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## Author

Owen, Suzzanne J, Massa, Helen H, Rose'Meyer, Roselyn B



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**Loss of adenosine $A_{2B}$ receptor mediated relaxant responses in the aged female rat bladder; effects of dietary phytoestrogens**

Suzzanne J. Owen, Helen H. Massa, Roselyn B. Rose'Meyer

School of Medical Sciences, Griffith University, Gold Coast Campus, Queensland, Australia

Corresponding author

Roselyn Rose'Meyer
School of Medical Sciences
Griffith University
Gold Coast Campus
Queensland Australia, 4222
Ph 617 5552 8938
Fax 617 5552 8908
Email. r.rosemeyer@griffith.edu.au

**Abstract**

This study examined the effect of age, ovariectomy and dietary phytoestrogen ingestion on adenosine $A_{2B}$ receptor mediated relaxant responses and mRNA expression of adenosine receptor subtypes in the rat isolated bladder. Female Wistar rats (8 weeks) were anaesthetised and the ovaries were removed (ovx) or left intact (sham). Rats were fed either normal rat chow (soy, phytoestrogens) or a non-soy (phytoestrogen free) diet. Isolated bladder from rats aged 12, 24 or 52 wks were precontracted with 3 μM carbachol prior to a concentration response curve to 5'-(N-ethylcarboxamido) adenosine (NECA) being obtained. In 12 wk old rats, the bladder exhibited enhanced relaxant responses to NECA in soy-fed rats ($P<0.05$), whilst at 24 weeks of age, the relaxant responses to NECA were attenuated in all the groups studied except soy treated sham rat bladders in which the relaxant responses were enhanced. At 52 wks of age, no relaxant effects were observed in any of the treatment groups and NECA induced contractile responses occurred. In all bladders the adenosine $A_{2B}$ receptor was the most abundantly expressed. In bladders from young and mature female rats, the mRNA expression of adenosine receptors ($A_1$, $A_{2A}$ and $A_{2B}$) was lowest in the bladder from non soy fed ovariectomised animals and the use of phytoestrogens in the diet increased the mRNA expression of these receptors ($P<0.05$). While a soy diet improves the relaxant effects to the adenosine analogue via adenosine $A_{2B}$ receptors in bladders from younger rats, the benefits are lost with advancing age.

**Key words** bladder, ageing, adenosine receptors, phytoestrogens

## Introduction

In females, menopause is one aspect of the ageing process, where lower urinary tract symptoms (incontinence, frequency urgency, dysuria and nocturia) are generally considered to be part of the menopausal syndrome (Rekers et al. 1992). Other urinary changes associated with the loss of oestrogen include atrophy of the lower urinary tract, reduced collagen content of connective tissue and reduced sensitivity of urethral smooth muscle to α-adrenergic stimulation (Andersson and Arner, 2004). Ovariectomy has been recently reported to increase voiding frequency and decrease bladder capacity by 25% in conscious rats (Kullman et al. 2009). Furthermore, reduced capacity for detrusor relaxation can lead to detrusor overactivity of the muscle which increases its contractile responsiveness, including response to stretch during filling (Frazier et al. 2008). In humans, increased detrusor *overactivity* leads to increased frequency and urgency of urination (Brading and Turner, 1994).

Normal physiological contraction of the bladder is mediated by the muscarinic $M_3$ receptor (Frazier et al. 2008; Michel and Parra, 2008). During the storage phase of the micturition cycle, the urinary bladder must accommodate increasing amounts of urine without major elevation of intravesical pressure. This enhancement of bladder compliance requires relaxation of smooth muscle cells of the detrusor and regulation by the sympathetic nervous system (Frazier et al. 2005; Otsuka et al. 2008; Yamanishi et al. 2003;) which primarily acts upon β-adrenergic receptors in the urinary bladder to promote relaxation during the storage phase. Therefore, adrenergic receptor activation is considered to be the most important physiological mechanism mediating urinary bladder relaxation during the filling/storage phase of the micturition cycle (Yamaguchi, 2002; Andersson and Arner, 2004; Yamaguchi and Chapple, 2007).

Adenosine is a metabolic product of ATP which is released from nonadrenergic, noncholinergic (NANC) nerves innervating the bladder *(Tong et al. 1997)* or from the uroepithelium in response to stretch (Sun et al. 2001; Yoshimura et al. 2008). Adenosine

signalling is initiated *via* the adenosine group of purinergic G protein-coupled receptors of which there are four sub-types: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Burnstock, 2007; Ruggieri, 2006). All of these adenosine receptor subtypes have been identified to be present in the bladder (Dixon et al., 1996).

Prejunctional adenosine $A_1$ receptors are present on the NANC nerves that innervate the rat urinary bladder (Fujji, 1988; Namasivayam et al. 1999). Adenosine $A_{2B}$ receptors are located post-junctionally and mediate relaxation of bladder smooth muscle (Nicholls et al. 1992; Aronsson et al. 2010), whilst adenosine $A_3$ receptors are reported to induce contraction of the bladder (Vesela et al. 2011). A role for adenosine $A_{2A}$ receptors in the bladder has not been elucidated (Namasivayam et al. 1999).

The adenosine receptors responsible for relaxation (adenosine $A_{2B}$ receptor) share similar signalling pathways to the β–adrenoceptors via the Gs protein and cAMP production. Most evidence of adenosine receptor mediated relaxation of the bladder has been obtained using the male rat and little is known regarding the role of adenosine and its receptors in the female bladder.

*Female rats are usually fertile by 7 weeks and sterile by 11-12 months of age (Merry and Holehan, 1979). Ovariectomised rats are commonly used as a model to emulate human menopause (Diep and Contantinou, 1999). The phytoestrogens that are present in soy bind both oestrogen receptors α and β (Morito et al. 2001) and phytoestrogens have been shown to be effective as β-estradiol for improving bladder function in ovariectomised rats (Thielemann et al. 2010).*

This study aimed to determine the effects of reduced ovarian hormone secretion or phytoestrogen diet on relaxant responses of the female rat isolated bladder to adenosine analogue 5'-(N-ethylcarboxamido) adenosine (NECA) and isoprenaline and bladder expression of adenosine receptor subtype mRNA.

## Methods

### Animal husbandry

Female Wistar rats, five to six weeks old, were obtained from the Central Animal Breeding House, University of Queensland and housed in a regulated environment (Griffith Gold Coast Animal Facility) at a mean temperature of 22 $^{o}$C (17-24 $^{o}$C) with 12-hour on/off light schedule and food and water *ad libitum*. Rats were caged in groups of 4 and oestrus cycles monitored through daily vaginal smears, recorded for at least two consecutive cycles prior to surgery to confirm cycle regularity. This project was approved by the Griffith University Animal Experimentation Ethics Committee and all protocols were conducted in accordance with the Guidelines for Animal Experimentation determined by the National Health and Medical Research Council of Australia.

### Diet

Rats were maintained on either a normal rat chow or a non-soy diet from 6 weeks of age. The commercial rat chow contains soy meal (meat free, rat and mouse, provided by Specialty Feeds, WA). The constituents of the non-soy diet are listed in Owen et al., 2011. The commercially obtained rat chow contains ingredients including wheat, barley, soya meal, fish meal and mixed vegetable oils while the non soy feed comprised casein purified whey protein, corn starch, corn or sunflower oil. Both mixes contained added vitamins such as vitamins A, $D_3$, K, E, $B_1$ and $B_2$, biotin and folic acid and trace minerals including magnesium iron, copper, iodine, manganese, zinc and selenium.

### Animal age and treatment groups

Three age groups were used in this study: young (12 weeks), mature (24 weeks) and aged (52 weeks). All rats were transferred to and maintained on their specific diet from the age of 6 weeks and underwent anaesthesia and either bilateral ovariectomy or sham bilateral

ovariectomy at 8 weeks of age. Bladder function was examined at harvest at 12 weeks of age (young), 24 weeks (mature) and 52 weeks (aged) of age.

Ovariectomy

Animals were anaesthetised using ketamine (60 mg/kg) and xylazine (8 mg/kg), IP at 8 weeks of age. Additional ketamine (30 mg/kg) was administered to maintain the plane of anaesthesia, if required. Ovaries were accessed through a dorsolateral flank incision and located through visualization of the peri-ovarian fat, prior to removal (Massa and Bruce, 1999). Care was taken to ensure that anastomotic blood vessels supplying the uterus remained intact during and following the procedure (Massa and Bruce, 1999). Silk ligatures, 4.0, were used to close the wound internally and stainless steel staples (Autoclip 9mm Wound Clips; Clay Adams, USA) used externally. During recovery from anaesthesia, all animals were continuously monitored, warmed under incandescent light and their eyes protected with physiological saline moistened swabs. Post-surgical oral rehydration was initiated and monitored until first micturition occurred. To minimise wound damage, operated animals were isolated from the rest of the group for 2 days, afterwhich they were housed together in cages of 4, until tissue harvest. A vaginal smear was taken and the morphology of the vaginal epithelium used to determine the stage of the oestrus cycle on the day of tissue harvest.

Tissue and plasma collection

Tissues were collected, 4, 16 or 44 weeks post-surgery, when the animals were aged 12, 24 or 52 weeks respectively. At harvest, animals were anaesthetised with pentobarbitone (60 mg/kg, IP) prior to collection of blood samples and tissues. Tissues were used immediately in experiments to determine bladder function or fixed in 10% neutral buffered formalin for

histological evaluation. Collected blood samples were stored frozen -20$^{o}$C until defrosted for assay and analysed for oestradiol and progesterone levels using competitive immunoassays by Queensland Medical Laboratory (Brisbane, Australia).

Plasma hormone measurement

To determine plasma oestrogen and plasma progesterone concentrations, the ADVIA Centaur Estradiol-6 III assay and the ADVIA Centaur Progesterone assay, respectively, were used.

Functional studies on isolated bladder strips

The urinary bladder was dissected from the euthanised animal, weighed and placed immediately in McEwans buffer (NaCl 130 mM, KCl l.5 mM, $NaHCO_3$ 25 mM, Glucose 11 mM, $CaCl_2$ $2H_2O$ 2.2 mM, $MgCl_2$ 0.5 mM, $NaH_2PO_4$, 1.0 mM, Sucrose 13.1 mM). The dorsal and ventral sides of the bladder were identified and the bladder divided into two longitudinal strips of equivalent width, which were attached to force transducer hooks in a 25 ml isolated organ bath. Bladder strips were allowed to equilibrate in McEwans buffer solution at 32℃, bubbled with carbogen (95% $O_2$; 5% $CO_2$) for 45 minutes.

After equilibration, a cumulative concentration-response curve to carbachol was obtained using a step-wise addition of carbachol (concentration range $10^{-9}$ to $10^{-4}$ M). Contractile responses to carbachol were not consistent in all animals, however, carbachol 3x $10^{-6}$ M was an effective submaximal concentration for all groups studied.

After the contraction plateau, cumulative concentration-response curves to isoprenaline ($10^{-9}$-$10^{-4}$ M) were applied to the tissue and relaxant responses were established over 2-3 mins. Upon completion of the isoprenaline response curve a concentration-response curves to NECA ($10^{-9}$-$10^{-4}$ M) was then obtained. Increasing concentrations of agonists were added

cumulatively to the bath, at 5 minute intervals, in ½ $\log_{10}$ concentrations (1 nM, 3 nM, 10 nM, 30 nM), however the total volume of the drug added did not exceed 5% of total organ bath volume (Kitchen, 1984).

Bladder contractile responses were measured using grams tension developed and data was expressed as mean ± SEM. Comparisons between groups were analysed using the Statistica programme with multivariant analysis (Manova) (Statistica, New York) *was* used to determine significant differences between and within groups. Where $P<0.05$, individual comparisons were *performed* using the post-hoc Neuman Keuls test.

Data Analysis

*As the contractile responses to carbachol do reduce over time the second bladder section served as a control for contractile tension for the duration of the concentration-response curve.* Relaxant action of the agonists used were calculated as the difference between the tension measured prior to the agonist administration and the tension measured following agonist administration as a percentage of the original contractile tension induced by $3x10^{-6}$M carbachol, for each detrusor strip as follows;

$$\text{\% relaxation} = \text{absolute value} \left[\frac{\text{tension (after agonist)} - \text{tension (before agonist)}}{\text{tension (before agonist)}}\right] \times 100$$

Similar to the carbachol contraction data, significance was determined by multi-way analysis of variance (MANOVA) with a post hoc Newman-Keuls test (Statistica, New York). Data were plotted using Graphpad Prism®. The maximum relaxation as % response of pre-contracted tension of the bladder strips to isoprenaline and NECA for each group are presented, with $P < 0.05$, considered significant.

Adenosine receptor gene expression in bladder tissue

Bladder tissue was dissected from the euthanised animal, weighed, sliced and placed into a 2ml microcentrifuge tube prior to homogenisation in 300 μl RLT buffer (Qiagen) containing guanidine thiocyanate. Total RNA was isolated using Qiagen Rnaeasy mini-columns, in accordance with manufacturers' instruction. Total RNA was then treated with chloroform, the aqueous phase removed and subjected to DNAse I treatment (0.4 μl, 37 $^{o}$C, 15 min, Sigma). First strand cDNA synthesis was carried out using 1-5 μg of total RNA, 5 U/μL reverse transcriptase (Superscript$^{TM}$, Invitrogen), 1 x RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) (Invitrogen), 100 μM dNTP mix (equimolar, dGTP, dCTP, dATP and dTTP), 10 μM DTT (Invitrogen) and 25 ng/μL random decamers (Geneworks) at 42$^{o}$C for 1 hr. Each representative cDNA pool was then aliquotted and stored at –20 $^{o}$C until required. Eight samples from each age group were analysed in quadruplicate for each oestrogen and adenosine receptor gene. Adenosine receptor primer sets used in this study have been published (Rose'Meyer et al. 2003). Each master mix contained a single receptor-specific primer set (forward and reverse, 0.5 μM each, Table 1), 100 ng of cDNA, Rox$^{TM}$ Fluorescein Calibration Dye (15 nM), 1 x PCR buffer II (50 mM KCl and 10 mM Tris HCl, pH 8.3), $MgCl_2$ (see Table 1), 200 μM dNTP mix, 1 x BSA, 0.5 x SYBR Green I, and *Taq* DNA polymerase (0.05 U/μL). Each 96 well plate assay contained 3-replicates and β-actin primers as a reference gene. The following cycling conditions were used for all Q-PCR SYBR Green I PCR assays with the IQ iCycler (Bio-rad): Cycle 1, 95$^{o}$C for 5 min (x 1); Cycle 2, 95$^{o}$C for 30 sec; 57$^{o}$C for 1 min; 72$^{o}$C for 30 sec (45 cycles); and Cycle 3, 72$^{o}$C for 4 min. At the end of each Q-PCR assay, the CT at linearity was determined for each primer set.

Q-PCR assays that showed non-specific product or primer dimer at end point were not included in further data analysis. A combination of both 10% PAGE and melt-curve analysis was used to discriminate between specific target and non-specific product amplification at end point. Following Q-PCR analysis of receptor gene expression, CT values are collected at linearity and used to calculate the β-actin mRNA corrected CT (or ΔCT) for each receptor

subtype in the bladders from rats that have undergone either ovariectomy (ΔCT ) and/or sham-operation (ΔCT) and/or fed different diets (soy or non-soy), and/or of different age groups.

Repeat determinations of age matched ΔCT ± SE values were then used to calculate the mean corrected difference in CT for each receptor subtype (or ΔΔCT ± SE). The extent of the response is determined by $2^{\text{mean }(\Delta\Delta CT)}$, while a negative value suggests repression of receptor expression, so the relative degree of response is calculated by $2^{-\text{mean }(\Delta\Delta\ CT)}$. All the values are related to a common reference standard which was cDNA from brain for adenosine receptor gene expression (Dixon et al. 1996) from soy fed, sham operated animals. Data was then expressed as a percentage compared to the reference standard.

These data were subsequently analysed using a MacLab Data Acquisition System *and* results produced for each group are reported as mean ± standard error of the mean (SEM). Differences between groups were examined using Neumann Keuls test for multiple comparisons (Statistica, New York).

Chemicals

NECA, isoprenaline and carbachol (carbamyl chloride) were purchased from Sigma Chemicals (Castle Hill, Australia). All solutions were prepared as stock solutions ($10^{-3}$M) in distilled water.

**Results**

Effect of ovariectomy or phytoestrogen diet on reproductive hormone levels

In aged rats (52 weeks), plasma oestrogen levels were over 400% higher than those recorded from the young and mature rats, but did not show additional significant variability with either diet or ovariectomy treatments (P>0.05, data not shown, see Owen et al. 2011).

In comparison, plasma progesterone levels were reduced with increasing age of the rats. Plasma progesterone concentrations, derived from ovarian sources, were significantly reduced (~30-50%) as a result of ovariectomy, regardless of diet, in both young and mature age groups (P<0.05, data not shown, see Owen et al. 2011).

Effect of ovariectomy or phytoestrogen diet on carbachol induced contraction

*At 12 weeks of age, the potency or maximal contractile response to carbachol did not change as a result of dietary phytoestrogens or loss of oestrogen (data not shown, P>0.05 see Owen et al. 2011). However, at 24 weeks of age, carbachol had reduced potency in detrusor strips from non-soy ovx treated rats when compared to age-matched non-soy sham (P<0.05). In bladders from aged rats, a reduction in contractile response to carbachol was observed in all aged groups studied except the soy fed sham group which retained contractile responses to carbachol (P<0.05). The potency of carbachol in aged bladders did not differ between the groups (P>0.05). Further experiments utilised the submaximal contraction dosage of 3 μM carbachol to pre-contract the bladder for relaxant agonist studies.*

NECA induced relaxation of bladder strips.

Data from NECA induced relaxant responses in carbachol pre-contracted bladder strips from all age, diet and treatment groups are shown in Table 1 and Fig. 1. *As NECA concentration-response data when analysed were not sigmoidal,* $EC_{50}$ values for this compound have not been included. NECA induced a concentration-dependent relaxation of carbachol pre-

contracted detrusor strips in all treatment groups of the young animals (see Fig. 1). Mature animals exhibited reduced responsiveness to NECA-mediated relaxation compared to the young group with exception of the soy sham group ($P<0.05$). In contrast, in aged animals there was no significant relaxation of pre-contracted detrusor strips in response to increasing concentrations of NECA.

Examination of bladder strip relaxation with regard to age showed that all diet and treatment groups exhibit a reduced capacity for relaxation with increasing age *with the exception of the mature soy sham group which maintained relaxant responses to NECA similar to those observed in the young soy sham group.* Indeed, bladder strips from aged animals from each group exhibited contractile responses to NECA, with the greatest effect observed in bladders from aged soy sham rats. Thus overall, relaxation of pre-contracted bladder strips was greatest in young rats and progressively fell with age.

Isoprenaline induced relaxation of bladder strips.

Data from isoprenaline induced relaxant responses in carbachol pre-contracted bladders strips is presented in Table 2 and Fig. 2 for all age, diet and treatment groups

Bladders from young animals showed a progressive relaxation of pre-contracted detrusor strips with increasing concentrations of isoprenaline in all treatment groups studied. Mature rats showed there was a clear difference in relaxant response to isoprenaline in bladders from sham-operated rats when compared to ovariectomised animals ($P<0.05$). Bladders from ovariectomised rats had attenuated relaxant responses to isoprenaline when compared to control bladder, indicating a potentially additive effect of age and ovariectomy in mature animals. *Bladders from all aged treatment groups demonstrated reduced relaxant responses to isoprenaline when compared to relaxant responses observed in bladders from young rats ($P<0.05$).*

Comparison of data from the non-soy fed ovx and soy fed ovx groups demonstrated a reduced responsiveness of mature and aged bladders to isoprenaline when compared to young rats within each treatment group. *Data from both sham groups did not support this trend, since the mature groups maintained relaxant responses observed in bladders from young animals, perhaps indicative of an effect of oestrogen.*

Effect of age diet or ovariectomy on adenosine receptor mRNA expression

mRNA expression of the adenosine $A_1$ receptor was observed in all bladder groups tested (see Fig. 3). In young rats a decrease in the expression of adenosine $A_1$ receptor mRNA was observed in non soy-fed ovariectomised and soy fed sham rats when compared to aged matched bladders from non-soy fed sham rats ($P<0.05$). In mature rats, a reduction in the expression of adenosine $A_1$ receptor mRNA was seen in all other dietary and treatment groups when compared to aged matched bladders from non-soy fed sham rats ($P<0.05$). Analysis of mature rat bladders from the non soy-fed ovariectomised and soy fed sham rats showed the expression of adenosine $A_1$ receptors increased to levels similar to the aged non-soy fed sham rats ($P>0.05$).

The mRNA expression of the adenosine $A_{2A}$ receptor was measured in all bladder groups tested (see Fig. 3). However, in bladders from young soy fed sham rats no expression of adenosine $A_{2A}$ receptor mRNA was detected. Overall, similar changes in adenosine $A_{2A}$ receptor expression to the adenosine $A_1$ receptor was observed with reduction in non soy-fed ovariectomised, soy fed sham and ovariectomised rats when compared to aged matched bladders from non-soy fed sham rats from young and mature rats ($P<0.05$). Similar levels of adenosine $A_{2A}$ receptor mRNA expression appeared across the dietary and treatment groups in bladders from aged rats ($P>0.05$).

Adenosine $A_{2B}$ receptor mRNA expression was present in bladders from all groups (see Fig. 4). Similar patterns of mRNA expression occurred in the young and mature groups to adenosine $A_1$ and $A_{2A}$ receptors. An increase in adenosine $A_{2B}$ receptor mRNA was observed in bladders from mature rats when compared to young rats *from the non soy-fed sham group* ($P<0.05$). Similar levels of adenosine $A_{2B}$ receptor mRNA expression was observed across the dietary and treatment groups in bladders from aged rats ($P>0.05$).

Although similar trends were observed in the adenosine $A_3$ receptor expression (see Fig. 4) no significant differences were determined with age, diet or ovariectomy ($P>0.05$).

**Discussion**

The effect of age, diet and ovariectomy was studied on bladder relaxant responses to agonists, isoprenaline and NECA on carbachol pre-contracted strips from female rats. Most commonly, ovariectomy is assumed to simply result in reduced plasma oestrogen levels, particularly in the short term post surgery, and physiological changes are usually attributed to reduced circulating oestrogen concentrations and perhaps subsequent effects on tissue maintenance. *In this study, plasma oestrogen levels were reduced in short term post-ovariectomy but were not reduced over the long term. In the rat model, ovariectomy has been previously reported to reduce plasma oestradiol levels by only 30-50% (Alagwu and Neli, 2005) as the remainder is derived from adipose tissue (Grodin et al., 1973). This implies that as a model for early menopause in humans, ovariectomised rats can only be used for short term studies or need to be calorie restricted to prevent increases in body fat and adipose tissue derived oestrogen.*

In this study investigating bladders from female rats, consistently reduced relaxation to both NECA and isoprenaline was exhibited by aged animals compared to the young group. Attenuated responses to β-adrenoceptor agonists have been previously observed in bladders from male rats (Derweesh et al. 2000).

Ovariectomy increases voiding frequency and decreased bladder capacity by 25% in awake rats (Kullman et al. 2009), potentially indicating a role for hormonal changes in bladder function. β-adrenergic receptor activation plays an important role in facilitation of urine storage and a reduction in the β-adrenoceptor mediated relaxation of the bladder has been observed with increasing age (Nishimoto et al. 1995).

In our study, ovariectomy resulted in attenuated relaxant responses to isoprenaline with maturation as seen in bladders from 24 week old rats. Thus a link between ovarian function and β-adrenoceptor function in mediating relaxation is indicated, however, the signalling mechanism involved requires further investigation. Interestingly, β-adrenoceptor gene

expression, as determined by RT-PCR, is reportedly unchanged in ovariectomised and sham-operated rats (Kullmann et al. 2009), thus the effects observed in our study may not result from a decrease in the receptor population, but a reduction in the capacity of the receptor to respond to isoprenaline. Stimulation of β-adrenoceptors activates the stimulatory G-protein, Gs, which activates the catalytic component of adenylyl cyclase. Age related alterations in β-adrenoceptor mediated relaxant responses to isoprenaline have been reported in bladders from male rats, being attributed to changes in the G protein content that cause an overall reduction in cAMP mediated relaxation (Derweesh et al. 2000), however other studies have indicated no change in G protein content in aged bladders (Schneider et al, 2003).

Induction of relaxation by NECA demonstrates a role for adenosine receptors in bladder relaxation, at least in male rats (Nicholls et al. 1992), where there is also a marked reduction of relaxation with age. Similar effects of ovariectomy on bladder responses to isoprenaline were observed with maturational decreases in the relaxant response of the bladder from 24 week old rats to NECA. In bladders from sham treated groups, relaxant responses to NECA were maintained in soy-fed rats, while attenuated responses occurred in tissues from non soy fed animals. Thus, phytoestrogens may offset a combined effect of aging and ovariectomy, until in aged animals (52 weeks old) where the capacity for relaxation is abolished *despite a significant rise in endogenous oestrogen, suggesting that age related changes can not be reversed by elevated oestrogen levels.* These results indicate the importance of considering age, as well as gender in animal studies examining bladder function.

The uroepithelium expresses adenosine receptor $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ subtypes and adenosine is synthesised and released by both cell surfaces of uroepithelium, but particularly the serosal surface of stretched epithelium, and stimulates membrane turnover in umbrella cells (Yu et al. 2006). Adenosine $A_{2B}$ receptors are located post-junctionally in the bladder and mediate relaxation of the tissue (Nicholls et al. 1992; Aronsson et al. 2010), whereas adenosine $A_3$ receptors are reported to induce contraction of the bladder (Vesela et al. 2011). The signalling

pathways downstream of adenosine (i.e. following adenosine receptor signalling) in the bladder are not currently well understood but may involve changes in $Ca^{2+}$ and cAMP levels (Yu et al. 2006; Apodaca et al. 2007). In other tissues, adenosine $A_1$ and $A_3$ receptors are coupled to Gi/o to inhibit cAMP production while adenosine $A_2$ receptor subtypes, similar to β-adrenoceptors are coupled to Gs and stimulation of adenylyl cyclase activity. The mRNA expression of the G-proteins alpha subunits do not differ between male and female bladders (Kories et al, 2003). These receptors have also been reported to activate a range of signalling pathways depending on tissue type and pathological state.

With respect to adenosine receptor mRNA expression in female rat bladder, all four adenosine receptors subtypes have been observed, which is consistent with a previous report by Dixon et al. (1999). Similar to this study, Dixon reported that among the adenosine receptor subtypes present in the bladder, the highest expression occurred in the adenosine $A_{2B}$ receptors. The current study, using young and mature female rats, determined that expression of adenosine receptors ($A_1$, $A_{2A}$ and $A_{2B}$) was lowest in the bladder from non soy fed ovariectomised animals and that the use of phytoestrogens in the diet increased the mRNA expression of these receptors. These results are similar to the effect of ovariectomy on brain adenosine receptors (Rose'Meyer et al. 2003) which utilised ovariectomised rats three months post surgery, similar in age to the mature group in the current study. In general, for sham operated female rats, the soy diet appeared to reduce expression of these receptors, which may indicate that phytoestrogens negate the role of endogenous oestrogen and expression of adenosine receptor subtypes. *With increasing age, the expression of the adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptor mRNA in bladders were maintained or increased.* While similar trends can be observed in the bladder mRNA expression of the adenosine $A_3$ receptors, no significant differences were shown with age, diet or ovariectomy. With respect to relative expression of the adenosine receptor subtypes within bladder groups the most highly expressed gene was the adenosine $A_{2B}$ receptor. Adenosine $A_{2B}$ receptors are

responsible for relaxation of the bladder while the adenosine $A_3$ receptors have been implicated in bladder contraction (Vesela et al. 2011). Adenosine $A_1$ receptors can cause contraction of smooth muscle in blood vessels (Jenner and Rose'Meyer, 2006), vas deferens (Hourani and Jones, 1994) and lungs (Nadeem et al. 2006). The relative expression of the adenosine receptor subtypes was not significantly altered with age, diet or ovariectomy, indicating no significant shift in the expression ratio of relaxant versus contractile receptors subtypes.

Hence, despite the fact that the adenosine $A_{2B}$ receptor expression varies considerably within the young age group, functional responses to NECA were similar, suggesting a considerable receptor reserve for this receptor in the bladder. *As adenosine receptor mRNA expression does not decrease with age while relaxant responses decline, suggests changes in second messenger systems and not receptor numbers are occurring, assuming the mRNA expression translates proportionally to protein. In the aged bladders, contractile responses to NECA were observed. As indicated earlier, the adenosine $A_3$ receptor has been reported to induce contraction of the detrusor. As both receptors are expressed in the bladders from all age and treatment groups, the loss of adenosine $A_{2B}$ receptor mediated relaxation with age allows the contractile response induced by adenosine $A_3$ receptors to be observed.*

Overall, the adenosine receptor expression in the aged animals within the ovariectomised groups recovers, implicating a potential role for oestrogen, since aged animals have very high circulating concentrations of oestrogen, but not progesterone in their plasma, relative to the younger animals. Adenosine receptor subtype mRNA expression tends to increase *in aged bladders* in all the treatment groups studied, thus NECA may cause contractile responses in the aged female rat bladder in response to the loss of NECA induced bladder relaxant responses (probably downstream of the adenosine $A_{2B}$ receptor) while contractile responses (via the adenosine $A_3$ receptor) are maintained or enhanced.

Fig. 1. Concentration-response curves to NECA in female rat isolated detrusor muscle strips pre-contracted with carbachol ($3x10^{-6}$M) following dietary intervention and/or following ovariectomy or sham operated procedures. Tissues are from young (upper panel), mature (centre panel) and aged (lower panel) rats. n= 8 per group, *P < 0.05 vs non soy sham.

Fig. 2. Concentration-response curves to isoprenaline in female rat isolated detrusor muscle strips pre-contracted with carbachol ($3x10^{-6}$M) following dietary intervention and /or following ovariectomy or sham operated procedures. Tissues are from young (upper panel), mature (centre panel) and aged (lower panel) rats. n= 8 per group, *P < 0.05 vs non soy sham.

Fig. 3. The effect of age on mRNA expression of adenosine $A_1$ receptors (left panel) and adenosine $A_{2A}$ receptors (right panel) in bladders from non soy fed sham from young (12 weeks), mature (24 weeks) and aged (52 weeks) female rats, n=6-7 per group *P < 0.05 vs non-soy fed sham within each age group; † P<0.05 vs young within each treatment group.

Fig. 4. The effect of age on mRNA expression of adenosine $A_{2B}$ receptors (left panel) and adenosine $A_3$ receptors (right panel) in bladders from non soy fed sham from young (12 weeks), mature (24 weeks) and aged (52 weeks) female rats, n=6-7 per group *P < 0.05 vs non-soy fed sham within each age group; † P<0.05 vs young within each treatment group.

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| Treatment | Young (12 weeks) $E_{max}$ (%) | Mature (24 weeks) $E_{max}$ (%) | Aged (52 weeks) $E_{max}$ (%) |
|---|---|---|---|
| Non soy sham | 25 ± 6 | 14 ± 6* | -20 ± 15* |
| Non soy ovx | 27 ± 9 | 12 ± 8* | -3 ± 4* |
| Soy sham | 32 ± 10# | 40 ± 12# | -50 ± 25* |
| Soy ovx | 41 ± 10# | 1 ± 0* | -22 ± 15* |

**Table 1.** Maximal relaxation response to 5'-(N-ethylcarboxamide) adenosine (NECA) in pre-contracted female rat detrusor muscle strips with diet, treatment and age. Data represents Mean ± SEM, n = 8, $p < 0.05$, *vs young, #vs non soy age-matched sham.

| Treatment | Young (12 weeks) $E_{max}$ (%) | Mature (24 weeks) $E_{max}$ (%) | Aged (52 weeks) $E_{max}$ (%) |
|---|---|---|---|
| Non-soy sham | 29 ± 9 | 30 ± 11 | 10 ± 5* |
| Non-soy ovx | 33 ± 7 | 3 ± 10*# | 15 ± 8* |
| Soy sham | 26 ± 3 | 27 ± 7 | 14 ± 6 * |
| Soy ovx | 46 ± 4 | 3 ± 6*# | 19 ± 3* |

**Table 2.** Maximal relaxant responses to isoprenaline in pre-contracted female rat detrusor muscle strips with diet, treatment and age. Data represents Mean ± SEM, n = 8; $p < 0.05$, *vs young, #vs non soy sham.