Acute exposure of adult male rats to dietary phytoestrogens reduces fecundity and alters epididymal steroid hormone receptor expression

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Abstract

Phytoestrogens are plant-derived compounds with oestrogenic activity. They are common in both human and animal diets, particularly through soy-based foods. This study assessed whether exposure of adult male rats to a high phytoestrogen diet for 3–25 days affected their fertility, and assessed possible mechanisms through which phytoestrogens may disrupt fertility. Adult males, fed a high phytoestrogen diet for 3 days, demonstrated significantly reduced fecundity. This effect was transient, with fecundity returning to control levels by day 12. The expression of oestrogen receptor- α and androgen receptor

mRNA was increased in the initial segment of the epididymis, but decreased in the cauda epididymis following 3 days on the high phytoestrogen diet. Epididymal sperm counts cannot account for the reduction in fertility at day 3. However, lipid peroxidation of epididymal sperm was significantly increased in animals fed a high phytoestrogen diet for 3 days. Disruption of the steroid regulation of the epididymis by phytoestrogens may alter its function, resulting in decreased quality of sperm, and thereby reducing fecundity.

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Introduction

Phytoestrogens are plant-derived, non-steroidal compounds, able to activate oestrogen receptor- α (ER α) and ER β (Kuiper *et al.* 1997, 1998). Phytoestrogens can be divided into three main classes – isoflavones, coumestans and lignans. Soy beans and foods derived from them are rich sources of isoflavones, such as genistein.

Oestrogen is vital for the development, maintenance and function of the male reproductive system. Administration or deprivation of oestrogen, both during development and in the adult, has been shown to cause structural and functional changes in the male reproductive tract, including infertility. Neonatal treatment of male rats with oestrogenic chemicals reduces testicular and epididymal sperm concentration, plasma testosterone (Goyal *et al.* 2003, Sharpe *et al.* 2003), Sertoli cell number (Atanassova *et al.* 2005), alters testicular gene expression (Adachi *et al.* 2004) and causes rete tubule distension and reduced epithelial height in the efferent ducts (Aceitero *et al.* 1998, Fisher *et al.* 1998, 1999). The absence of a functional ER α also causes distension of the rete testes, efferent ducts and epididymides, and causes infertility (Lubahn *et al.* 1993, Eddy *et al.* 1996, Hess *et al.* 2000). In adult rats, very similar structural and functional abnormalities to those seen in

ER α knockout mice can be induced by the antioestrogen ICI 182,780 (Oliveira *et al.* 2001), whilst the administration of the synthetic oestrogen diethylstilboestrol (DES) to adult rats reduces reproductive organ weights, epididymal sperm numbers and fertility (Goyal *et al.* 2001).

Phytoestrogen exposure during the neonatal period causes reproductive abnormalities similar to those induced by other oestrogenic chemicals, including the down-regulation of testicular gene expression (Adachi *et al.* 2004). The effect of exposure to phytoestrogens in the adult has received very little attention.

The epididymis, a steroid-dependent organ, is responsible for the post-testicular maturation and storage of sperm. Because of the composition of the sperm plasma membrane and their lack of cytoplasm, sperm in the epididymis are susceptible to damage from reactive oxygen species (Aitken & Vernet 1998). The epididymis protects sperm from oxidative damage by secretion of antioxidant enzymes (Zini & Schlegel 1997) under steroid regulation (Schwaab *et al.* 1998).

The following experiments tested our hypotheses that exposure to high levels of dietary phytoestrogens will reduce fertility, and that this is due to altered steroid regulation of the reproductive tract, increasing oxidative stress of sperm.

Materials and Methods

Diets

Two diets were used in this study: a low phytoestrogen diet (control) and a high phytoestrogen diet (treatment). The low phytoestrogen diet was Diet 86 (Sharpe, Palmerston North, New Zealand) containing (w/w) 78.8% cereal, 1.5% skimmed milk, 7% fish meal, 6% bone meal, 0.5% NaCl, 0.1% rodent premix and 1% soy meal. The total phytoestrogen content of the low phytoestrogen diet was 112 µg/g comprised of 53.5 µg/g genistein, 32.5 µg/g daidzin and 26 µg/g glycitein. The high phytoestrogen diet was Diet RMH 3500 (Reliance Stockfoods, Dunedin, New Zealand) and contained (w/w) 61% cereal, 3.5% skimmed milk, 2.5% fish meal, 7.5% meat/bone meal, 0.4% NaCl, 0.3% rodent premix and 25% soy meal. The total phytoestrogen content of the high phytoestrogen diet was 465 µg/g made up of 225 µg/g genistein, 180 µg/g daidzin and 60 µg/g glycitein. Concentrations of isoflavones are the sum of individual isomers, as determined by an independent analysis by the Department of Food Science and Human Nutrition, Iowa State University, Iowa, IO, USA.

Animals

This study was approved by the University of Otago Animal Ethics Committee. To exclude developmental effects of phytoestrogen exposure, all male and female Wistar rats used in the experiments were bred from females fed the low phytoestrogen diet prior to mating and during pregnancy and lactation. The offspring were weaned onto the low phytoestrogen diet and maintained on this diet until adulthood (90 days of age) when they were included in the study. The rats were group housed with others of the same sex and kept under a 12 h light:12 h darkness cycle and had food and water available *ad libitum*. At the conclusion of experiments, rats were killed by CO₂ inhalation.

Experiment 1: Effects of a high phytoestrogen diet on fertility

All rats (male and female) were mated to provide a baseline litter size for each female. Females fed a low phytoestrogen diet were housed for 4 days and nights with a male, also fed a low phytoestrogen diet, to allow mating to occur. The subsequent litter size for each female was recorded. Any female or male that failed to produce a litter was excluded from the study.

Following the birth of the first litters, females ($n=48$) were continually fed the low phytoestrogen diet whilst males were randomly assigned to a control ($n=6$) or treatment group ($n=6$). The control group was continued on the low phytoestrogen diet. The treatment group was transferred to the high phytoestrogen diet (day 0).

All males were then mated 3, 6, 12 and 25 days after the initiation of dietary regimes, overnight with a pro-oestrous female (as determined by vaginal smear histology) in individual cages with the low phytoestrogen diet provided. Males were removed and the presence of sperm in vaginal smears confirmed that mating had occurred. Females were housed individually until parturition and the number and sex of the pups were determined. The number of pups in the first (baseline) litter of each female was compared with the number in the second litter after mating with either a male continually fed the low phytoestrogen diet, or a male which had been transferred to the high phytoestrogen diet. After the final mating (day 25) the males were killed.

Experiment 2: Effects of a high phytoestrogen diet on fertility parameters

A second study assessed the effect of increasing periods of dietary phytoestrogen exposure on epididymal sperm counts, epididymal steroid hormone receptor expression and testicular testosterone and plasma gonadotrophin levels. Adult male rats were randomly allocated to either the control group or the treatment group. The treatment group was transferred to the high phytoestrogen diet (day 0) while the control group remained on the low phytoestrogen diet. After 3, 6, 12 and 25 days rats ($n=8$ each time-point) from both the control and treatment groups were killed and tissues collected. One epididymis from each rat was immediately frozen in liquid nitrogen and subsequently stored at -80°C . The other was immersed in Bouin's fixative for 24 h and then stored in 70% ethanol at room temperature until dehydrated and embedded in paraffin wax prior to histological examination. Trunk blood was collected in heparinised tubes and centrifuged to separate plasma from the haematocrit. The plasma was removed and stored at -30°C .

Experiment 3: Effects of a high phytoestrogen diet on oxidative stress

To determine if reduced litter size is associated with oxidative damage, a third study was carried out to assess the effects of phytoestrogens on oxidative stress of epididymal sperm after 3 days of exposure to a high phytoestrogen diet. Adult male rats were raised on the low phytoestrogen diet and randomly assigned to either the control ($n=10$) or treatment ($n=10$) groups. The treatment group was transferred to the high phytoestrogen diet. After 3 days the rats were killed by CO₂ inhalation and epididymides were removed and stored on ice for the lipid peroxidation experiment.

Sperm counts and morphology

Counts of epididymal sperm were performed on the four regions of one epididymis per rat. Each segment was

thawed on ice and minced in 1 ml 0.9% (w/v) saline for 90 s using two razor blades according to the method of Taylor *et al.* (1985). The average count for four separate aliquots was calculated. The mean coefficient of variation was 11.53%.

The diluted homogenates used for sperm counts were also used to assess sperm morphology. The sperm suspension was further diluted to allow individual sperm to be clearly assessed. Ten microlitres of the sperm suspension were placed on a glass slide and covered with a glass coverslip. The slide was left to sit at room temperature for 1 min to allow the saline and sperm to settle.

Under the $\times 40$ objective lens a minimum of 200 sperm was classified as being of normal morphology, or as having a head defect or a tail defect. Head defects included a sideways, misshapen or double head. Examples of tail defects are the tail being significantly curled or bent up towards the head and sperm having a double tail. The percentage of normal and abnormal sperm was determined for each animal.

Plasma gonadotrophin levels

RIAs were performed to measure the plasma concentration of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as previously described (Nicholson *et al.* 1991). The limits of detection for the LH and FSH assays were 0.12 ng/ml and 0.5 ng/ml respectively. The mean intra-assay coefficient of variation for the two LH assays was 14.06%, while the interassay coefficient of variation was 18.07%. For the FSH assays, the mean intra-assay coefficient of variation was 17.54%, and the variation between assays was 30.29%.

Testicular testosterone levels

Testosterone was extracted from testes of rats from experiment 2 and experiment 3 at 3 days from a known amount of tissue by homogenisation with 70% methanol in glass tubes. The tubes were left at 4 °C overnight and then centrifuged at 3000 *g* for 30 min at 4 °C. The supernatant was transferred to a fresh glass tube, dried and extract resuspended in 1 ml testosterone buffer (0.158 mol/l Na_2HPO_4 , 0.044 mol/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.154 mol/l NaCl, 0.015 mol/l NaN_3 and 0.1% (w/v) gelatine; pH 7.0–7.2) overnight at 4 °C. Testosterone was measured by RIA as previously described (Yeung *et al.* 1988). The antiserum used was 85/6 (Department of Anatomy, University of Bristol, Bristol, UK). The limit of detection of the assay was 40 pg/ml and the interassay coefficient of variation was 10.8%.

Steroid hormone receptor expression

Epididymides were divided into the four morphologically distinct regions – the initial segment, caput, corpus

and cauda – according to Serre & Robaire (1998). ER α , ER β and androgen receptor (AR) expression was measured in the four epididymal regions of 3- and 6-day rats. Tissue was crushed under liquid nitrogen and total RNA extracted from each region using TriZol (Invitrogen Life Technologies, Inc) following the manufacturer's protocol. The RNA pellet was resuspended in RNase/DNase free water and the concentration and purity of the sample was measured. Total RNA (1 μg) was reverse transcribed with random hexamers into cDNA in a 20 μl reaction according to the manufacturer's protocol (MultiScribe II reverse transcription kit; Applied Biosystems, Branchburg, NJ, USA).

Steroid receptor expression was quantitated by real-time PCR of cDNA using the Sequence Detection System 7000 (Applied Biosystems). Primer sets and fluorescent TaqMan MGB probes were designed, using the Applied Biosystems Primer Express program, to detect rat ER α (X61098), ER β (NM_012754) and AR (NM_012502). Forward primer, reverse primer and probe sequences for ER α were 5'-CCACCGAGT CCTGGACAAGA-3', 5'-CGGATATGGGAAAGGA TGA-3' and 5'-CACAGACACTTTGATCCACTTG ATGGCC-3' respectively. For ER β , forward primer, reverse primer and probe were 5'-CACTGCACCTT CCCAGGAGTCA-3', 5'-CTTGGCATTTCGGTGGT ACATC-3' and 5'-TGGTTGTGTGGCGGTGTTCC TCATA-3' respectively. The ER β primers and probe recognise both isoforms of ER β -ER β 1 (NM_012754) (Kuiper *et al.* 1996) and ER β 2 (AB190770) (Chu & Fuller 1997). AR forward and reverse primer sequences were 5'-GGGACATGCGTTTGGACAGT-3' and 5'-CCACAGATCAGGCAGGTCTTC-3', with probe sequence 5'-CCAGGACCACGTTTTTACCCATCG ACTA-3'. Absolute standards (2×10^{-4} to 4×10^{-10} ng) prepared from purified cDNA identical to the real-time PCR products of the targets (ER α , ER β and AR) were included on each plate, to ensure equal efficiency of amplification between standards and sample products. No template controls and no reverse transcriptase controls were used as negative controls. The reaction mixture contained 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.9 $\mu\text{mol/l}$ of the forward and reverse primers, 0.25 $\mu\text{mol/l}$ of the 5' FAM labelled TaqMan MGB probe and 50 ng sample cDNA, in a final reaction volume of 25 μl . The standards, samples and negative controls for each target were run in triplicate with a thermal cycling profile of 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The concentration of target cDNA in each sample was calculated using the linear equation of the appropriate standard curve and normalised to the amount of total RNA used in the reverse transcription reaction.

Tubule and lumen diameters in proximal epididymis

Fixed proximal epididymides were dehydrated, wax embedded, cut into 4 µm sections and stained with haematoxylin and eosin. Sections were viewed on a light microscope under the ×4 objective lens and photos were taken of the sections using a Spot digital camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). A modified version of Cruz-Orive's (1987) selector method was used to select profiles of the epididymis. A grid of parallel, point-sampled, intercept lines (the points being 4 cm apart on lines that are 2 cm apart) was orientated by randomly selected angles over the displayed images using a sine-weighted angle reference frame. The lumen and tubule diameters of tubules selected by the intercept lines were measured along the parallel lines using the measure tool in the Spot program.

Sperm lipid peroxidation

Each epididymis was placed in 2.5 ml 0.9% saline and cut lengthwise with a razor blade to release sperm. Sperm counts were made for each sample as described above. The thiobarbituric acid reactive substances assay, measuring malondialdehyde (MDA), a by-product of lipid peroxidation, was based on the method of Ohkawa *et al.* (1979). MDA standards were made by acid hydrolysis of tetraethoxypropane and serially diluted (0–0.25 µmol/l). The thiobarbituric acid (TBA) reaction mix was comprised of 3 ml H₂O, 3 ml 70% perchloric acid (BDH Chemicals Ltd, Poole, Dorset, UK) and 0.03 g TBA. Two hundred microlitres of TBA reaction mix were added to 800 µl sperm suspension or 800 µl MDA standard in 15 ml plastic screw top tubes. The tubes were incubated in a boiling waterbath for 15 min and cooled on ice for 3 min. The coloured adduct was extracted by vortexing with 6 ml 67% (v/v) butanol, and centrifuged at 2740 g for 5 min at 4 °C. Two hundred microlitres of the butanol phase were measured fluorometrically with an excitation wavelength of 510 nm and an emission wavelength of 544 nm. The concentration of MDA in the epididymal sperm samples was calculated using the linear equation from the standard curve and normalised to 2×10^7 sperm.

Statistics

All data given are means ± S.E.M. Paired *t*-tests were used to compare litter sizes between the first and second litters of females. Differences in litter incidence was tested by Fisher's exact test. Sex ratios, body and epididymal weights, sperm counts, plasma gonadotrophin levels, real-time PCR, tubule measurements and lipid peroxidation results were compared by ANOVA. $P < 0.05$ was deemed statistically significant. Statistical analysis was carried out using GraphPad Prism 4.0 Software (GraphPad Software Inc,

San Diego, CA, USA). Datum points determined as outliers by Dixon's test were excluded from analyses (Sokal & Rohlf 1981).

Results*Litter sizes and sex ratios*

Five female rats were excluded from the study because they did not become pregnant or had only one pup following the initial mating. After they had been killed it was discovered that a control male had significantly smaller than average reproductive organs and below average testicular and epididymal sperm counts. Data from this male, including that of litter size, were excluded from analyses.

Five females did not become pregnant following the second mating. Three were housed with high phytoestrogen males and two were housed with low phytoestrogen males. The two females in the low phytoestrogen group had ambiguous vaginal smears prior to mating and subsequently failed to mate as determined by the absence of sperm. Therefore they were most likely not in pro-oestrus and were unreceptive. Of the three females in the high phytoestrogen group, one mated with a 12-day male had an ambiguous smear prior to mating, but did mate. This mating did not result in a litter. One female, housed with a 25-day treatment male, whilst pro-oestrus, as determined by vaginal cytology, failed to mate. The third female did mate, did not have an ambiguous smear but failed to produce a litter. This female was mated with a male after 6 days of treatment. Whilst all failures to produce litters following a successful mating occurred in pairings with males fed the high phytoestrogen diet, litter incidence was not significantly different.

There was no significant difference between the number of pups in the first and second litters at any of the four time-points when the females were mated only with males on a low phytoestrogen diet (Fig. 1A). However, litter sizes were significantly reduced ($P = 0.017$) for females mated with a male fed a high phytoestrogen diet for 3 days prior to mating (Fig. 1B). There was no significant change in litter size after the male was fed a high phytoestrogen diet for 6, 12 or 25 days (Fig. 1B). There was no difference in the incidence of litters, irrespective of litter size, between treatments or time-points and hence no difference in outright fertility.

The average ratios of male to female pups in the second litters ranged from 0.570 to 1.693, and were not significantly different between the control and treatment groups.

Body and epididymal weights

The average total body weight for the day-3 control ($n = 8$) and treatment groups ($n = 7$) were 368 ± 8 g and

Figure 1 Mean $\pm$ S.E.M. differences between the first and second litters when males were fed (A) a low or (B) a high phytoestrogen diet for 3, 6, 12 and 25 days. The number of replicates for each determination (n) are given on or above the bars. * $P=0.017$.

378 ± 8 g respectively. The average epididymal weight for the control group was 440 ± 11 mg while that for the high phytoestrogen treatment group was 471 ± 10 mg. There were no significant differences in body or epididymal weights.

Sperm counts and morphology

Total epididymal sperm concentration in 3-day control ($n=8$) and treatment groups ($n=7$) were $1.94 \times 10^{-9} \pm 1.22 \times 10^8/\text{g}$ and $1.93 \times 10^9 \pm 1.02 \times 10^{-8}/\text{g}$ respectively. These were not significantly different. Sperm morphology (Table 1) did not significantly differ between the treatment group and the control group at day 3.

Table 1 Average percentage ($\pm$ S.E.M.) of epididymal sperm with normal morphology of day-3 control ($n=8$) and treatment rats ($n=6$)

	Initial segment	Caput	Corpus	Cauda
Diet				
Control	96.0 ± 0.7	96.3 ± 0.8	95.8 ± 2.2	95.6 ± 1.4
High phytoestrogen	96.2 ± 0.8	96.5 ± 1.3	98.2 ± 0.6	95.8 ± 1.5

Table 2 Mean plasma LH and FSH (ng/ml $\pm$ S.E.M. (n)) and testicular testosterone (ng/ml per g $\pm$ S.E.M. (n)) in day-3 control and treatment groups

	LH	FSH	Testosterone
Diet			
Control	1.00 ± 0.28 (4)	8.52 ± 0.83 (8)	42.81 ± 5.9 (16)
High phytoestrogen	1.48 ± 0.31 (5)	7.48 ± 0.60 (7)	39.83 ± 4.5 (16)

Plasma gonadotrophins and testicular testosterone

No statistically significant differences were found in the plasma concentrations of either LH or FSH or in testicular testosterone between the low phytoestrogen-fed control group and the high phytoestrogen-fed group at any time-point (Table 2).

Steroid hormone receptor expression

ER α , ER β and AR mRNA were all detected in each of the epididymal regions. However, the levels of expression differed between regions. Statistically significant differences were detected in the expression of ER α and AR mRNA between the low phytoestrogen and high phytoestrogen groups. In rats fed the high phytoestrogen diet for 3 days before they were killed, ER α expression in the initial segment was significantly increased compared with that in rats maintained on the control low phytoestrogen diet ($P=0.0007$) while, in the cauda, ER α mRNA was significantly reduced ($P=0.0003$) (Fig. 2A). No differences were detected in ER β expression in any of the epididymal regions after 3 days on the high phytoestrogen diet (Fig. 2A). After 3 days on the high phytoestrogen diet, AR expression was increased in the initial segment ($P=0.0199$) and decreased in the cauda ($P=0.015$) (Fig. 2A). No statistically significant differences were detected in the expression of ER α or AR in the caput and corpus regions following 3 days on the high phytoestrogen diet. In the rats fed the high phytoestrogen diet for the 6 days before they were killed, no statistically significant differences were detected in the expression of ER α or AR in the initial segment or caput regions between the control and treatment groups (Fig. 2B). However, ER α expression was decreased in the corpus whilst ER β expression was significantly lower in the initial segment and corpora epididymis.

Tubule and lumen diameters in proximal epididymis

The sections were confirmed as being from the proximal epididymis by the presence of tall pseudostratified or tall columnar epithelium. There were no significant differences

Figure 2 Mean $\pm$ S.E.M. ER α , ER β and AR cDNA in the four regions of the epididymis for rats fed the low phytoestrogen diet (control, ■) or rats fed a high phytoestrogen diet (treatment, □) for (A) 3 days or (B) 6 days. The number of replicates for each determination (n) are given on the bars. * $P<0.05$; ** $P<0.001$; *** $P<0.0005$.

in lumen diameter, tubule diameter or tubule:diameter ratio between the day-3 control and treatment groups. The average measurements are shown in Table 3.

Lipid peroxidation

The concentration of MDA, as a marker for lipid peroxidation, was significantly higher ($P=0.041$) in the sperm samples from rats fed a high phytoestrogen diet for 3 days

compared with the samples from rats maintained on the low phytoestrogen diet (Fig. 3).

Discussion

This study assessed the reproductive effects of exposing adult male rats to high dietary phytoestrogens for 3–25 days. The diets used in this study were chosen to be

Table 3 Average tubule and lumen diameters ($\mu\text{m} \pm \text{S.E.M.}$) and ratios between the tubule and lumen diameters in the proximal epididymis for day-3 control ($n=7$) and high phytoestrogen-fed rats ($n=6$)

Diet	Tubule diameter	Lumen diameter	Tubule:lumen
Control	250 ± 12	163 ± 10	1.68 ± 0.08
High phytoestrogen	228 ± 7	140 ± 6	1.78 ± 0.03

comparable with those used in previous studies (Weber *et al.* 2001, Wang *et al.* 2002) where the low and high diets generated plasma levels of phytoestrogens similar to those of western and Japanese men (Adlercruetz *et al.* 1993). In the study of Wang *et al.* (2002), rats that had a total daily phytoestrogen intake of 1.8 mg/kg and 19.25 mg/kg body weight, comparable with our study where intake was estimated at 3 mg/kg body weight (low diet) and 14 mg/kg (high diet), generated total phytoestrogen plasma concentrations of 60 and 861 mmol/l respectively. These levels were much lower than those generated in men by dietary supplements available over the counter (Rannikko *et al.* 2006). Exposure to a high phytoestrogen diet for 3 days reduced fecundity. This is a transient effect as rats exposed to the high phytoestrogen diet for a longer period of time did not show a reduction. The absence of changes following 6 or more days on the high phytoestrogen diet was consistent with previous long-term phytoestrogen exposure studies in men and adult rats. Men taking an isoflavone dietary supplement daily for 2 months showed no changes in blood hormone levels, testicular volume or semen parameters (Mitchell *et al.* 2001). Similarly, adult rats fed phytoestrogen-containing diets for 5 weeks or 12 months showed no differences in reproductive organ weights and testicular sperm numbers

Figure 3 Mean $\pm$ S.E.M. concentration of MDA, a marker of lipid peroxidation, in epididymal sperm from rats fed a low phytoestrogen diet or a high phytoestrogen diet for 3 days. The number of replicates for each determination (n) are given on the bars. * $P=0.041$.

when compared with low or no phytoestrogen controls (Ashby *et al.* 2003, Faqi *et al.* 2004).

The reduction in litter size cannot be attributed to a depression of sexual behaviour following exposure to the high phytoestrogen diet. There was no difference in the failure to mate, as determined by sperm in vaginal smears, between the control and treatment groups. Goyal *et al.* (2001) induced infertility in adult male rats via the administration of low levels of DES for 12 days and attributed this result to depressed sexual behaviour or problems with epididymal sperm. The results of this study suggest that the latter is most likely. DES has a much greater oestrogenic potency than phytoestrogens (Kuiper *et al.* 1997) which would explain the more profound effect on fertility seen in that study compared with the present study.

No differences in plasma LH, FSH or testicular testosterone were determined between groups at any time-point, indicating that the hypothalamo–pituitary–gonadal axis was not affected.

Furthermore, total epididymal sperm counts and the percentage of sperm with normal morphology were not altered after 3 days of exposure and therefore do not account for the reduction in fecundity.

It is most likely that the reduced fecundity seen in the 3-day treatment group was a result of altered epididymal function caused by disruption of steroid receptor expression. This was supported by the fact that after 6 days on the high phytoestrogen diet, fecundity was no longer reduced and there were no differences in expression of steroid receptors. After 3 days on a high phytoestrogen diet, both ER α and AR expression were decreased in the cauda epididymis. These changes were similar to those induced by the phytoestrogen genistein in the adult rat prostate (Fritz *et al.* 2002). In contrast, expression of both ER α and AR are up-regulated in the initial segment after 3 days of a high phytoestrogen diet. This suggests that there is differential regulation of these steroid receptors in the epididymis. ER β expression remained unchanged throughout the epididymis, in contrast to the prostate where it was down-regulated (Fritz *et al.* 2002). These findings further highlight the differential regulation of the two oestrogen receptors previously described in the male reproductive tract (Oliveira *et al.* 2004). The present study demonstrates, for the first time, differential regulation of the ERs in the adult epididymis. It should also be noted that it describes, for the first time, fully quantitative regional differences in gene expression of AR, ER α , and ER β in the adult rat epididymis. Regional differences in gene expressions are well documented in the rat epididymis (Jervis & Robaire 2001) and reflect the complex changing environment that sperm experience in the epididymis in order to mature and gain their fertilising ability. The regional expression patterns of AR in the caput, corpus and cauda epididymis are similar to relative levels determined by cDNA array analysis (Jervis & Robaire 2001). Conflicting reports make the distribution

of ER α and ER β less clear. Fisher *et al.* (1997), Atanassova *et al.* (2001) and Yamashita (2004) did not detect ER α in any region of the adult rat epididymis by immunohistochemistry, while Sar & Welsch (2000) detected ER α in the initial segment only. In contrast, Hess *et al.* (1997b) demonstrated that immunoreactive ER α was found throughout the epididymis with the initial segment strongest, similar to the expression profile measured in the 3-day high phytoestrogen group. This may reflect the formula of the rat chow used in that study.

Disruption of oestrogen action, by the removal of functional ER α in mice (Eddy *et al.* 1996, Hess *et al.* 1997a) or the administration of an anti-oestrogen to adult rats (Oliveira *et al.* 2001) causes reduced fluid absorption in the excurrent duct system, leading to lumen distention and flattening of epithelial cell height. This did not appear to be a factor in this study. No change in tubule or lumen diameter was determined. Furthermore, the ratio of lumen to tubule diameters, an indication of epithelial cell height, was not affected.

During storage in the epididymis, sperm must be protected from damage caused by reactive oxygen species (Aitken & Vernet 1998). The epididymis provides protection by the secretion of antioxidant enzymes (Zini & Schlegel 1997) under steroid regulation (Schwaab *et al.* 1998). As it takes 8–9 days for sperm to transit the epididymis of the rat, the last 5 days being spent in the cauda epididymis (Robb *et al.* 1978), it is likely then that disruption of caudal sperm is responsible for the decreased fertility seen after 3 days on the high phytoestrogen diet. Sperm in the cauda epididymis are particularly susceptible to oxidative stress (Tramer *et al.* 1998). This study has shown that sperm lipid peroxidation is significantly increased in epididymal sperm of rats fed the high phytoestrogen diet for 3 days. This coincides with the changes in ER α and AR in the cauda epididymis. Lipid peroxidation has detrimental effects on sperm function, reducing motility and the ability to fuse with an oocyte (Aitken *et al.* 1993). The reduction of epididymal protective strategies by oestrogenic chemicals has been previously demonstrated. Bisphenol A, a chemical with known oestrogenic activity (Kuiper *et al.* 1997, Steinmetz *et al.* 1998), has been shown to reduce antioxidant enzyme activity in the epididymis and to increase lipid peroxidation of epididymal sperm (Chitra *et al.* 2003). To demonstrate unequivocally that lipid peroxidation of sperm is the cause of reduced litter size, one would need to demonstrate that lipid peroxidation was not induced by a high phytoestrogen diet for those time-points where litter size was unaltered. However, the increase in sperm lipid peroxidation seen after 3 days of exposure coincides with induced changes in ER α and AR expression in the cauda epididymis. No changes in these steroid hormone receptors occurred after 6 days and hence we predict that sperm lipid peroxidation would also be unaltered.

In conclusion, acute exposure of adult male rats to a diet of high phytoestrogen content transiently reduces their fecundity. This effect coincides with altered expression of epididymal AR and ER α , indicating disrupted epididymal function. We propose that the reduction in fecundity results from reduced steroid-regulated antioxidant protection in the epididymis, leading to oxidative damage and loss of sperm function.

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