenosine receptor agonists and antagonists. As observed in the proliferation assay, DMSO (vehicle control) significantly decreased cell proliferation in EA.hy926. This study also showed that the adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor agonists stimulated the proliferation of EAhy926 endothelial cells, an effect inhibited by their respective adenosine receptor antagonists. The adenosine receptor subtypes have been reported to stimulate cell proliferation and wound healing in other in vitro studies, as well as in vivo studies. The adenosine A\textsubscript{1} and A\textsubscript{2B} receptors have been reported to regulate proliferation and cell differentiation in vascular smooth muscle cells and to promote cell proliferation and migration of endothelial cells in wound healing studies using mice\textsuperscript{17, 91, 278}. Moreover, the selective adenosine A\textsubscript{1} receptor agonist (CPA) has been shown to significantly increase the proliferation of keratinocytes, fibroblasts and endothelial cells using bromodeoxyuridine labelled cells in an in vivo wound healing study in mice\textsuperscript{245}. The non-selective adenosine A\textsubscript{2B} receptor agonist (NECA) stimulates retinal angiogenesis, cell proliferation and cell migration as well as stimulating cAMP response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK) signalling pathways associated with cell survival and proliferation\textsuperscript{113}. This same study also reported that selective adenosine A\textsubscript{2B} receptor antagonists blocked the effect of NECA and attenuated the proliferation of
human retinal endothelial cells, leading to abnormal angiogenesis as observed in diabetic retinopathy. Western blotting results of this study determined that the protein expression of adenosine A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptor subtypes varies in the EAhy926 endothelial cells. This study showed that the adenosine A<sub>2A</sub> receptors are predominantly expressed in EAhy926 endothelial cells when compared to the adenosine A<sub>1</sub> and A<sub>2B</sub> receptors. The expression of adenosine receptor subtypes have been reported to vary in different cell lines such as isolated cerebral arterial muscle cells (CAMCs), dermal fibroblast and human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells-1 (HMEC-1) and human and porcine coronary artery endothelial cells. The presence of the A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors subtypes were observed in cultured dermal fibroblasts and human umbilical vein endothelial cells using the Western blotting technique and reverse transcriptase–PCR were expressed in both cell lines. In contrast, the adenosine A<sub>1</sub> receptor was only expressed in human umbilical vein endothelial cells (HUVECs) but not in dermal fibroblasts. Previous studies investigating the expression of adenosine A<sub>2A</sub> and A<sub>2B</sub> receptors reported that the adenosine A<sub>2A</sub> receptors were the predominant subtype and highly expressed in human umbilical vein endothelial cells (HUVECs), human and porcine coronary artery endothelial cells. In contrast, the expression of adenosine A<sub>2B</sub> receptors was higher in human retinal microvascular endothelial cells and human microvascular endothelial cells-1 (HMEC-1). Feoktistov et al., 2002 suggested that the adenosine receptor populations might depend on location in the vasculature, with the adenosine A<sub>2A</sub> receptor subtypes predominantly
expressed in endothelial cells lining large conduit vessels while the adenosine $A_{2B}$ receptor subtypes were mainly expressed in endothelial cells lining capillaries.\textsuperscript{71} These experiments were completed in high glucose cell culture media and the results observed in this study may be relevant to the high glucose environment observed in humans with Diabetes Mellitus. A comparison of the effects of high and low glucose concentrations in the media on EAhy926 endothelial cell function in the wound healing assay can be seen in Chapter 6.

### 3.6. CONCLUSION

In summary, the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptors agonists (CPA, CGS21680 and NECA) significantly increased the rate of the wound healing and cell proliferation in EAhy926 endothelial cells. In contrast, the selective adenosine receptors antagonists DPCPX ($A_1$), ZM241385 ($A_{2A}$) and MRS1754 ($A_{2B}$) considerably reduced wound healing rates and proliferation of EAhy926 endothelial cells and blocked the effect of their relevant adenosine receptor agonists. Furthermore, the selective adenosine $A_{2A}$ receptor agonist (CGS21680) was more potent than the other adenosine receptor agonists but less efficacious than selective adenosine $A_1$ receptor agonists (CPA). This study reported the protein levels of the adenosine $A_{2A}$ and $A_1$ receptors are highly expressed in EAhy926 endothelial cell lines. However, the adenosine $A_{2B}$ receptor protein is also expressed in EAhy926 endothelial cells but in reduced levels compared to the other two subtypes. This work suggests that all three adenosine receptors can individually stimulate cell proliferation and
wound healing in the EA.hy926 endothelial cells and probably work together when the non-selective physiological agonist adenosine is released.
CHAPTER 4

EFFECT OF ACUTE CAFFEINE ON

ADENOSINE RECEPTOR MEDIATED

WOUND HEALING
CHAPTER 4: EFFECT OF ACUTE CAFFEINE ON ADENOSINE RECEPTOR MEDIATED WOUND HEALING

4.1. INTRODUCTION

4.1.1. Acute caffeine

Caffeine (1,3,7-trimethylxanthine) is one of the most commonly used psychostimulants that is consumed in the form of beverages and food by almost 70% of world’s population with an average consumption of 200-250 mg per day per person. Following ingestion of a single cup of coffee (which contains 80 mg of caffeine) by a 70 kg adult, the peak plasma caffeine concentration will occur between 15 and 120 minutes after oral ingestion and equates to a plasma concentration 8 to 10 mg/L. Caffeine is mostly present in coffee, which includes trace amount of theophylline. Studies have reported that caffeine acts as a non-selective antagonist of all the adenosine receptor subtypes. Caffeine blocks the actions of endogenously released adenosine which can tonically activates its receptors. Therefore, caffeine potentially affects all areas of the brain where adenosine receptors are expressed and endogenous adenosine can exert its effects. Caffeine can also reduce adenosine A2A receptor mediated activity during various pathological conditions and has been reported to attenuate brain damage in Parkinson's disease, ischaemic brain injury associated excitotoxicity and experimental allergic encephalitis. Conversely, caffeine can exacerbate acute liver damage and elevate the level of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-4.
Previous studies have demonstrated that caffeine has many actions other than blocking the adenosine receptors (especially adenosine A2A receptor) \( \textsuperscript{40, 45, 175, 176} \). Caffeine can act on many other targets such as cAMP phosphodiesterase and phosphatidylinositol 3-kinase (PI3K) \( \textsuperscript{40, 45, 175, 176} \). Caffeine, at higher concentrations, is a phosphodiesterase inhibitor and through increasing cAMP content in immune cells can exert immunosuppressive effects to attenuate tissue damage during an acute inflammatory injury \( \textsuperscript{45, 175-177} \). Studies have reported that cAMP is a key cellular regulator that mediates various effects including cell adhesion, cytoskeletal dynamics and cell migration and it is a unique biofactor associated with tissue repair and regeneration \( \textsuperscript{286-289} \). Caffeine can increase cytosolic \( \text{Ca}^{2+} \) and cAMP via ryanodine receptor activation and phosphodiesterase (PDE) inhibition, respectively \( \textsuperscript{286-289} \). Moreover, studies have demonstrated that intracellular cAMP levels can be increased by forskolin which associates with adenylyl cyclase while, cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL-12330A) is an adenylyl cyclase inhibitor that can decrease the levels of cAMP \( \textsuperscript{290-294} \).

It has been reported that the protective effects of caffeine on tissues occurs via inhibition of the adenosine A2A receptors \( \textsuperscript{45} \). The therapeutic or detrimental effects of caffeine are significantly different, depending on whether it is consumed chronically or acutely \( \textsuperscript{152} \). For instance, high doses of acute caffeine suppresses liver damage via inhibition of cAMP-phosphodiesterase \( \textsuperscript{45} \). Also, chronic caffeine consumption can be neuroprotective by elevating plasma adenosine levels and up-regulating the adenosine A1 receptors. This is in direct contrast to the effects of acutely antagonising the adenosine A1 receptors \( \textsuperscript{295, 296} \).
Several researches have demonstrated that caffeine can have an effect on cutaneous wound healing procedures, particularly wound epithelialisation and closure, through its antioxidant properties and antagonism of the adenosine receptors. Human skin fibroblasts pre-treated with caffeine at low doses (0.01 mM) and intermediate doses (0.1 mM) were protected from apoptosis and necrosis induced by hydrogen peroxide. Caffeine also stimulated an increase in cell number and altered cell morphology, an effect which was mediated by a mechanism other than its antioxidant property. Furthermore, an in vivo study reported that caffeine increased cell proliferation in the ventral prostatic lobe in 5-week-old male Wistar rats.

The varied results observed following caffeine treatment may be caused by differences in tissue types, use of in vivo or in vitro models, differences in caffeine concentrations or duration of treatment.

Several in vivo and in vitro studies have reported that caffeine can attenuate angiogenesis in human umbilical vein endothelial cells (HUVEC), the chorioallantoic membranes (CAM) of chick embryos and zebrafish embryos. These studies demonstrated that caffeine inhibited in vitro proliferation of HUVECs in a time and dose dependent manner through the stimulation of apoptosis. This suggested that the inhibitory effect of caffeine on angiogenesis could be related to its ability to induce apoptosis in these cells. Angiogenesis is regulated via a balance between pro-angiogenic and anti-angiogenic factors. Another study reported that adenosine and adenosine receptor agonists can stimulate angiogenesis through down regulating the production of the anti-angiogenic matrix protein thrombospondin-1 (TSP-1). Caffeine, through blocking adenosine receptors, increased the expression of TSP-1 in
HUVECs. Consequently, this study reported that caffeine mediated inhibition of angiogenesis was associated with cell apoptosis related to the elevated expression of TSP-1, an effect mediated by the adenosine A<sub>2B</sub> receptor subtype. High doses of caffeine (4,200–5,800 mg/kg) were able to inhibit the angiogenesis in zebrafish embryos at a dose which is much higher than standard daily consumption of caffeine in the adult human (150–300 mg per day). This study also indicated that daily caffeine consumption would not cause defects in angiogenesis. Another study reported that exposure to caffeine (5–15 µmol per egg) reduced angiogenesis in chick embryo chorioallantoic membranes and chick yolk sacs by altering the expression of angiogenesis-associated genes, some of which are involved in the regulation of excess ROS production. For example, caffeine treatment was found to down-regulate the expression of vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGFR-2), placental growth factor (PlGF), insulin-like growth factor-2 (IGF-2) and neuropilin-1 (NRP1) in chick embryo chorioallantoic membranes and chick yolk sacs, causing a decrease in angiogenesis with an associated negative impact on fetal development.

The purpose of this work was to investigate the effects of caffeine (applied acutely) on the EA.h926 endothelial cells in wound healing, cell proliferation and tube formation (angiogenesis) assays. Furthermore, this study investigated whether acute caffeine modified the cell function assays through affecting adenosine receptor function or cAMP levels.
4.2. AIMS

The aims of this study were as follows;

1. To determine the effect of acute caffeine treatment alone and on adenosine A₁, A₂A and A₂B receptor mediated stimulation of wound closure using the EA.h926 endothelial cells in the wound healing scratch assay.
2. To study the role of adenylyl cyclase in the wound healing scratch assay.
3. To investigate the effects of acute caffeine treatment on EA.hy926 endothelial cell proliferation and angiogenesis in vitro using the cell proliferation assay and tube formation assay.

4.3. METHODS AND MATERIALS

4.3.1. Treatment preparations (drugs)

All drugs were freshly prepared from stock solution (1 mM) as described in Chapter 3. Caffeine solutions were prepared from stock solution by serial dilutions with high glucose complete media to reach a final concentration of 30 µM. The selective adenosine A₁, A₂A and A₂B receptor agonists (CPA, CGS21680 and NECA) were prepared from stock solution by diluting with caffeine mixture to reach a final concentration of 30 nM and 10 nM, respectively (Table 4.3.1). The 1 mM stock solutions of MDL-12330A and forskolin (Sigma Aldrich, NSW, Australia) were dissolved in DMSO and stored as aliquots in the -20 °C freezer until further use. Then, MDL-12330A and forskolin were prepared from stock solution by diluting with high glucose complete media to reach a final concentration of 10 µM (Table 4.3.1). Also, a
mixture of MDL-12330A and caffeine was prepared through dilution of MDL-12330A from stock solution into caffeine 30 µM treated high glucose complete media to reach a final concentration of 10 µM (Table 4.3.1).

4.3.2. *Wound healing scratch assay*

EA.hy926 endothelial cells (2x10^5 cells) were seeded into the 12-well plates, in triplicate in high glucose complete media and incubated until the cells reached 80-85% confluence. The cell layer was then scratched with a 200 µL pipette tip and high glucose complete media with caffeine (30 µM) were loaded into the wells (Table 4.3.1). The wells were photographed at the starting point of the treatment (0 hours) and at 2, 4 and 6 hours (end of the treatment). For more detail, refer to Chapter 2. The data from the wound healing scratch assay was obtained by measuring the width of the scratch at 2 hours intervals, using Image J software. The average speed of wound healing was examined by comparing the effect of acute caffeine treatment on adenosine receptor agonists. The results were analysed using One Way ANOVA via GraphPad Prism software (CA, USA -version 6.0).
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Cells + high glucose Complete Media</td>
</tr>
<tr>
<td>Acute Caffeine</td>
<td>Caffeine 30 µM + high glucose Complete Media</td>
</tr>
<tr>
<td>CPA</td>
<td>CPA 30 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>CGS21680</td>
<td>CGS21680 30 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>NECA</td>
<td>NECA 30 nM + ZM241385 10 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>MDL-12330A &amp; Caffeine</td>
<td>MDL-12330A 10 µM + Caffeine Mixture (30 µM)</td>
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<td>MDL-12330A 10 µM + high glucose Complete Media</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Forskolin 10 µM + high glucose Complete Media</td>
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<tr>
<td>MDL-12330A &amp; Forskolin</td>
<td>MDL-12330A 10 µM + Forskolin 10 µM</td>
</tr>
</tbody>
</table>

Table 4.3.1. Preparation of the acute caffeine treatment and test drugs for the wound healing scratch assay
4.3.3. *Cell proliferation assay*

The EA.hy926 endothelial cells were seeded in triplicate at 5000 cells/ well in 96 well plates. Additionally, 4000, 5000, 6000, 7000, 8000 and 9000 cells per well, were seeded in duplicate respectively, to form a standard curve. The plates were then incubated for 24 h at 37 °C, 5% CO₂ as described in Chapters 2 and 3. The adenosine receptor agonists were prepared with the caffeine mixture 30 µM (caffeine + high glucose complete media) at final concentrations of 30 nM and incubated for 4 hours. In addition, this experiment included the control conditions, where cells were treated with normal high glucose complete media and vehicle controls conditions using DMSO at two different dilutions of 0.004% and 0.03% (Table 4.3.2). The plate was then placed into the micro-plate reader and the absorbance at 450 nm measured. For more detail refer to Chapter 2.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Caffeine treatment</td>
<td>Caffeine 30 µM + high glucose Complete Media</td>
</tr>
<tr>
<td>CPA</td>
<td>CPA 30 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>CGS21680</td>
<td>CGS21680 30 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>NECA</td>
<td>NECA 30 nM + Caffeine Mixture (30 µM)</td>
</tr>
<tr>
<td>Control</td>
<td>Cells + high glucose Complete Media</td>
</tr>
<tr>
<td>Vehicle Control 1</td>
<td>0.004% DMSO + high glucose Complete Media</td>
</tr>
<tr>
<td>Vehicle Control 2</td>
<td>0.03% DMSO + high glucose Complete Media</td>
</tr>
<tr>
<td>Negative Control</td>
<td>No cells + high glucose Just Complete Media</td>
</tr>
</tbody>
</table>

Table 4.3.2. Acute caffeine treatment conditions and concentrations
4.3.4. Tube Formation Assay

Geltrex (50 µL) was loaded into each well of the plate and incubated at 37 ºC, 5% CO₂, for 30-40 minutes to allow the gel to solidify. Afterwards, 50,000 cells were loaded gently into each well of a 96 well plate and the drug treatment added to give a final volume of 100 µL. Suramin (0.3%) inhibits angiogenesis in the chick chorioallantoic membrane assay and was used as a negative control in this study. The positive control for this work was the basic fibroblast growth factor (bFGF, 25%), which is reported to stimulate angiogenesis in isolated cells. Therefore, this experiment comprised the following groups: control group containing cells treated with DMEM without FBS; positive control group prepared from 25% basic fibroblast growth factors (bFGFs) diluted in high glucose complete media with 10% FBS; a negative control group prepared from 0.3% Suramin sodium salt, diluted in high glucose complete media with 2% FBS; and acute caffeine treatment (30 µM) diluted in high glucose complete media with 2% FBS (Table 4.3.3). FBS (2%) was used instead of 10% FBS to prepare acute caffeine conditions as FBS can stimulate tube formation. Therefore, the FBS levels were reduced to prevent interference of effect of FBS on acute caffeine treatment.

The plate was incubated for a maximum of 18 hours at 37 ºC, 5% CO₂. At the end of the incubation period tube formation was analysed using the Nikon ECLIPSE TS100 light microscope (Nikon, USA) with attached camera. The tube networks were photographed in twelve randomly selected microscopic fields. The total number of junctions, nodes and branches in each individual image was detected using the Angiogenesis Analyzer Image J software. Statistical analysis was conducted using a two-way ANOVA to determine
significance of differences between groups and each mean of each group was compared to the control group. Values are presented as mean± SEM. Differences were considered statistically significant at P < 0.05.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentrations</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>Cells + DMEM (No FBS)</td>
</tr>
<tr>
<td>Positive Control</td>
<td>25% bFGF + high glucose Complete Media (10% FBS)</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0.3% Suramin Sodium Salt + high glucose Complete Media (2% FBS)</td>
</tr>
<tr>
<td>Acute Caffeine treatment</td>
<td>Caffeine 30 μM + high glucose Complete Media (2% FBS)</td>
</tr>
</tbody>
</table>

Table 4.3.3. Acute caffeine treatment conditions and concentrations
4.4. RESULTS

4.4.1. The Effect Of Caffeine Treatment On Adenosine Receptor Mediated Rates Of Wound Closure In Ea.hy926 Endothelial Cells

The results of the wound healing scratching assay show that acute caffeine treatment did not alter the rate of wound closure in EA.hy926 endothelial cells in comparison with the control group (Figures 4.4.1 and 4.4.2).

The effect of caffeine (30 μM) was determined on the three selective adenosine receptor agonists. The results showed that caffeine significantly decreased the effect of the selective adenosine A₁, A₂A and A₂B receptor agonists (CPA, CGS21680 and NECA 30 nM) on wound healing rates by 28%, 16% and 12%, respectively when compared with the untreated conditions (P < 0.0001) (Figure 4.4.2). Therefore, acute caffeine treatment can block the effect of selective adenosine receptor agonists (CPA, CGS21680 and NECA) to significantly reduce the rate of wound healing (Figure 4.4.2).
Figure 4.4.1. Images showing the wound healing scratch assay in the absence (panel 1) and presence of acute caffeine (30 μM, panel 2). The effect of caffeine (30 μM) on selective adenosine receptor agonists (CPA, CGS21680 and NECA) (30 nM) on the rate of wound healing in EA.hy926 endothelial cells at 0 and 6 hrs are also presented (panels 3 to 5 respectively). Magnification x 40
Figure 4.4.2. Graph comparing the effects of adenosine receptor agonists on the rate of wound healing in untreated (dark blue bars; control, CPA, CGS21680 and NECA (30 nM)) and caffeine (30 µM) treated (light blue bars; control, CPA, CGS21680 and NECA (30 nM)) EA.hy926 endothelial cells. Two-Way ANOVA was used to analyse data. Data are presented as mean ± SEM, n= 6. **** P < 0.0001
4.4.2. The Role Of Adenylyl Cyclase On Caffeine Treatment And Rates Of Wound Closure In Ea.hy926 Endothelial Cells

The effects of forskolin and MDL-12330A were studied on the rate of wound healing. The purpose of this work was to determine the potential role of cAMP in the cellular effects of acute caffeine in wound closure. In this experiment the EA.hy926 endothelial cells were treated with different drug treatments as follows: acute caffeine treatment (30 µM); acute caffeine (30 µM)+ MDL-12330A (10 µM); MDL-12330A group (10 µM); forskolin group (3 µM); and forskolin (3 µM) and MDL-12330A group (10 µM).

The results demonstrated that the rate of wound healing in EA.hy926 endothelial cells increased by 32% with the caffeine treated control group (T/control) when compared to the untreated control group (U/control), however it was not significant (P > 0.05) (Figures 4.4.3 and 4.4.4). Also, the wound-healing rate in EA.hy926 endothelial cells significantly increased by 85±4 µM/hour with the forskolin treatment in comparison with U/control group (40±3 µM/hour) and T/control group (54±5 µM/hour) (P < 0.0001) (Figure 4.4.4). In comparison with U/control and T/control group, the rates of wound healing did not have any significant changes with the other treatment group (P> 0.05) (Figure 4.4.4).

Consequently, while these results showed a non-significant increase in wound healing rate with acute caffeine treatment, they also demonstrated a decrease in caffeine stimulated rate of wound closure in the presence of the adenylyl
cyclase inhibitor MDL-12330A (which alone did not affect wound closure rates. Additionally, MDL-12330A significantly decreased the effect of forskolin on wound closure rate by 40% in EA.hy926 endothelial cells (P < 0.0001) (Figures 4.4.3 and 4.4.4). Therefore, the results with forskolin show that stimulation of adenyl cyclase increases rates of wound closure in EA.hy926 endothelial cells.
Figure 4.4.3. The effect of various treatments on EA.hy926 endothelial cells in the wound healing scratch assay at 0 and 6 hr.

Panel 1 illustrates control conditions. Panel 2 shows the effect of acute caffeine treatment (30 μM) while Panel 3 demonstrates the effect of adenylyl cyclase inhibitor MDL-12330A (10 μM) alone and with acute caffeine (Panel 4). Panel 5 demonstrates the effect of adenylyl cyclase activator forskolin (3 μM) alone and in the presence of MDL-12330A (Panel 6). Magnification x 40.
Figure 4.4. Graph showing the effect of acute caffeine, MDL-122330A and forskolin treatments on the rates of the wound healing.

Data are presented as mean ± SE, n= 6, **** P < 0.0001 versus Untreated control (U/Control) and Caffeine Treated control (T/Control) as indicated.
4.4.3. The Effect Of Caffeine On Adenosine $A_1$, $A_{2A}$ And $A_{2B}$ Receptor Mediated Cell Proliferation

A standard curve was generated to convert sample absorbance values into cell numbers (4000, 5000, 6000, 7000, 8000 and 9000 cells) as described in Chapters 2 and 3. The calibration curve shows that absorbance values rose with increasing cell numbers. Data for the proliferation assay were analysed using one-way ANOVA and multiple comparisons.

EA.hy926 endothelial cell proliferation was considerbly decreased by vehicle control treatments (0.004% and 0.03% DMSO) when compared with the control group ($P < 0.0001$) (Chapter 3, Figure 3.4.9). Also acute caffeine treatment did not alter the effects of DMSO in this assay (data not shown).

The effect of acute caffeine treatment (30 $\mu$M) on selective adenosine receptor agonists CPA, CGS21680 and NECA (30 nM) mediated proliferation of EA.hy926 endothelial cells was studied. The results show that acute caffeine significantly decreased the cell proliferation stimulated by all the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists (CPA, CGS21680 and NECA 30nM) when compared to the control (untreated conditions), ($P < 0.0001$) (Figure 4.4.5). Consequently, these results show that the adenosine receptor agonist mediated proliferation of EA.hy926 endothelial cell was non-selectively blocked by acute caffeine.
Figure 4.4.5. Graph comparing the effect of acute caffeine on adenosine receptor mediated cell proliferation as indicated by absorbance for: control group (no drug) (Ctrl); Adenosine A\textsubscript{1} receptor agonist (CPA 30 nM); adenosine A\textsubscript{2A} receptor agonist (CGS 30nM); and adenosine A\textsubscript{2B} receptor agonist (NECA 30 nM). The data are presented as mean ± SEM, n= 6 and analysed using two-Way ANOVA, **** P < 0.0001
4.4.4. The Effect Of Acute Caffeine Treatment On Angiogenesis And Tube Formation In EA.hy926 Endothelial Cells

Tube formation was monitored using the Nikon ECLIPSE TS100 light microscope (Nikon, USA) with attached camera as described in Chapter 2 (Figure 4.4.6). The total number of junctions, nodes and branches in each individual image was determined using Angiogenesis Analyzer of Image J software.216

The addition of the positive control, 25% bFGF to EA.hy926 endothelial cells considerably increased the formation of tubes when compared to the control group (P < 0.0001) (Figure 4.4.7). Similarly, the addition of the negative control suramin (0.3%) significantly decreased tube formation when compared with the control group (P < 0.001). In these experiments, however, suramin induced morphological changes in the EA.hy926 endothelial cells and the cells did not survive in the presence of suramin after 18 hours incubation.

The results of the tube formation assay with acute caffeine treatment demonstrated that caffeine significantly increased the number of nodes, junction and branches when compared to control conditions, indicating that acute caffeine induces angiogenesis and tube formation in EA.hy926 endothelial cells (Figure 4.4.7).
Figure 4.4.6. Images showing the tube formation assay with control (DMEM, Panel 1), acute caffeine treatment (30 μM, Panel 2), positive control (25% bFGF, Panel 3) and negative control (0.3% Suramin, Panel 4). Magnification x 40
Figure 4.4.7. The effect of drug treatments on tube formation in EA.hy926 endothelial cells for: Control (DMEM); acute caffeine (30 µM); positive control (25% bFGF); and negative control 0.3% Suramin. Data are presented as mean ± SEM, n= 12. Data were analysed using two-way ANOVA *P < 0.05, **P < 0.01, *** P < 0.001 and **** P < 0.0001 versus control (DMEM)
4.5. **DISCUSSION**

This study determined the effects of acute caffeine treatment (30 µM) on wound healing, cell proliferation and tube formation using EA.hy926 endothelial cells.

In this study using the immortalised EA.hy926 endothelial cell line, it was found that acute caffeine (30 µM) did not significantly alter wound healing rates or cell proliferation. Acute caffeine however did inhibit the stimulatory effects of all three adenosine receptor agonists on the wound healing rate and cell proliferation assays used in this study. Previous studies have reported that adenosine is able to up-regulate the expression of vascular endothelial growth/vascular permeability factor $^{245, 304}$. Selective adenosine A$_1$, A$_{2A}$ and A$_{2B}$ receptor agonists have been shown to stimulate the proliferation of various cell types *in vivo* mice models and *in vitro* studies investigating keratinocytes, fibroblasts, and endothelial cells as well as in normal and genetically modified healing-impaired mice $^{5, 71, 113, 245}$.

This study demonstrated that the effects of all the selective adenosine receptor agonists (CPA, CGS21680 and NECA, 30 nM) on rates of wound healing and cell proliferation were reversed following caffeine treatment. Consequently, these results support previous literature indicating the adenosine-antagonist activity of caffeine. Caffeine has been reported to competitively inhibit binding to adenosine receptors in brain membranes $^{51, 305}$. Another study showed that caffeine was associated with reduced collagen deposition and decreased fibrosis in the liver and lung, an effect due to the antagonist actions of caffeine on adenosine receptors $^{41, 42, 262}$. Sarobo et al., 2012 reported that inhibition of adenosine activity occurs with high doses of caffeine (25 mg/kg
per day) and that no changes were observed on adenosine activity at lower doses (2.5 mg/kg per day) \(^{42}\).

In this study, the role of adenylyl cyclase in the wound healing assay and its effects on acute caffeine treatment were investigated. Forskolin was implemented as a positive control in these experiments. Forskolin directly stimulates the catalytic subunit of adenylyl cyclase to stimulate cAMP formation, which can affect cellular growth and morphology \(^{306, 307}\). Also, forskolin is well recognised as a potent tool to study this enzyme and the role of cAMP in physiological and pathological processes \(^{306, 307}\). A significant increase in the rate of wound closure was observed with the addition of forskolin to the assay. The effect of forskolin was reversed by the compound MDL12330A, indicating that increased rates of wound healing were linked to stimulation of adenylyl cyclase and increases in intracellular cAMP levels. Other studies have shown that exposure to forskolin considerably increased intracellular cAMP concentrations in different cell types such as endothelial cells, smooth muscle cells and glial cells \(^{306}\). MDL-12330A, as an adenylyl cyclase inhibitor, significantly decreased the wound healing rate with acute caffeine treatment. Thus, the non-significant rise in rate of wound closure may be mediated in some part by cAMP in EA.hy926 endothelial cells. Another study has reported that caffeine inhibits cell proliferation in endothelial cells in both a time and dose dependent manner by inducing apoptosis \(^{68}\). Moreover, a different study showed caffeine, in a dose-and time dependent manner, suppresses cell proliferation and impedes migration in a human keratinocyte HaCaT cell line \(^{6}\). Interestingly, other in vivo and in vitro studies have reported that caffeine can increase cell proliferation in human skin fibroblast, epithelial
cells and cell proliferation in the prostate gland of rats. The results of tube formation assay demonstrated that acute caffeine treatment (30 µM) significantly increased tube formation and angiogenesis in EA.hy926 endothelial cells and the changes observed were similar to the effect of 25% bFGF on EA.hy926 endothelial cells. These results are contrary to several other studies, which reported that caffeine treatment can inhibit angiogenesis in different cell types. For example, adenosine receptor activation stimulates the release of angiogenic factors to promote angiogenesis in zebrafish embryos, an effect that was inhibited by caffeine. Moreover, a study using cultured human umbilical vein endothelial cells (HUVECs) reported that caffeine at concentrations of 250 µM and 500 µM, inhibited HUVEC proliferation and angiogenesis but when caffeine was applied at concentrations lower than 100 µM, it had no effect on cell proliferation. Similarly, Li et al., 2013 observed that caffeine had no effect on HUVEC induced tube formation on Matrigel or cell migration in vitro.

4.6. CONCLUSION

In conclusion, caffeine did not affect the rate of wound healing or cell proliferation of EA.hy926 endothelial cells. However, caffeine by its adenosine receptor antagonist role blocked the effect of selective adenosine A1, A2A and A2B receptor agonists and decreased the rate of wound healing in EA.hy926 endothelial cells. The second messenger molecule cAMP does have a role in increased wound healing rates, however caffeine at the concentration tested in our assay did not increase wound healing rates. Nevertheless, caffeine treatment did increase angiogenesis by stimulation of tube formation.
in EA.hy926 endothelial cells. As this work has been completed on cells in a high glucose environment, acute caffeine may have a role in stimulating angiogenesis in diabetic patients.
CHAPTER 5

EFFECT OF CHRONIC CAFFEINE ON

ADENOSINE RECEPTOR MEDIATED

WOUND HEALING
CHAPTER 5: EFFECT OF CHRONIC CAFFEINE ON ADENOSINE RECEPTOR MEDIATED WOUND HEALING

5.1. INTRODUCTION

5.1.1. Chronic Caffeine

A chronic caffeine treatment is defined as a continuous treatment of animal models and/or cell lines with caffeine, lasting a few days up to a few weeks using in vivo and in vitro conditions. For example, an in vitro cell proliferation study used primary human keratinocytes that were cultured and treated with caffeine chronically for 3 and 5 days. Also, several in vivo studies have investigated chronic caffeine treatments, administered to different animals including rats and mice, with water containing caffeine for various timeframes including 3 days, 14 days, 23 days and 6 or 12 weeks.

As described in Chapter 4, caffeine is a non-selective antagonist for all subtypes of the adenosine receptor. This Chapter will investigate the role of adenosine A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> receptors following chronic caffeine treatment of EA.hy926 endothelial cell on cell proliferation, migration and protein expression.

Caffeine is ingested in beverages consumed daily and is taken chronically in many humans. A single cup of coffee contains around 100 mg of caffeine. Studies have examined the effect of chronic caffeine treatment on organ and tissue function. An in vivo study demonstrated that chronic caffeine inhibited the development of hepatic fibrosis in two mouse models of hepatic injury and fibrosis. Another study using mice, reported that chronic caffeine treatment reduced lung injury and inhibited inflammation in an adenosine A<sub>2A</sub>...
receptor independent manner. This study also showed that the adenosine A2A receptors were significantly up-regulated following chronic caffeine treatment when compared with the other adenosine receptor subtypes.

Moreover, another study showed that chronic caffeine consumption decreased liver tissue damage in mice, due to up-regulation of the adenosine A2A receptor and increasing adenosine mediated anti-inflammatory actions. It has been shown that the chronic consumption of caffeine can protect the brain from ischaemic damage as well. Chronic caffeine consumption can be neuroprotective by decreasing neuronal damage in the rat hippocampus in a model of global forebrain ischaemia. Chronic adenosine receptor antagonism with xanthines led to upregulation of the adenosine A1 receptor in the rat brain, with no changes in the level of adenosine A2A receptor expression, leading to a shift in the balance of adenosine A1 and A2A receptor populations with prolonged caffeine consumption. Furthermore, following chronic caffeine treatment in rats, a modified function of the adenosine A1 and A2A receptor heteromer was reported. The authors suggested this altered function to be the cause of the potent tolerance to the psychomotor effects observed following the chronic consumption of caffeine. As a result, upregulation of adenosine receptors by chronic caffeine treatment can cause increased levels of adenosine and adenosine mediated actions in the rat brain.
5.2. AIMS

Chronic caffeine is well documented to affect adenosine receptor subtype populations in a range of cells and tissues. The purpose of this study was to determine whether chronic caffeine affects endothelial cell function with respect to wound healing and cell proliferation. This study will investigate the effect of chronic caffeine treatment on adenosine A1, A2A and A2B receptor populations in EA.h926 endothelial cell lines. As described previously, the adenosine A3 receptor is not expressed in the EA.hy926 endothelial cells and was not therefore studied in this Chapter. This study used a high glucose cell culture solution, as previously described. While studies have reported the effect of chronic caffeine treatment on adenosine receptors and their properties in different types of endothelial cell lines, the EA.hy926 endothelial cells have yet to be investigated. As a result, the aims of this study are to:

1. Determine the effect of chronic caffeine treatment on selective adenosine A1, A2A and A2B receptor agonist stimulation of wound healing in EA.hy926 endothelial cells.

2. Examine the effect of chronic caffeine treatment on cAMP levels and the rate of wound closure in EA.hy926 endothelial cells, using the wound healing scratch assay and selective adenylyl cyclase compounds.

3. Compare the effect of chronic and acute caffeine treatment on the wound healing rate in EA.hy926 endothelial cells using the wound healing scratch assay.

4. Establish the protein expression level of adenosine A1, A2A and A2B receptor subtypes in EA.hy926 endothelial cells under chronic caffeine treatment, using Western-blotting assays.
5.3. METHODS AND MATERIALS

5.3.1. Treatment Preparations (Drugs)

All drugs were freshly prepared from stock solution (1mM) as described in Chapter 3. Caffeine was prepared from stock solution by several dilutions with high glucose complete media to reach a final concentration of 30 µM. Selective adenosine A$_1$, A$_{2A}$ and A$_{2B}$ receptor agonists (CPA, CGS21680 and NECA) were prepared from stock solution by diluting with high glucose complete media to reach a final concentration of 30 nM, as described in Chapter 3 and presented in the following Table 5.3.1. MDL-12330A and forskolin were prepared from stock solution by diluting with high glucose complete media to reach a final concentration of 10 µM and 3 µM respectively as explained in Chapter 4 (Table 5.3.1).
Table 5.3.1. Preparation of drug treatments for the wound healing scratch assay
5.3.2. **Wound Healing Scratch Assay**

EA.hy926 endothelial cells (2x10^5 cells) were seeded into 12-well plates, in triplicate in high glucose complete media, to reach 60-65% confluence. High glucose complete media was then replaced with high glucose complete media and caffeine (30 µM) and the cells underwent chronic caffeine treatment for 48 hours. At the end of the incubation period, the high glucose complete media containing caffeine (30 µM) was removed from the wells. The cell layer was then scratched with a 200 µL pipette tip, and subsequently the various drug treatments were loaded into the wells (Table 5.3.1). The wounds were photographed at the starting point of the treatment (0 hours) and at 2, 4 and 6 hours (end of the treatment). For more detail, refer to Chapter 2. The data for the wound healing scratch assay was obtained by measuring the width of the scratch at 2 hour intervals, using Image J software. The average speed of wound healing was examined by comparing the effect of chronic caffeine treatment on adenosine receptor agonists. The results were analysed using one way ANOVA via GraphPad Prism software (CA, USA) (version 6.0).

5.3.3. **Western Blotting Technique**

Two groups of EA.hy926 endothelial cells (the control group and chronic caffeine treated group) were harvested, then lysed and prepared for the Western blotting technique as explained in Chapters 2 and 3. Protein samples (50 µg) were loaded into the gels and run at 150 volts for approximately 50 minutes until the samples reached the bottom of the gel. The protein samples
were then transferred to PVDF membranes using 75 volts for 1 hour and 30 minutes. The membranes were treated with a blocking buffer for 1 hour. Subsequently, they were incubated overnight at 4 °C with rabbit polyclonal primary antibody for adenosine A₁ receptor (1:500), adenosine A₂A receptor (1:500) and adenosine A₂B receptor (1:200); details are in Chapter 3. Afterward, the membranes were incubated in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000) for adenosine A₁ and A₂A receptors and (1: 500) for adenosine A₂B receptor for 1 hour at room temperature. Beta (β)-Actin antibody was used as a loading control (1:1000) for all three adenosine receptor subtypes and horseradish peroxidase-conjugated goat anti-rabbit antibody was chosen for the secondary antibody (1:3000) as described in Chapter 3.

The protein size was determined through comparison to the protein ladder or molecular weight marker. The data were analysed using the unpaired student’s t-test and two-way ANOVA via GraphPad Prism software (version 6.0).

5.4. RESULTS

5.4.1. The Effect Of Selective Adenosine Receptor Agonists On Rate Of Wound Closure In Ea.hy926 Endothelial Cells Following Chronic Caffeine Treatment

The results from the wound healing scratch assay showed that the rate of wound closure in EA.HY926 endothelial cells after the 48 hours caffeine treatment increased significantly to 74±3.0 μM/hour when compared with the
control group (no caffeine treatment, 48±0.5 µM/hour, P < 0.0001) (Figures 5.4.1 and 5.4.2).

A comparison of wound healing rates between the chronic caffeine treatment and untreated EA.hy926 endothelial cells, showed that the rates of wound closure with all the selective adenosine A₁, A₂A and A₂B receptor agonists (CPA, CGS21680 and NECA 30nM) increased significantly by 11%, 14% and 25% (P < 0.0001), respectively (Figure 5.4.2). However, compared to the control caffeine treated cells, the rate of wound closure did not change with the agonists CPA and NECA and was in fact reduced in the CGS21680 treated cells (P<0.05). Consequently, chronic caffeine treatment alone enhanced wound healing in the high glucose condition but had no effect on adenosine A₁ or A₂B receptor mediated increases in wound healing rates in EA.hy926 endothelial cells, and it attenuated the ability of adenosine A₂A receptors to mediate wound closure.
Figure 5.4.1. Images showing the effects of selective adenosine receptor agonists (CPA, CGS21680 and NECA) (30nM) on the rate of wound healing following chronic caffeine treatment (30 µM) in EA.hy926 endothelial cells at 0 and 6 hours using a wound healing scratch assay. Panel 1 control untreated cells; panels 2-5 have been treated with caffeine (30 µM for 48 hours) prior to the experiments. Panel 3 shows CPA (30 nM), panel 4 CGS21680 (30 nM) and panel 5 NECA (30 nM +ZM241385 10 nM). Magnification x 40.
Figure 5.4.2. Graph comparing the effects of adenosine receptor agonists (CPA, CGS21680 and NECA) (30 nM) on the rate of wound healing with 48 hours caffeine pre-treatment and compared to untreated conditions. Two-way ANOVA was applied to analyse data. The data are presented as mean ± SEM, n= 6. ****P < 0.0001, *P < 0.05 versus 48 hours caffeine pre-treated control.
5.4.2. Effect Of Chronic Caffeine Treatment On Rates Of Wound Closure In Ea.Hy926 Endothelial Cells; Role Of cAMP

Forskolin and MDL-12330A were used to determine the effect of chronic caffeine treatment on adenylyl cyclase activity in EA.hy926 endothelial cells undergoing the wound healing assay. The wound healing rates in EA.hy926 endothelial cells in the wound healing assay following caffeine treatment (48 hours) were 74±3.0 µM/hour (control) compared to forskolin treatment (3 µM) 67±3.0 µM/hour showing that forskolin treatment had no effect in chronic caffeine treated cells in this assay (Figures 5.4.3 and 5.4.4). Also, MDL-12330A significantly attenuated the effect of chronic caffeine and reversed the rate of wound healing by 47% (P < 0.0001) (Figures 5.4.3 and 5.4.4). Additionally, MDL-12330A decreased the effect of forskolin on wound closure rate by 32% in EA.hy926 endothelial cells (P < 0.001) (Figures 5.4.3 and 5.4.4). These results indicate that chronic caffeine treatment increases wound healing rates through increased cAMP levels.
Figure 5.4.3. Image showing effect of MDL-12330A (10 µM) and forskolin (3 µM) on wound healing rates following chronic caffeine treatment in EA.hy926 endothelial cells at 0 and 6 hours using wound healing scratch assay. Conditions from left to right: Control, chronic Caffeine (30 µM), MDL-12330A (10 µM), chronic caffeine MDL-12330A (10µM), forskolin (3 µM), Mixture of forskolin (3 µM) and MDL-12330A (10 µM). Magnification x40
Figure 5.4.4. Graph showing the effect of the 48 hours caffeine Pre-treatment (P, top panel) or Untreated cells (U, lower panel) on rates of wound healing and the effect of MDL-12330A and Forskolin (versus their respective control group). Data are presented as mean ± SEM, n= 6, ***P < 0.001, ****P < 0.0001 versus control.
5.4.3. Comparing The Effect Of Chronic And Acute Caffeine Treatment On The Rate Of Wound Healing In EA.hy926 Endothelial Cells

Figure 5.4.5 shows that the wound healing rate in the control group with 48 hours caffeine pre-treatment significantly increased compared to the control group with no caffeine pre-treatment in EA.hy926 endothelial cells (P < 0.0001). Also, the addition of acute caffeine on EA.hy926 endothelial cells with the 48 hours caffeine pre-treatment (30 µM) did not significantly alter the rate of wound healing in chronic caffeine treated or no caffeine treated cells (P > 0.05) (Figure 5.4.5).
Figure 5.4.5. Graph comparing the effect of acute caffeine (30 µM) on chronic caffeine treated cells (48 hours) and no caffeine pre-treatment on rate of the wound healing. Two-way ANOVA was applied to analyse data. Data are presented as mean ± SEM, n= 6, **P < 0.01, ****P < 0.0001
5.4.4. Expression Of The Adenosine Receptor Subtypes On EA.hy926 Endothelial Cells Following Chronic Caffeine Treatment

The Western blotting technique showed the expression of the adenosine A₁, A₂A and A₂B receptors following the chronic caffeine treatment (48 hours) and in untreated conditions (Figure 5.4.6). The expected molecular size of adenosine receptor subtypes is A₁ (≈ 37 kDa), A₂A (≈ 45 kDa) and A₂B (≈ 50-52 kDa) in EA.hy926 endothelial cells as described in Chapter 3. Moreover, β-actin (molecular size ≈ 42 kDa) was used as a loading control and used to normalize the data for protein expression of receptors across the blots (Figure 5.4.6).

The expression of adenosine A₁ and A₂B receptors on EA.hy926 endothelial cells following chronic caffeine treatment did not show any significant changes compared to the untreated or control group (P > 0.05) (Figure 5.4.7). However, the chronic caffeine treatment significantly decreased the expression of the adenosine A₂A receptor on EA.hy926 endothelial cells when compared with the control group (P < 0.01) (Figure 5.4.7).

Furthermore, the expressions of the adenosine receptor subtypes (A₁, A₂A and A₂B) were compared between the chronic caffeine treatment and control groups (Figure 5.4.8). This data showed that the expression of adenosine A₂A receptors was significantly higher than the other adenosine receptor subtypes (A₁ and A₂B) in the control group (P < 0.05) and (P < 0.0001), respectively (Figure 5.4.8). However, the expression of the adenosine A₂A receptors decreased with chronic caffeine treatment and was similar in expression levels to the adenosine A₁ receptor (Figure 5.4.8). Moreover, the adenosine A₁ and
A$_{2B}$ receptor expression did not change with chronic caffeine treatment (P > 0.05) (Figure 5.4.8).
Figure 5.4.6. Western blot images showing protein bands of adenosine receptors (A) $A_1 \approx 37$ kDa, (B) $A_{2A} \approx 45$ kDa, (C) $A_{2B} \approx 50-52$ kDa and (D) β-actin $\approx 42$ kDa in the control / untreated condition (Ctrl) and (48 hours) chronic caffeine treatment (Caffeine). Also in all images, a lane contains the molecular weight marker.
Figure 5.4.7. Histogram showing the expression of adenosine receptor subtypes $A_1$, $A_{2A}$ and $A_{2B}$ on EA.hy926 endothelial cells in untreated conditions (control) and (48 hours) chronic caffeine treatment. The data was analysed using unpaired student’s t-test. **($P < 0.01$), caffeine treatment versus control. Data are presented as mean ± SEM ($n=6$)
Figure 5.4.8. Graph showing a comparison of the protein expression of adenosine receptor subtypes $A_1$, $A_{2A}$ and $A_{2B}$ on EA.hy926 endothelial cells in untreated condition (control) and (48 hours) chronic caffeine treatment. Two-way ANOVA was applied to analyse data. Data are presented as mean ± SE, n= 6
5.5. DISCUSSION

This study determined the effect of chronic caffeine treatment on selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonist stimulation of wound healing rates in EA.hy926 endothelial cells. It also investigated the effect of acute caffeine treatment on cells. Furthermore, the protein expression of adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptors in EA.hy926 endothelial cells was examined following the chronic caffeine treatment.

The results of this study showed that chronic caffeine treatment significantly increased the rate of wound healing in EA.hy926 endothelial cells when compared with the untreated group. An in vivo study using humans and animals (rats) reported that topical coffee powder treatment for 4 weeks and 8 weeks respectively increased wound tissue healing without causing adverse effects. Studies have reported that coffee has numerous polyphenols that act as antioxidants. High levels of radical oxygen species are produced in the wound area during the early stages of the wound healing process. Thus antioxidants are required to scavenge free radicals to increase the rate of wound healing. In addition, another study has shown that long-term low dose caffeine consumption can stimulate modifications in the morphology and homoeostasis of the rat ventral prostate. Also, chronic caffeine consumption from puberty to adulthood elevated androgenic signalling and cell proliferation in the prostate gland and could be related to the development of benign prostatic hyperplasia (BPH).

The results of this PhD study show that chronic caffeine treatment did not significantly accelerate the rate of wound healing by the selective adenosine
A<sub>1</sub> and A<sub>2B</sub> receptor agonists (CPA and NECA) and reduced the effect of the adenosine A<sub>2A</sub> receptor agonist (CGS21680) in the EA.hy926 endothelial cells. Chronic caffeine treatment of nigrostriatal neurons causes up-regulation of adenosine A<sub>1</sub> receptors and reduces the toxic effects of psychostimulant drugs such as methamphetamine in the mouse<sup>296</sup>. However, the same study also reported that methamphetamine-induced toxicity was exacerbated in the mouse with acute caffeine treatment<sup>296, 315</sup>. Another in vivo study observed that chronic caffeine consumption up-regulated adenosine receptors and increased the levels of adenosine and adenosine actions in the rat brain<sup>152</sup>. Other studies have reported that caffeine decreases collagen deposition and reduced fibrosis in the liver and lung, an effect attributed to the antagonistic effects of caffeine on adenosine receptors<sup>41, 42, 44, 311</sup>.

This recent study showed that chronic caffeine treatment significantly increased the rates of wound healing in the EA.hy926 endothelial cells, however the effect of selective adenosine receptor agonists was not enhanced and was depressed with the adenosine A<sub>2A</sub> receptor agonist CGS21680. Protein levels of the adenosine receptors showed a reduction of the adenosine A<sub>2A</sub> receptor expression with chronic caffeine treatment which may explain why there was a reduced rate of wound closure with EA.hy926 endothelial cells. The protein expression of the adenosine A<sub>1</sub> and A<sub>2B</sub> receptors did not change with chronic caffeine treatment, however the selective agonist induced stimulation of wound closure rates did not increase further when compared to the chronic caffeine untreated cells. It is feasible that there is a maximum rate of wound closure (around 80 µM/hour) and further increases may not be possible.
The present study showed that acute caffeine had no effect on caffeine pretreated cells, which may be an indication of tolerance occurring with the chronic caffeine treatment.

In the current study, MDL12330A, an inhibitor of adenylyl cyclase, significantly reduced the rates of wound healing in caffeine pretreated cells. Forskolin, on the other hand, caused no increase in wound healing rates in caffeine treated EA.hy926 endothelial cells. Caffeine is a nonselective competitive inhibitor of the phosphodiesterase enzyme. This enzyme has the ability to degrade the phosphodiesterase bond in cAMP and cyclic guanosine monophosphate (cGMP) \(^{316, 317}\). Therefore, through inhibition of cyclic nucleotide phosphodiesterase activity, caffeine causes an accumulation of cAMP \(^{316, 318, 319}\). Also, it has been reported that inhibition of phosphodiesterase activity occurs at extremely high concentrations of caffeine such as plasma concentration 0.1–1 mM or caffeine doses 1520–15,200 mg \(^{319}\).

A study investigating chronic caffeine and its effects in the mouse hippocampus reported that the G\(_i\) and G\(_s\) proteins were down-regulated in the hippocampus and suggested that chronic caffeine treatment could induce adaptive increases in G-protein stimulated adenylyl cyclase \(^{320}\). While these effects occur in brain tissue, a similar effect may be occurring in the endothelial cells.

Western blotting results of the present study demonstrated that the protein expression of adenosine A\(_1\) and A\(_{2B}\) receptors did not significantly change with chronic caffeine treatment in EA.hy926 endothelial cells. However, the expression of the adenosine A\(_{2A}\) receptor subtype in EAhy926 endothelial cells was significantly decreased with chronic caffeine treatment when compared to
the control group. On the other hand, an *in vivo* study reported that adenosine receptors significantly increased following chronic caffeine in the mice brain stem. Also, their results suggested that the adenosine receptors required longer exposure to caffeine to significantly change the expression of these receptors in the cerebral cortex and the thalamus. For instance, in both these areas, the expression of adenosine receptors was enhanced at 16 days caffeine treatment but just reached statistical significance at 23 days. The results from this study suggest that chronic caffeine does not increase adenosine receptor subtype population in the endothelial cells. This may be due to differences in cell type and changes in adenosine receptor population that can be seen only in neuronal cells or alternatively, the duration of caffeine treatment was not long enough to cause a change in adenosine receptors.

### 5.6. CONCLUSION

In summary, the 48 hours caffeine treatment significantly elevated the rate of wound healing in EA.hy926 endothelial cells compared with untreated group and acute caffeine treatment. Therefore, this shows that chronic caffeine treatment may alter cAMP signalling pathways in EA.hy926 endothelial cell lines. The protein expression of adenosine A₁ and A₂B receptors did not significantly change with chronic caffeine treatment in EA.hy926 endothelial cell lines but the expression of the adenosine A₂A receptor chronic caffeine treatment was considerably lower than untreated group.
CHAPTER 6

EFFECT OF LOW GLUCOSE CONCENTRATION ON ADENOSINE RECEPTOR MEDIATED WOUND HEALING
CHAPTER 6: EFFECT OF LOW GLUCOSE CONCENTRATION ON ADENOSINE RECEPTOR MEDIATED WOUND HEALING

6.1. INTRODUCTION

6.1.1. Diabetes Mellitus and Wound Healing

Diabetes mellitus is a chronic metabolic disorder that is characterized by hyperglycaemia and caused by insulin deficiency and/or insulin resistance \(^3,321\). Diabetes is now present in all countries and its prevalence continues to grow in the world’s population \(^3,321,322\). According to the World Health Organization, the number of people with diabetes has increased from 108 million in 1980 to 422 million in 2014 \(^53\). Also, the USA National Diabetes Statistics report has predicted that diabetes mellitus will affect approximately 600 million of the population worldwide by the year 2035 \(^322,323\). Diabetic patients are prone to prolonged multifaceted complications such as cardiovascular diseases, retinopathy, neuropathy, nephropathy and impaired wound healing \(^3,322\).

Normal wound healing in non-diabetic conditions is a complex coordinated sequence of processes including cell migration into the wound area, inflammation, cell proliferation, angiogenesis, formation of extracellular matric components, remodeling and finally wound healing \(^3\). However, the wound healing sequelae in diabetes is dysregulated at several levels causing, increased inflammation, impaired tissue repair and eventually loss of function.
Furthermore, it has been confirmed that several process in normal wound healing are disrupted by diabetes, such as changed bactericidal actions of granulocytes and thickening of the extracellular matrix basement membrane together with poor tissue perfusion \textsuperscript{1,2}. Therefore, most diabetic patients suffer from chronic ulceration due to vascular disease, peripheral neuropathy and immune dysfunction \textsuperscript{1-3,37}. Diabetes is reported to be the number one cause of non-traumatic amputation \textsuperscript{1-3,37}.

\textbf{6.1.2. Adenosine Receptors and Diabetes}

Adenosine has a critical role in the regulation of glucose homeostasis and the pathophysiology of diabetes \textsuperscript{322,324-328}. The concentration of adenosine increases during energy starvation or environmental stress conditions to regulate cellular energy metabolism and prevent cellular damage \textsuperscript{321}. Furthermore, adenosine via activation of adenosine A\textsubscript{2A} and A\textsubscript{3} receptors affects insulin secretion and also regulates β-cell homeostasis via controlling cell proliferation and β-cell regeneration \textsuperscript{322,329,330}. In addition, adenosine is a regulator of cellular responses to insulin as it controls insulin signaling in adipose tissue, muscles and liver \textsuperscript{171,322,327,331}. Adenosine can indirectly mediate the actions of inflammatory cells and/or immune cells in the above tissues \textsuperscript{171,322,327,331}. These studies also show that diabetes is associated with altered expression of adenosine receptors and their function \textsuperscript{322}. For example, the levels of adenosine A\textsubscript{1} and A\textsubscript{3} receptors were elevated in isolated cardiac myocytes of streptozotocin-treated diabetic rats and the levels of adenosine A\textsubscript{2A} receptors increased in diabetic rat whole hearts but decreased in isolated
cardiac myocytes\textsuperscript{322, 332}. Several studies have reported that adenosine can protect cells from damage and also adenosine receptors effectively improve diabetic ischaemic wound repair\textsuperscript{9, 97, 106, 321}.

All experiments in the previous chapters were performed in diabetic conditions, as the EA.hy926 endothelial cells were seeded in high glucose cell culture solution (4500 mg/L glucose) as recommended in EA.hy926 (ATCC\textsuperscript{®} CRL-2922\textsuperscript{TM}) product sheet information (ATCC, VA. USA). However, this Chapter will examine the effect of low glucose or normal conditions (1000 mg/L) on EA.hy926 endothelial cell proliferation and rate of wound healing and compare the results with EA.hy926 endothelial cell function in high glucose conditions, as reported in Chapters 3 to 5.

6.2. AIMS

The purpose of this study is to determine the rate of wound healing and proliferation of EA.h926 endothelial cells in low glucose cell culture solution (1000 mg/L or 5.5 mM) and high glucose (4500 mg/L or 25 mM) conditions. The aims of these experiments were to:

1. Determine the effect of selective adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor agonists and antagonists on the rate of the wound closure in EA.h926 endothelial cells using wound healing scratch assay in vitro.
2. Measure the actions of selective adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} receptor agonists and antagonists on the proliferation of EA.h926 endothelial cells.
3. Investigate the effects of high and normal glucose levels on wound healing rates and cell proliferation.
6.3. METHODS AND MATERIALS

6.3.1. Treatment preparations (drugs)

The low glucose complete media were supplemented with the same mixture as high glucose complete media in Chapter 2 however high glucose DMEM was replaced with low glucose DMEM (1000 mg/L or 5.5 mM) (Dulbecco’s Modified Eagle’s Medium) (ThermoFisher Scientific, VIC, Australia) for more detail refer to Chapter 2.

All drugs were freshly prepared from stock solution (1 mM) by diluting with low glucose complete media (1000 mg/L) to reach a final concentration of 30 µM for caffeine, 30 nM and 10 nM for selective adenosine A1, A2A and A2B receptor agonists (CPA, CGS21680 and NECA) and antagonists (DPCPX, ZM241385 and MRS1754), respectively (Table 6.3.1). Also, it is noteworthy that due to NECA being non-selective for the adenosine A2B receptor agonist, 10 nM ZM241385 was added to exclude the adenosine A2A receptor as described in Chapter 3.

6.3.2. Wound healing scratch assay

EA.hy926 endothelial cells (2x10^5 cells) were seeded into the 12-well plates, in triplicate in low glucose complete media, and incubated until the cells had reached 80-85% confluence.

The EA.hy926 endothelial cells are not able to remain viable and functional under low glucose conditions after 48 hours. Cell morphology and confluency
changes were observed after 48 hours cell growth in EA.hy926 endothelial cells under the low glucose condition.

The cell layer was then scratched with a 200 µL pipette tip and prepared caffeine (30 µM), agonists (30 nM) and antagonists (10 nM) were loaded into the wells. The EA.hy926 endothelial cells exhibited less adhesion to the plate in low glucose condition and the scratch process caused a wider wound scratch.

The wells were photographed at the starting point of the treatment (0 hours) and at 2, 4 and 6 hours (end of the treatment). For more detail refer to Chapter 2. The data for the wound healing scratch assay was obtained by measuring the width of the scratch at 2 hour intervals, using Image J software. The average speed of wound healing was examined by comparing the effect of caffeine and adenosine receptor agonists on EA.hy926 endothelial cells in the low glucose condition with the diabetes condition (high glucose condition).

The results were analysed using unpaired student’s t-test, One Way ANOVA and Two Way ANOVA via GraphPad Prism software (CA, USA) (version 6.0).

6.3.3. Cell proliferation assay

The EA.hy926 endothelial cells were seeded in triplicate at 5000 cells/well in 96 well plates. Also, 4000, 5000, 6000, 7000, 8000 and 9000 cells were seeded in duplicate per well respectively to form a calibration curve and all incubated for 24 h at 37 °C, 5% CO₂ as described in Chapters 2 and 3. Selective adenosine receptor agonists (30 nM) and antagonists (10 nM) were
prepared with low glucose complete media. In addition, this experiment comprised the control conditions where cells were treated with normal complete media and vehicle controls conditions using DMSO at two different dilutions of 0.004% and 0.03% (Table 6.3.1). The plate was placed into the micro-plate reader and the absorbance at 450 nm measured. For more detail refer to Chapter 2.
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<tr>
<th>Treatments</th>
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<tr>
<td>CPA</td>
<td>CPA 30 nM + Low Glucose Complete Media</td>
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<tr>
<td>DPCPX</td>
<td>DPCPX 10 nM + Low Glucose Complete Media</td>
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<tr>
<td>CPA + DPCPX</td>
<td>CPA 30 nM + DPCPX 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>CGS21680</td>
<td>CGS21680 30 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>ZM 241385</td>
<td>ZM241385 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>CGS21680 + ZM 241385</td>
<td>CGS21680 30 nM + ZM241385 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>NECA</td>
<td>NECA 30 nM + ZM241385 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>MRS1754</td>
<td>MRS1754 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>NECA + MRS1754</td>
<td>NECA 30 nM + ZM241385 10 nM + MRS1754 10 nM + Low Glucose Complete Media</td>
</tr>
<tr>
<td>Control</td>
<td>Cells + Low Glucose Complete Media</td>
</tr>
<tr>
<td>Vehicle Control 1</td>
<td>0.004% DMSO + Low Glucose Complete Media</td>
</tr>
<tr>
<td>Vehicle Control 2</td>
<td>0.03% DMSO + Low Glucose Complete Media</td>
</tr>
<tr>
<td>Negative Control</td>
<td>No cells + Just Low Glucose Complete Media</td>
</tr>
</tbody>
</table>

Table 6.3.1. Treatment conditions and their concentrations
6.4. RESULTS

6.4.1. The effect of selective adenosine A\textsubscript{1} receptor agonist and antagonist on rate of wound closure in the low glucose condition

The results of the wound healing scratch assay showed that the selective adenosine A\textsubscript{1} receptor agonist (CPA 30 nM) significantly increased the rate of wound healing by 51\% in EA.hy926 endothelial cells when compared to the control group in the low glucose condition (P < 0.0001) (Figures 6.4.1 and 6.4.2). However, there were no significant changes in wound healing rate with selective adenosine A\textsubscript{1} receptor antagonist (DPCPX 10nM) and combined group (CPA+DPCPX) in comparison with the control group (P > 0.05) (Figures 6.4.1 and 6.4.2).

Furthermore, the results showed that the effect of the selective adenosine A\textsubscript{1} receptor agonist (CPA 30 nM) on wound healing rate was considerably decreased by the selective adenosine A\textsubscript{1} receptor antagonist (DPCPX 10 nM) by 32\% in the combined group (CPA+ DPCPX) (P < 0.0001) (Figures 6.4.1 and 6.4.2).
Figure 6.4.1. Images showing wound healing scratch assay in low glucose conditions. Effect of selective adenosine A$_1$ receptor agonist (CPA 30 nM), antagonist (DPCPX 10nM) and its combination (CPA+ DPCPX) on the rate of wound healing in EA.hy926 endothelial cells at 0 and 6 hrs. Magnification x 40.
Figure 6.4.2. Graph showing the effect of selective adenosine A₁ receptor agonist (CPA 30 nM), antagonist (DPCPX 10 nM) and its combination (CPA+ DPCPX) on rate of wound healing in EA.hy926 endothelial cells in the low glucose condition, One-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, **** P < 0.0001 versus control and versus CPA treated cells as indicated.
6.4.2. The effect of selective adenosine $A_{2A}$ receptor agonist and antagonist on rate of wound closure in the low glucose condition

The results show that the selective adenosine $A_{2A}$ receptor agonist (CGS21680 30 nM) significantly enhanced the rate of wound healing in EA.hy926 endothelial cells in the low glucose condition by 16% in comparison with control group ($P < 0.01$) (Figures 6.4.3 and 6.4.4). However, the selective adenosine $A_{2A}$ receptor antagonist (ZM241385 10 nM) and combined group (CGS21680+ZM241385) considerably reduced the wound healing rate in EA.hy926 endothelial cells compared with control group ($P < 0.001$) and ($P < 0.05$) (Figures 6.4.3 and 6.4.4). Moreover, the data shows that the selective adenosine $A_{2A}$ receptor antagonist (ZM241385 10nM) significantly decreased the effect of CGS21680 (30 nM) on the rate of wound closure by 25% in the low glucose condition ($P < 0.0001$) (Figures 6.4.3 and 6.4.4).
Figure 6.4.3. Image showing the wound healing scratch assay in the low glucose condition. Effect of selective adenosine A$_{2A}$ receptor agonist (CGS21680 (CGS) 30 nM), antagonist (ZM241385 (ZM) 10 nM) and its combination (CGS21680+ZM241385) on the rate of wound healing in EA.hy926 endothelial cells at 0 and 6 hrs. Magnification x40.
Figure 6.4.4. Graph showing the effects of selective adenosine A$_{2A}$ receptor agonist (CGS21680 (CGS) 30 nM), antagonist (ZM241385 (ZM) 10 nM) and its combination (CGS21680+ZM241385) on rate of wound healing in EA.hy926 endothelial cells in the low glucose condition. One-Way ANOVA was applied to analyse data. Data are presented as mean ± SEM, n= 6, *P < 0.05, **P < 0.01, *** P < 0.001 and **** P < 0.0001 versus control and versus CGS21680 treated cells as indicated.
6.4.3. The effect of selective adenosine $A_{2B}$ receptor agonist and antagonist on rate of wound closure in the low glucose condition

The data shows that the rate of wound healing in the low glucose condition significantly increased by 30% with the selective adenosine $A_{2B}$ receptor agonist (NECA 30 nM) when compared with control group ($P < 0.0001$) (Figures 6.4.5 and 6.4.6). In comparison with the control group, the selective adenosine $A_{2B}$ receptor antagonist (MRS1754) considerably decreased the wound healing rate in EA.hy926 endothelial cells in the low glucose condition ($P < 0.0001$) (Figures 6.4.5 and 6.4.6). Furthermore, the results demonstrated that MRS1754 significantly reversed the effect of NECA and decreased the rate of wound healing by 30% in EA.hy926 endothelial cells ($P < 0.0001$) (Figures 6.4.5 and 6.4.6).
Figure 6.4.5. Image showing the wound healing scratch assay in the low glucose condition. Effect of selective adenosine A$_{2B}$ receptor agonist (NECA 30 nM + ZM241385 10 nM), antagonist (MRS1754 (MRS) 10 nM) and its combination (NECA+ ZM + MRS) on the rate of wound healing in EA.hy926 endothelial cells at 0 and 6 hrs. Magnification x40.
Figure 6.4.6. Graph showing the effects of selective adenosine A\textsubscript{2A} receptor agonist (NECA 30 nM), antagonist (MRS1754 (MRS) 10 nM) and its combination (NECA+ MRS) on the rate of wound healing in EA.hy926 endothelial cells in the low glucose condition. One-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, **** P < 0.0001 versus control and NECA treated cells as indicated.
6.4.4. *Effect of adenosine agonists and anagonists on cell proliferation in the low glucose condition*

The calibration curve was created for converting sample absorbance values into cell numbers such as 4000, 5000, 6000, 7000, 8000 and 9000 cells per well. The calibration curve demonstrated that absorbance values were proportional to cell numbers see Figure 2.3.2 in Chapter 2.

The data shows that cell proliferation was significantly decreased by the vehicle control (0.004% DMSO) and the selective adenosine A$_{2A}$ receptor antagonist (ZM241385 10 nM) when compared with the control group (P < 0.05) and (P < 0.01), respectively (Figure 6.4.7). However, in comparison with control group, the other treatment groups did not demonstrate any significant changes (P > 0.05). During treatment, the other selective adenosine agonists and antagonists did not affect cell proliferation in the low glucose condition (P > 0.05).
Figure 6.4.7. Graph showing the effect of different treatments on cell proliferation in the low glucose conditions, Control group or untreated (Ctrl), vehicle control group (0.004% and 0.03% DMSO), adenosine receptor agonists 30 nM (CPA, CGS21680 and NECA), Adenosine receptor antagonists 10 nM (DPCPX, ZM241385 and MRS1754) and combined group (mixture of adenosine receptor agonist 30 nM with antagonist 10 nM). One-Way ANOVA applied to analyse data. Data are presented as mean ± SEM n= 6, *P < 0.05, **P < 0.01 versus control
6.4.5. **Comparing the effect of adenosine $A_1$ receptor agonist and antagonist on the rate of wound healing in low and high glucose conditions**

The results show that the selective adenosine $A_1$ receptor agonist and antagonist in the low glucose condition are more effective than in the high glucose condition in the wound healing process. Also, comparison of the two control groups showed that the rate of wound healing significantly increased in the low glucose condition in contrast to high glucose conditions by 78% ($P < 0.0001$) (Figure 6.4.8). Furthermore, the selective adenosine $A_1$ receptor agonist (CPA 30 nM) significantly enhanced the wound healing rates by 51% in the low glucose condition when compared with its control group in a same condition ($P < 0.0001$). Also, the CPA treatment significantly increased wound healing rates by 88% in the low glucose condition in comparison with wound healing rates in high glucose ($P < 0.0001$) (Figure 6.4.8). Moreover, the selective adenosine $A_1$ receptor antagonist (DPCPX 10 nM) considerably reduced the effect of the selective adenosine receptor agonist and decreased the rate of wound closure in both conditions (low glucose and high glucose) (Figure 6.4.8). However, the data showed that the rate of wound healing with the selective adenosine $A_1$ receptor antagonist (DPCPX 10 nM) in the high glucose condition decrease significantly by 60% when compared to rates in the low glucose condition ($P < 0.0001$) (Figure 6.4.8).
Figure 6.4.8. Graph comparing the effect of selective adenosine $A_1$ receptor agonist and antagonist (CPA 30 nM and DPCPX 10 nM), respectively on the rate of wound healing in EA.hy926 endothelial cells in low glucose and high glucose conditions, Two-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, **** P < 0.0001. CPA versus Control in the low glucose condition as indicated
6.4.6. Comparing the effect of adenosine $A_{2A}$ receptor agonist and antagonist on the rate of wound healing in low and high glucose conditions

The results show that the selective adenosine $A_{2A}$ receptor agonist and antagonist (CGS21680 and ZM241385, respectively) in the low glucose condition are more effective than in the high glucose condition in the wound healing process. A comparison of the two control groups demonstrated that the rate of wound healing significantly decreased by 57% in the high glucose condition in contrast to the low glucose condition ($P < 0.0001$) (Figure 6.4.9). Furthermore, the selective adenosine $A_{2A}$ receptor agonist (CGS21680 30 nM) considerably increased the wound healing rates by 18% in the low glucose condition when compared with its control group of cells under the same conditions ($P < 0.01$). Also, CGS21680 significantly increased the wound healing rate by 78% in the low glucose condition in comparison with high glucose’s wound healing rate ($P < 0.0001$) (Figure 6.4.9). Moreover, the selective adenosine $A_{2A}$ receptor antagonist (ZM241385 10 nM) significantly reduced the effect of CGS21680 and decreased the rate of wound closure in both conditions (low and high glucose) (Figure 6.4.9). However, the results showed that the rate of wound healing with the selective adenosine $A_{2A}$ receptor antagonist (ZM241385 10 nM) in the high glucose condition significantly decreased by 45% when compared to its rate in the low glucose condition ($P < 0.0001$) (Figure 6.4.9).
Figure 6.4.9. Graph comparing the effect of selective adenosine A$_{2A}$ receptor agonist and antagonist (CGS21680 (CGS) 30 nM and ZM241385 (ZM) 10 nM), respectively on the rate of wound healing in EA.hy926 endothelial cells in low and high glucose conditions. Two-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, ** P < 0.01, **** P < 0.0001. CGS21680 versus Control in the low glucose condition as indicated.
6.4.7. Comparing the effect of adenosine $A_2B$ receptor agonist and antagonist on the rate of wound healing in low and high glucose conditions

Similar to the other two adenosine receptors, these results show that the rate of wound healing with the selective adenosine $A_2B$ receptor agonist and antagonist (NECA and MRS1754, respectively) was higher in the low glucose condition than in the high glucose condition. For the control groups, the wound healing rate in the low glucose condition was significantly increased by 75% compared to the wound healing rate in the high glucose condition ($P < 0.0001$) (Figure 6.4.10). In addition, the selective adenosine $A_2B$ receptor agonist (NECA 30 nM) significantly increased the wound healing rate by 31% in the low glucose condition when compared with its control group in the same conditions ($P < 0.0001$). Also, NECA significantly increased the wound healing rate by 89% in the low glucose condition compared to the high glucose condition ($P < 0.0001$) (Figure 6.4.10). The selective adenosine $A_2B$ receptor antagonist (MRS1754 10 nM) significantly reduced the effect of NECA and decreased the rate of wound closure in both conditions (low and high glucose) (Figure 6.4.10). However, the results showed that the rate of wound healing with the selective adenosine $A_2B$ receptor antagonist significantly reduced in high glucose condition by 44% when compared to the wound healing rate in the low glucose condition ($P < 0.0001$) (Figure 6.4.10).

Overall, the results show that the effects of the selective adenosine $A_1$ receptor agonist (CPA) and its ability to improve wound healing in the low glucose condition was greater than the selective adenosine $A_{2A}$ and $A_{2B}$ receptor agonists (CGS21680 and NECA, respectively). The order of efficacy in the
low glucose condition was CPA< NECA< CGS21680 with no differences in the order of efficacy in the high glucose condition.
Figure 6.4.10. Graph comparing the effects of selective adenosine A1 receptor agonist and antagonist (NECA 30 nM and MRS1754 (MRS) 10 nM), respectively on the rate of wound healing in EA.hy926 endothelial cells in low and high glucose conditions, Two-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, **** P < 0.0001. NECA versus Control in the low glucose condition as indicated.
6.4.8. Comparing the effect of adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists and antagonists on EA.hy926 endothelial cell proliferation in low and high glucose conditions

The cell proliferation rates not have any significant differences in the low glucose condition treatments ($P > 0.05$) (Figure 6.4.11). However, the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists (CPA, CGS21680 and NECA) significantly increased cell proliferation in the high glucose compared with the low glucose condition ($P < 0.0001$) (Figure 6.4.11). Moreover, the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor antagonists (DPCPX, ZM241385 and MRS1754, respectively) reduced the effect of the selective adenosine receptor agonists and decreased proliferation of EA.hy926 endothelial cells in both low and high glucose conditions, however the data were not significantly different ($P > 0.05$) (Figure 6.4.11).
Figure 6.4.11. Graphs comparing the effect of selective adenosine A₁, A₂A and A₂B receptor agonists 30 nM (CPA, CGS21680 and NECA), antagonists 10 nM (DPCPX, ZM241385 and MRS1754), respectively and combined group (mixture of adenosine receptor agonists 30 nM with antagonists 10 nM) on EA.hy926 endothelial cell proliferation in low and high glucose conditions. Two-Way ANOVA applied to analyse data. Data are presented as mean ± SEM, n= 6, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 as indicated.
This study determined the effect of selective adenosine A₁, A₂A and A₂B receptor agonists and antagonists on the rate of wound closure and proliferation of EA.hy926 endothelial cells in a low glucose condition (1000 mg/L or 5.5 mM glucose). The results showed that the rates of wound healing in the EA.hy926 endothelial cells were significantly lower in the high glucose condition compared to the low glucose environment. However, the proliferation rates of the EA.hy926 endothelial cells were slightly higher (not significant) in cells under the high glucose condition.

Endothelial cell proliferation and migration are essential steps in angiogenesis and wound healing processes. This study also reported that endothelial cells were sensitive to hyperglycemia due to their poor ability to modulate intracellular glucose levels. Another study observed that high glucose concentrations (15-30 mmol/L) significantly inhibited proliferation and migration of human umbilical vein endothelial cells (HUVECs) and that angiogenesis and wound healing rates were reduced in high glucose conditions. In diabetes mellitus, increased plasma glucose causes endothelial dysfunction, which is characterized by the destruction of vasoactive factors including nitric oxide (NO)-dependent vasorelaxation and this leads to comorbidities such as retinopathy, peripheral neuropathy, renal failure, atherosclerosis and heart disease.

The results of the current study showed that the wound healing rates of EAhy926 endothelial cells in the low glucose condition were increased significantly by the selective adenosine A₁, A₂A and A₂B receptor agonists.
(CPA, CGS21680 and NECA respectively) when compared with their control group and selective adenosine receptor agonists in the high glucose condition (4500 mg/L or 25 mM glucose). In addition, these results demonstrated that the selective adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{2B} antagonists (DPCPX, ZM241385 and MRS1754, respectively) in the low glucose condition significantly reduced the effect of selective adenosine receptors agonists on the rate of wound healing. Similar outcomes were observed in the high glucose condition. It should be noted that there are no other studies on the effect of the adenosine receptors on wound healing in the EA.hy926 endothelial cell lines to compare with the current study results.

The results of the current study show that EA.hy926 endothelial cell proliferation did not show any significant changes under the low glucose compared to the high glucose condition, although generally the proliferation was lower in the low glucose condition. Therefore, due to this issue, the EA.hy926 endothelial cell lines were grown in the high glucose condition as recommended, to improve the cell proliferation in studies using these cells. Another study demonstrated that under prolonged (48 hour) high glucose (25 mM) conditions, modification of EA.hy926 endothelial cell morphology is accompanied by initiation of apoptosis\textsuperscript{336}. Moreover, previous studies showed that increased cell death, rather than reduced cell proliferation, is one of the main consequences associated with high glucose conditions\textsuperscript{336-338}. 

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In conclusion, the low glucose condition can accelerate the wound healing in EA.hy926 endothelial cells when compared to the high glucose condition. Under the low glucose condition, the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists significantly increased the rate of wound healing in EA.hy926 endothelial cells. Also, similar to high glucose conditions, the selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor antagonists reversed the effect of their agonist and decreased the wound healing rates in low glucose conditions indicating that all three receptors can be involved in stimulating wound healing. Furthermore, under the low glucose condition, the efficacy of selective adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor agonists and antagonists on EA.hy926 endothelial cell wound healing were greater than their effects under the high glucose condition.

EA.hy926 endothelial cell proliferation was decreased in the low glucose condition. Cell morphology and confluency changes in EA.hy926 endothelial cells were observed after 48 hours of cell growth in the low glucose condition. These experiments showed that EA.hy926 endothelial cells do not remain viable and functional in the low glucose condition after 48 hours.
CHAPTER 7

DISCUSSION
Diabetes mellitus is a chronic metabolic disorder that is characterized by hyperglycaemia and caused by insulin deficiency and or insulin resistance. Diabetic patients develop complications with this condition, which include cardiovascular disease, retinopathy, neuropathy, nephropathy and impaired wound healing. Normal wound healing in non-diabetic conditions is a complex coordinated sequence of processes including cell migration into the wound area, inflammation, cell proliferation, angiogenesis, formation of extracellular matrix components, remodeling and finally wound healing. Consequently, many diabetic patients suffer from chronic ulceration due to vascular disease, peripheral neuropathy and immune dysfunction and diabetes is known to be a main cause of non-traumatic amputation. The permanent human endothelial hybrid cell line (EA.hy926), which was used throughout this study, was created through the hybridisation of primary human umbilical vein endothelial cells (HUVEC) and a human lung carcinoma cell line (A549/8) in 1983. After the establishment of the EA.hy926 endothelial cell line, several studies have utilised these cell lines for research related to vascular function. For instance, it was reported that vascular endothelial growth factor (VEGF) stimulated angiogenesis by elevating EA.hy926 endothelial cell proliferation, migration and micro-vascular hyperpermeability. Adenosine is a ubiquitous purine nucleoside, which is generated through the dephosphorylation of ATP and adenine nucleotides. Adenosine and adenosine receptors have essential roles in the processes of
matrix production and neovascularization or angiogenesis that are important for wound healing and tissue repair. This study showed that the selective adenosine A₁, A₂A and A₂B receptor agonists, CPA, CGS21680 and NECA, significantly increased the rate of wound healing in EA.hy926 endothelial cells in both a high (4500 mg/L or 25 mM) and a low (1000 mg/L or 5.5 mM) glucose condition. Several in vivo studies have demonstrated that the selective adenosine A₁ receptor agonist CPA reduced tissue damage associated with ischaemia and stimulated the proliferation of endothelial cells, keratinocytes and fibroblasts in mouse models. Also, CPA has a potent vasodilator property in conjunction with cell proliferation and wound healing activities. Another in vivo study reported that the topical application of the selective adenosine A₂A receptor agonist, CGS21680, significantly accelerated wound healing rates in mice. Moreover, in vitro studies show that selective adenosine receptor agonists of the adenosine A₂A and A₂B receptors enhanced migration of both human dermal fibroblasts and human umbilical vein endothelial cells (HUVEC) in an in vitro artificial wound via cAMP and PKA-dependent pathways. However, data from the wound healing scratch assay demonstrated that the selective adenosine A₁, A₂A and A₂B receptor antagonists (DPCPX, ZM 241385 and MRS1754) reversed the effects of the selective adenosine receptor agonists and considerably decreased the rate of the wound healing in EA.hy926 endothelial cells. Furthermore, an in vivo study showed that the topical application of the selective adenosine A₂ receptor antagonist DMPX (3,7-dimethyl-1- propargyl xanthine) blocked the effect of CGS21680 on the rate of wound closure in mice. Moreover, Western blot data from this study showed the expression of adenosine A₁, A₂A
and A$_{2B}$ receptors in the EA.hy926 endothelial cell line. The adenosine receptors in order of expression in EA.hy926 endothelial cells were A$_{2A}$ > A$_1$ > A$_{2B}$ from highest to lowest.

It has been well established that each adenosine receptor subtype has different effects on stimulation or inhibition of adenylyl cyclase. For instance, the adenosine A$_1$ and A$_3$ receptors inhibit adenylyl cyclase but adenosine A$_{2A}$ and A$_{2B}$ receptors stimulate adenylyl cyclase $^{245}$. Moreover, several studies reported that intracellular cAMP levels can be elevated by forskolin that associates with adenylyl cyclase, while MDL-12330A is an adenylyl cyclase inhibitor that can decrease the levels of cAMP $^{290-294}$. This study observed that forskolin significantly increased the rate of wound healing in EA.hy926 endothelial cells. However, the effect of forskolin was reversed by the compound MDL12330A, demonstrating the increased rate of wound healing was linked to stimulation of adenylyl cyclase and increases in intracellular cAMP levels.

Caffeine (1,3,7-trimethylxanthine) is synthesised from the purine nucleotide xanthosine and is one of the most commonly used psychostimulants, usually consumed in the form of beverages and food $^{22,45,130,131,175}$. Several studies have demonstrated that caffeine is a non-selective antagonist against all of the adenosine receptor subtypes $^{152}$. Also, caffeine affects the cutaneous wound healing process, especially wound epithelialisation and closure, through its antioxidant properties and antagonism of the adenosine receptors $^6$. Caffeine can have different effects if it is taken as an acute dose or chronically. This study found that acute caffeine significantly decreased the effect of the selective adenosine A$_1$, A$_{2A}$ and A$_{2B}$ receptor agonists (CPA, CGS21680 and
NECA 30 nM) on wound healing rates by 28% 16% and 12%, respectively in EA.hy926 endothelial cells. A study using mouse brain slices demonstrated that caffeine blocks the actions of endogenously released adenosine which can tonically activate its receptors\textsuperscript{152}. Furthermore, these studies demonstrated that the actions of caffeine are not only mediated through antagonism of adenosine receptors but also through increasing the second messenger cAMP levels by inhibition of phosphodiesterase activity and elevated cytosolic Ca\textsuperscript{2+} via activation of ryanodine receptors\textsuperscript{340-343}. Another study showed that high doses of acute caffeine suppressed liver damage by inhibition of cAMP-phosphodiesterase\textsuperscript{45}. These results demonstrated that acute caffeine treatment increased the rate of wound closure, but the value was not significant. Also, the effect of caffeine decreased the rate of the wound healing in the presence of the adenylyl cyclase inhibitor MDL-12330A in EA.hy926 endothelial cells. In this study, proliferation rates decreased in the acute caffeine treated group and also caffeine significantly reduced the effect of selective adenosine A\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{3B} receptor agonists (CPA, CGS21680 and NECA) on EA.hy926 endothelial cell proliferation. Another in vivo study demonstrated that caffeine enhanced cell proliferation in the ventral prostatic lobe in Wistar rats\textsuperscript{42}. The tube formation assay in this study showed that caffeine significantly increased tube formation, which promoted angiogenesis in the EA.hy926 endothelial cells. However, other studies have reported that caffeine attenuated angiogenesis in HUVEC, the chorioallantoic membranes (CAM) of chick embryos and in zebrafish embryos\textsuperscript{68,69,298}. These other studies demonstrated that caffeine inhibited the \textit{in vitro} proliferation of HUVECs, in a time and dose dependent manner, through the stimulation of apoptosis, suggesting that
the inhibitory effect of caffeine on angiogenesis could be related to its ability to induce apoptosis in these cells \(^68,299,300\).

In this study, the rate of wound healing after the 48 hour caffeine treatment was significantly increased in the EA.hy926 endothelial cell line. Also, the 48 hour caffeine pre-treatment considerably elevated the effects of adenosine A\(_1\), A\(_{2A}\) and A\(_{2B}\) receptor agonists (CPA, CGS21680 and NECA) when compared with untreated and acute caffeine treated groups. Furthermore, other studies have reported that chronic caffeine treatment is associated with up-regulation of adenosine receptors in mouse brain tissue \(^291,320\). However, chronic consumption of caffeine was shown to decrease lung injury and inhibit inflammation in an adenosine A\(_{2A}\) receptor independent manner in mice \(^175\). In this same study, chronic caffeine treatment significantly up-regulated the adenosine A\(_{2A}\) receptors \(^175\). Also, other researchers have reported that up-regulation of the adenosine A\(_{2A}\) receptor and increased adenosine levels mediated the anti-inflammatory actions through chronic consumption of caffeine to decrease liver tissue damage in mice \(^45,48\). Chronic caffeine consumption can protect the brain from ischaemic damage \(^45,49,50\). The studies have demonstrated that caffeine treatment, due to its chronic blocking of the adenosine A\(_1\) receptors, lead to down-regulation of adenylyl cyclase expression \(^40,344\). It has been reported that G\(_i\) and G\(_s\) proteins were down-regulated in the mouse hippocampus following chronic caffeine treatment which suggests that chronic caffeine treatment induces adaptive increases in G-protein stimulated adenylyl cyclase \(^320\). Furthermore, our Western blot study showed that following chronic caffeine treatment, the expression of adenosine A\(_1\), A\(_{2B}\) receptor subtypes did not change, but a significant decrease was
observed in the expression of the adenosine A_{2A} receptor in EA.hy926 endothelial cells. Also, data from the wound healing scratch assay showed that the effect of the selective adenosine A_{2A} receptor agonist, CGS21680, on wound healing rate was less than the effect of the other two selective adenosine A_{1} and A_{2B} receptor agonists, CPA and NECA, on the rate of EA.hy926 endothelial cell wound healing after chronic caffeine treatment. This may be due to the low protein expression of the adenosine A_{2A} receptor in EA.hy926 endothelial cells. Another in vivo study has reported that adenosine receptors significantly increased expression in the mouse brain stem with chronic caffeine treatment \textsuperscript{51}. In addition, their data suggested that the adenosine receptors required longer exposure to caffeine to significantly change the expression of these receptors in the cerebral cortex and the thalamus, with 16-23 days of treatment examined \textsuperscript{51}. Further research could investigate the effect of chronic caffeine on G protein, adenylyl cyclase or phosphodiesterase expression in EA.hy926 endothelial cells. In Chapters 3 to 5 of this PhD study, EAhy926 endothelial cells were seeded in a high glucose cell culture solution (4500 mg/L glucose) to mimic diabetic conditions. In contrast, as presented in Chapter 6, the EAhy926 endothelial cell lines were seeded in a low glucose cell culture solution (1000 mg/L) to mimic the ‘normal’ condition (non-diabetes) and its effect on EAhy926 endothelial cell proliferation and rate of wound healing. However, the EA.hy926 endothelial cells did not remain viable and functional under the low glucose condition after 48 hours. Also, some changes in the cell morphology and confluency were observed in the EA.hy926 endothelial cells under the low glucose condition. These results support the recommendation in the product
information sheet for EAhy926 endothelial cells, which advises that a high glucose cell culture solution should be used for seeding and growing this type of cell line.

Several studies have demonstrated that cell proliferation and migration are crucial stages in endothelial cell angiogenesis and wound healing processes. Also, they have reported that endothelial cells are very sensitive to hyperglycemia due to their poor ability to regulate intracellular glucose levels. This current study demonstrated that a low glucose condition significantly accelerated the rate of wound healing in the EAhy926 endothelial cell line.

Furthermore, stimulation of adenosine A₁, A₂A and A₂B receptor subtypes with selective agonists increased wound healing rates, similar to that in the high glucose condition. Moreover, the order of efficacy of selective adenosine A₁, A₂A and A₂B receptor agonists in stimulating wound healing under both low and high glucose conditions was CPA< NECA< CGS21680 (Table 7.1.1).
The rate of wound healing ($\mu$M/hour) as affected by selective adenosine $A_1$, $A_{2A}$, and $A_{2B}$ receptor agonists in low and high glucose conditions. Data is presented as mean ± SEM, n= 6. Also shown are the percentage increases achieved with the selective adenosine receptor agonists versus their control.

<table>
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<th>Selective Adenosine Receptor Agonists (30 nM)</th>
<th>Low Glucose Condition (1000 mg/L = 5.5 mM glucose)</th>
<th>High Glucose Condition (4500 mg/L = 25 mM glucose)</th>
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</thead>
<tbody>
<tr>
<td>CPA</td>
<td>130±2 $\mu$M/hour 51%</td>
<td>70± 0.5 $\mu$M/hour 46%</td>
</tr>
<tr>
<td>CGS21680</td>
<td>100±3 $\mu$M/hour 18%</td>
<td>56± 0.4 $\mu$M/hour 50%</td>
</tr>
<tr>
<td>NECA</td>
<td>111± 2 $\mu$M/hour 31%</td>
<td>60± 0.03 $\mu$M/hour 23%</td>
</tr>
</tbody>
</table>

Table 7.1.1. The rate of wound healing ($\mu$M/hour) as affected by selective adenosine $A_1$, $A_{2A}$, and $A_{2B}$ receptor agonists in low and high glucose conditions. Data is presented as mean ± SEM, n= 6. Also shown are the percentage increases achieved with the selective adenosine receptor agonists versus their control.
The cell proliferation assays in this study have shown that in the high glucose condition, increased cell proliferation occurred with the selective adenosine A_1, A_2A and A_2B receptor agonists in EA.hy926 endothelial cells. However, no changes were observed in cell proliferation rates under the low glucose condition. Several studies have reported that adenosine has a critical role in the regulation of glucose homeostasis and the pathophysiology of diabetes. Furthermore, the studies have shown adenosine protects cells from any damage, and additionally, activation of adenosine receptors can efficiently accelerate diabetic wound healing. Chen et al. 2002 showed that adenosine can increase diabetic ischaemic wound healing by elevating autophagy in endothelial progenitor cells (EPCs) grown on biomaterials. Also, they produced a degradable biomaterial with high biocompatibility. This material was seeded with adenosine-stimulated EPCs and transplanted onto the surfaces of diabetic ischaemic wounds to accelerate healing of the wounds. Limitations of this study include the use of an immortalised endothelial cell line which may not represent natural endothelial cells which would have a limited lifespan. These cells would also not represent the various types of endothelial cells that come from different parts of body and so the results of these studies can not be generalised. This work has also been in an in vitro environment away from the complexity of tissues and organ systems. The variable results of manual method of wound healing scratching assay caused issues and inconsistent outcomes as did the high sensitivity of EA.hy926 endothelial cells to low glucose conditions.
In conclusion, diabetes mellitus is a chronic disease that is characterized by hyperglycaemia and abnormalities in the metabolism of carbohydrates, proteins and lipids. Impaired wound healing is a common and serious complication of diabetes mellitus. It causes dysregulation of the process at multiple levels, increasing the risk of infection and tissue damage. The adenosine A₁, A₂A and A₂B receptors increase wound healing rates in high and low glucose conditions. Also, the effect of adenosine receptor on wound healing is antagonised by acute caffeine treatment and selective adenosine receptor antagonists. In diabetes, activation of adenosine receptors may enhance wound healing rates similar to the effect of chronic caffeine treatment on wound healing in EA.hy926 endothelial cells.
CHAPTER 8

REFERENCES
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