Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells

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Abstract

Apoptosis plays a critical role in regulating sperm production. Removal of androgens and gonadotropins, or estrogen administration induces germ cell apoptosis. It is hypothesized that dietary phytoestrogens increase apoptosis of developing germ cells, decreasing sperm production. This study aimed to test this in rats fed a high phytoestrogen diet only during adulthood. Male Wistar rats used in this study were offspring of females maintained on a low phytoestrogen diet prior to conception through to weaning. After weaning, juveniles were fed the same low phytoestrogen diet into adulthood. A cohort of males were transferred to a high phytoestrogen diet for 24 days and subsequently testes were collected from all animals. In the high phytoestrogen fed group, homogenization-resistant sperm counts were significantly decreased, as were epididymal sperm counts. Morphometric analysis determined round and elongated spermatid volumes to be significantly decreased, but seminiferous tubule lumen diameters to be significantly increased. TUNEL analysis determined that apoptosis of spermatocytes and round spermatids was significantly greater in the high phytoestrogen fed rats. Neither plasma gonadotropin concentrations nor testicular testosterone were altered. In conclusion, exposure of the adult male rat to a high phytoestrogen diet disrupts spermatogenesis, increasing germ cell apoptosis. This effect is independent of the hypothalamo–pituitary–testicular axis and is likely due to disruption of estrogen's actions in the testis.

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Introduction

Since phytoestrogens were associated with the disruption of mammalian fertility (Moersch *et al.* 1967), there has been considerable interest in their effects on sexual development and reproductive function. Phytoestrogens are plant-derived compounds and can be divided into three classes, namely isoflavones, coumestans, and lignans. Soybeans, and foods derived from soy, are a rich source of isoflavones that are able to bind and activate estrogen receptors (Kuiper *et al.* 1997, 1998). Particularly, the binding affinity of estrogen receptor β (ER β) for the isoflavonoid genistein is 87% that for estradiol, and genistein activates ER β with high potency (Kuiper *et al.* 1998).

Estrogen is essential for maintenance and normal activity of the male reproductive tract (Eddy *et al.* 1996, Hess *et al.* 1997). Exposure to exogenous estrogen or inhibition of endogenous estrogen, either during development or adulthood, induces structural and

functional changes in the male reproductive tract. Exposure of neonatal rats to estrogenic chemicals reduces sperm concentrations, plasma testosterone (Goyal *et al.* 2003, Sharpe *et al.* 2003), Sertoli cell number (Atanosova *et al.* 2005), gene expression (Adachi *et al.* 2004), rete tubule distension, and height of efferent duct epithelium (Aceitero *et al.* 1998, Fisher *et al.* 1998, 1999). Similarly, distension of the rete testes, efferent ducts, and epididymides, and subsequent infertility, is seen in ER α null mice (Lubahn *et al.* 1993, Eddy *et al.* 1996, Hess *et al.* 2000). Similar structural and functional abnormalities can be induced by anti-estrogen treatment of adult rats (Oliveira *et al.* 2001), while exposure to low levels of the potent synthetic estrogen diethylstilbestrol (DES) reduces weights of reproductive organs, decreases epididymal sperm, and decreases fertility (Goyal *et al.* 2001).

Apoptosis of select germ cells occurs normally in the testis and is required to maintain homeostasis (Huckins

1978, Blanco Rodriguez 1998, Print & Loveland 2000). Increased apoptosis may be induced following physical or toxicological insult (Richburg 2000). Apoptosis is induced by disruption of the endocrine actions of estrogen by the synthetic DES due to suppression of gonadal testosterone (Nonclerq *et al.* 1996). ER β inactivation decreases apoptosis of spermatogonia in neonatal mice (Delbès *et al.* 2004), while in adult human testes *in vitro* estradiol has been shown to inhibit apoptosis of spermatocytes and spermatids (Pentikäinen *et al.* 2000). Furthermore in aromatase-deficient mice, spermatid development is disrupted with increased germ cell apoptosis (Robertson *et al.* 1999). This mouse model has also demonstrated that dietary phytoestrogen exposure through a soy-containing rodent feed, influences spermatogenesis independently of the hypothalamo–pituitary–gonadal axis (Robertson *et al.* 2002).

While many studies have demonstrated the effects of fetal and neonatal phytoestrogen exposure on testis development and subsequent fertility of the adult male (e.g. Tou *et al.* 1999, Atanassova *et al.* 2000, Roberts *et al.* 2000), very few studies have investigated the effects of adult male exposure to dietary phytoestrogens on spermatogenesis, and none have controlled for effects of exposure during the fetal, neonatal, or pubertal periods. This study aimed to test the hypothesis that adult-only exposure of male rats to a diet of high phytoestrogen content disrupts spermatogenesis by increasing germ cell apoptosis.

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 $\mu\text{g/g}$ comprised of 53.5 $\mu\text{g/g}$ genistein, 32.5 $\mu\text{g/g}$ daidzin, and 26 $\mu\text{g/g}$ glycitein. The high phytoestrogen diet was Diet RMH 3500 (Reliance Stockfoods, Dunedin, New Zealand) and is the rat chow routinely used in our facilities. It 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 $\mu\text{g/g}$ made up of 225 $\mu\text{g/g}$ genistein, 180 $\mu\text{g/g}$ daidzin, and 60 $\mu\text{g/g}$ 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.

Animals

This study was approved by the Otago University Animal Ethics Committee. To exclude developmental effects of phytoestrogen exposure, all male Wistar rats used in the experiments were bred from females fed the low phytoestrogen diet for 3 weeks 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 old) when 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 90 days of age, the male rats were assigned either to the control group ($n=8$), and continued to be fed a low phytoestrogen diet, or to the experimental group ($n=6$) which were transferred to the high phytoestrogen diet for further 24 days. Animals were then killed by CO₂ inhalation. Trunk blood was collected in heparinized tubes, centrifuged to separate plasma from the hematocrit, and the plasma stored at $-30\text{ }^{\circ}\text{C}$. Testes and epididymides were removed and fat was discarded. Tissue was then weighed, flash-frozen in liquid nitrogen, and stored at $-70\text{ }^{\circ}\text{C}$. One testis from each animal was post-fixed by thawing in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol for 24 h. These were then embedded in paraffin wax in an orientation to yield cross-sections of seminiferous tubules and processed for morphological analysis and TUNEL assays.

Testicular and epididymal sperm counts

A sample of each frozen testis was excised on ice, fibrous capsule removed and remaining tissue weighed, and minced in 1 ml 0.9% (w/v) saline for 90 s using two razor blades according to the method of Taylor (Taylor *et al.* 1985). Epididymides were thawed on ice and minced as per testes samples. Homogenization-resistant sperm were counted in a hemocytometer, with the average count of ten individual $0.1\text{ }\mu\text{m}^3$ divisions for three separate aliquots of each suspension being determined, giving a count variation of $<5\%$.

Plasma gonadotropin determination

The concentrations of plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by RIA as previously described (Nicholson *et al.* 1991), with limits of detection of 0.12 and 0.5 ng/ml respectively. Both control and experimental samples were measured in triplicate with a coefficient of variation (CV) of 14.06% for LH and 17.54% for FSH.

Testicular testosterone determination

Samples from each testis were excised on ice and capsule removed, weighed, minced, and homogenized

in 70% (v/v) 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) gelatin (pH 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 was 50 pg/ml with a CV of 7.04%.

Morphometric analyses

Point counting was used to measure volume density (V_v) of seminiferous tubules and interstitium according to Ebling *et al.* (2000). The observer was blinded to all treatment groups prior to measures being made. Briefly, sections were cut at 5 μm , de-waxed in xylene, rehydrated in graded alcohol, stained with hematoxylin and viewed under bright field using a $\times 10$ objective with an Olympus BX50 microscope with SpotR digital camera. Sixteen fields of view per whole section from each animal were randomly selected by a pre-programmed snake pattern (3 $\times$ 3 mm, *x*- and *y*-axis widths) of automatic stage movement (Prior Optiscan, Prior Scientific Instruments, Cambridge, UK). Each field was overlain with a square grid (7 $\times$ 7) with 49 intersects, and the number of superimposed intersects for each component counted. V_v was determined as a percentage of the total number of superimposed intersects counted. Volumes per testis were then calculated from testis weight. Seminiferous tubule and lumen diameters were determined from the average distance across the *x*- and *y*-axes of at least 100 tubules per animal.

The volume density of Sertoli cell nuclei, spermatogonia, spermatocyte, and round and elongated spermatid nuclei was determined by the point counting method as mentioned previously, but with the following modifications. Five micrometer thin sections were stained with Hoescht 333 42 (10 mg/ml (w/v); Molecular probes, Eugene, Oregon, USA). Sections were illuminated at 340–385 nm and viewed under a $\times 40$ objective with an Olympus BX50 microscope with SpotR digital camera. At least ten fields of view were randomly chosen by a pre-programmed snake pattern (1 $\times$ 1 mm, *x*- and *y*-axis widths) of automatic stage movement (Prior Optiscan) and overlain with a 19 $\times$ 15 grid of 225 intersects.

Quantification of germ cell apoptosis

Apoptotic germ cells were quantified after the method of Sharpe *et al.* (1998). Briefly, apoptotic DNA fragmentation in germ cells was detected in 5 μm thin testes sections by TUNEL assay according to the manufacturer's

instructions (*In situ* Cell Death Detection Kit, peroxide detection POD: Roche Diagnostics GmbH). Negative controls were included by the omission of 3'-end labeling by terminal deoxynucleotidyl transferase. Positive controls were performed by treatment of sections with 1 U/ml DNase I in 10 mmol/l Tris-HCl (pH 7.4) at 37 °C for 30 min prior to terminal end labeling. Sections from each animal were viewed under bright field using a $\times 40$ objective, and scored by an observer blinded to the treatment groups. At least ten fields of view were randomly chosen by a pre-programmed snake pattern (1 $\times$ 1 mm, *x*- and *y*-axis widths) of automatic stage movement (Prior Optiscan) and overlain with a 19 $\times$ 15 grid of 225 intersects. Points falling over apoptotic or non-apoptotic spermatogonia, spermatocytes, and round and elongated spermatids were counted, expressed as a percentage of the 225 intersects and relative volumes per testis determined from testis weight. The proportion of total apoptotic germ cells was calculated as a ratio of germ cell volume and, similarly, ratios of apoptotic:non-apoptotic spermatogonia, spermatocytes, and round and elongated spermatids were determined (apoptotic indices).

To determine if any changes in germ cell apoptosis induced by dietary regimens was spermatogenic stage dependent, tubule cross-sections for each stage groupings of I–V, VII–VIII, and IX–XIV were scored by counting the number of apoptotic cells per tubule and the mean number of cells per tubule for each stage calculated (Atanassova *et al.* 1999). For each rat, ten tubules per stage group were scored.

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Significant differences between treatment groups were tested by one-way ANOVA furnished by VassarStats statistical computation website (<http://faculty.vassar.edu/lowry/vassarstats.html>). The significance level was set at $P \leq 0.05$.

Results

Testicular and epididymal sperm counts

The number of homogenization-resistant sperm was significantly reduced ($P < 0.001$) in testes of adult male rats that had been transferred to the high phytoestrogen diet (Fig. 1A). Epididymal sperm counts were also significantly lower ($P < 0.05$) in the high phytoestrogen fed group (Fig. 1B).

Gonadotropin and testosterone concentrations

No differences in testicular testosterone concentrations were measured between the two treatment groups. Similarly, plasma LH and FSH were not different (Table 1).

Figure 1 Mean ($\pm$ S.E.M.) number of homogenization-resistant sperm in testes (A) and ($\pm$ S.E.M.) epididymides (B) of adult male Wistar rats fed a low phytoestrogen (low; $n=8$) or a high phytoestrogen (high; $n=6$) diet for 24 days.

Morphometry

Dietary regimen neither altered the volume of the testis occupied by either the seminiferous tubules or the interstitium, nor did it affect tubule diameter (Table 2). However, tubule lumen diameter was significantly larger in the high phytoestrogen fed group ($P=0.05$; Table 2). Point counting determined no significant differences in nuclear volumes of Sertoli cells, spermatogonia, or spermatocytes between rats fed a low phytoestrogen diet and those fed a high phytoestrogen diet. In contrast, nuclear volume of round and elongated spermatids was significantly lower ($P=0.021$ and $P=0.007$ respectively) in those rats fed a high phytoestrogen diet (Table 2).

Apoptosis

TUNEL analysis was used to quantify apoptosis of distinct cell types. Immunoreactive cells were absent in negative control sections while many immunoreactive cells were determined in DNase-treated, positive control sections (data not shown). TUNEL-labeled germ cells were detected in both treatment groups. The ratio of

Table 1 Mean concentrations ($\pm$ S.E.M.) of testicular testosterone, plasma luteinizing hormone (LH), and plasma follicle-stimulating hormone of adult rats fed a diet of low phytoestrogen content (low; $n=8$) or high phytoestrogen content (high; $n=6$) for 24 days.

	Testosterone (ng/g tissue)	LH (ng/ml)	FSH (ng/ml)
Low	27.3 (3.9)	1.06 (0.47)	8.45 (0.82)
High	28.0 (4.4)	1.34 (0.29)	8.07 (0.85)

Table 2 Body, testes, and epididymal weights and morphometric measures of testes of adult male rats fed a low phytoestrogen diet (low; $n=8$) or high phytoestrogen diet (high; $n=5$) for 24 days.

	Low	High	<i>P</i>
Body weight (g)	565 $\pm$ 13	567 $\pm$ 15	0.461
Testis weight (g)	1.31 $\pm$ 0.04	1.30 $\pm$ 0.08	0.453
Epididymal weight (g)	0.49 $\pm$ 0.01	0.48 $\pm$ 0.02	0.469
Tubule volume (mm ³)	874 $\pm$ 24	880 $\pm$ 80	0.421
Tubule diameter (μ m)	342 $\pm$ 10	339 $\pm$ 10	0.419
Tubule lumen diameter (μ m)	169 $\pm$ 11	199 $\pm$ 10	0.050
Interstitial volume (mm ³)	406 $\pm$ 21	459 $\pm$ 40	0.116
Sertoli cell volume (mm ³)	12.0 $\pm$ 1.8	12.0 $\pm$ 1.5	0.500
Spermatogonia volume (mm ³)	45.9 $\pm$ 4.6	45.0 $\pm$ 3.9	0.444
Spermatocyte volume (mm ³)	69.1 $\pm$ 4.7	70.0 $\pm$ 4.3	0.447
Round spermatid volume (mm ³)	108 $\pm$ 7.8	77.8 $\pm$ 8.0	0.021
Elongated spermatid volume (mm ³)	116 $\pm$ 8.7	81.5 $\pm$ 4.8	0.007

Data are expressed as total volumes per testis occupied by structures or cell nuclei as calculated from volume densities determined by point counts. Values are given as mean $\pm$ S.E.M. Levels of significance are given, where values of $P \leq 0.05$ are deemed significantly different.

apoptotic germ cells (total) determined by point counting was significantly greater in the high phytoestrogen treatment group ($P < 0.001$; Fig. 2). Apoptotic indices calculated for spermatocytes and round spermatids were significantly greater in the high phytoestrogen fed animals ($P < 0.001$ and $P < 0.01$ respectively), while those of spermatogonia and elongated spermatids in the low and high phytoestrogen diet fed animals were similar (Fig. 2). The increase in apoptosis was not preferential for stage groupings with significantly greater numbers of apoptotic germ cells in seminiferous tubules of the high phytoestrogen fed rats. The numbers of TUNEL positive germ cell nuclei were 9.24 ± 0.36 and 5.32 ± 0.31 in stage I–V tubules of high and low phytoestrogen fed rats respectively ($P < 0.01$). In stage VII–VIII tubules, there were 6.08 ± 0.61 vs 3.46 ± 0.32 (high versus low, $P < 0.02$) and 9.0 ± 0.2 vs 5.17 ± 0.37 (high vs low; $P < 0.001$) TUNEL positive germ cell nuclei in stage IX–XIV tubules. These average numbers are not representative of a particular tubule at a given stage of the seminiferous cycle, but of the groupings scored.

Discussion

This study aimed to test if adult-only exposure of male rats to a diet of high phytoestrogen content would disrupt spermatogenesis by increasing germ cell apoptosis. The diets used in this study were chosen as they were comparable with those used in previous studies (Weber *et al.* 2001, Fritz *et al.* 2002, Wang *et al.* 2002) where low and high phytoestrogen diets generated plasma concentrations similar to those of Western or Oriental men respectively (Adlercreutz *et al.* 1993). In this study, the total daily phytoestrogen intake was estimated to be 3 mg/kg body weight (low diet) and 14 mg/kg body weight, and is comparable with the study of Wang *et al.* (2002) where total daily phytoestrogen intake was

Figure 2 Effects on apoptotic index of a low phytoestrogen (closed bars; $n=8$) or high phytoestrogen (open bars; $n=6$) containing diet fed to adult rats for 24 days. Values are means ($\pm$ S.E.M.) for all germ cell types counted (total), and for spermatogonia (Spg), spermatocytes (Spc), round spermatids (RS), and elongated spermatids (ES).

1.8 and 19.25 mg/kg body weight generating plasma concentrations of 60 and 861 nmol/l respectively. These levels are much lower than those generated in men by dietary supplements available over the counter (Rannikko *et al.* 2006).

This study has demonstrated that feeding a diet of high phytoestrogen content to adult rats, not previously exposed to elevated dietary phytoestrogens, disrupts normal spermatogenesis by increasing apoptosis of developing germ cells. Spermatogenesis describes a process of mitotic clonal proliferation, genetic reduction by meiosis, and cell differentiation and remodeling. In order to maintain homeostasis within the testis, apoptosis of developing sperm is required and occurs predominantly in populations of spermatogonia (Huckins 1978, Allan *et al.* 1992). It is well established that the development of germ cells is dependent on testosterone and FSH, the absence of both hormones increasing germ cell apoptosis (reviewed in McLachlan *et al.* 1996, 2002). FSH regulates spermatogonial development in the adult rat (Meachem *et al.* 1998, 1999), testosterone is essential for spermatid development, while both FSH and testosterone are required for spermatocyte development (McLachlan *et al.* 2002). In this study, neither were there changes in plasma levels of the gonadotropins LH and FSH, nor were there significant differences in testicular testosterone concentrations. A previous study using a comparable high phytoestrogen diet reported no effects on plasma LH but significantly decreased plasma testosterone (Weber *et al.* 2001). The discrepancy in testosterone between this study and that of Weber *et al.* may be due to the longer exposure period used there (35 vs 24 days). No changes in testosterone concentrations have been described in adult-only exposure studies for 3 days (Glover & Assinder 2006) or 14 days (Fritz *et al.* 2002). Therefore, increased apoptosis of spermatocytes and round spermatids did not appear to be due to disruption of the

hypogonadal–pituitary–testicular axis, consistent with mechanism of action of dietary phytoestrogen in the aromatase null mouse (Robertson *et al.* 2002). Furthermore, there was no evidence of stage-dependent changes in apoptosis between the high and low phytoestrogen fed groups. Increased germ cell apoptosis was not restricted to the testosterone sensitive stages VII and VIII (Russell & Clermont 1977, O'Donnell 1994, Sharpe 1994, Creasy 2001) as significantly increased numbers of apoptotic germ cells occurred in all stage groupings of the spermatogenic cycle in animals fed the high phytoestrogen diet. A similar effect has been demonstrated in rats exposed to estrogen neonatally whereby efficiency of spermatogenesis in adulthood is decreased as germ cell apoptosis, independent of spermatogenic stage, is increased (Atanassova *et al.* 1999). This was suggested to be an indirect action of estrogen due to decreased fluid resorption in the efferent ducts as evidenced by increased luminal volume of the seminiferous tubules (Atanassova *et al.* 1999). It is possible that decreased fluid resorption is a factor in this study as tubule lumen diameter was found to be significantly increased in the high phytoestrogen fed animals. Given that there was a decrease in post-meiotic germ cell numbers in this group, one may expect to see a decrease in the testis weight also. However, no difference in testis weight was determined. This may be explained by increased luminal fluid volume in the high phytoestrogen fed animals. Disruption of normal estrogen action by the removal of functional ER α in mice (Eddy *et al.* 1996, Hess *et al.* 1997) or administration of an anti-estrogen to adult rats (Oliveira *et al.* 2001) causes reduced fluid absorption in the excurrent ducts.

Paracrine and/or autocrine actions of estrogen in the rat testis are possible given that: aromatase is present in the rat Leydig cells, Sertoli cells, spermatocytes, and round and elongating spermatids (Levallet *et al.* 1998); ER α is expressed in Leydig cells (Pelletier *et al.* 2000); ER β in Sertoli cells (Saunders *et al.* 1998) and spermatogonia (Saunders *et al.* 1998, van Pelt *et al.* 1999); both receptor types are present in spermatocytes and round spermatids (Saunders *et al.* 1998, Pelletier *et al.* 2000). A direct role for estrogen in the prevention of human germ cell apoptosis has been described (Pentikäinen *et al.* 2000). *In vitro* incubation of seminiferous tubules in serum and hormone-free media induces apoptosis of spermatocytes and spermatids. This apoptosis is inhibited by 17 β -estradiol. This same population of cells exhibit increased apoptosis here when a diet of high phytoestrogen content is consumed. This induction of apoptosis suggests, therefore, that phytoestrogens are anti-estrogenic in this respect. Indeed, in adult aromatase null mice, there is an arrest at the meiotic stage of spermatogenesis causing a significant decrease in the number of round and elongated spermatids (Robertson *et al.* 1999). Aromatase deficiency does not alter spermatogonial numbers

(Robertson *et al.* 1999) nor is estrogen synthesis from testosterone responsible for the testosterone induced restoration of spermatogonial development following GnRH inactivation (Meachem *et al.* 2005). This pattern is identical to that induced by the high phytoestrogen diet here. Spermatogonia and spermatocyte volumes were not depleted. As there was no difference in the incidence of apoptotic spermatogonia, this was as expected. A greater incidence of apoptotic spermatocytes was consistent with fewer round spermatids in the high phytoestrogen fed animals. As this would lead to fewer round spermatids progressing to elongating spermatids, and given that there was increased apoptosis of round spermatids, a smaller population would proceed to the elongated spermatid stage. Hence, one would predict fewer elongating spermatids, and hence a reduced testicular sperm count in the high phytoestrogen group, as was found.

A recent study by Anway *et al.* (2005) has described transgenerational effects of endocrine disruption that are transferred through the male germ line in the rat. Exposure to either the anti-androgen vinclozolin or the estrogenic compound methoxychlor during the period of sex determination and testis development in the rat resulted in increased apoptosis of germ cells and sperm numbers in the F₁ generation and were still manifest in the F₄ generation. This transgenerational effect was coincidental to altered DNA methylation patterns of the male germ line induced by endocrine disruption during the re-methylation programming of the germ line that occurs during the period of sex determination (Reik & Walter 2001). As the male rats used in this study were only exposed to the high phytoestrogen diet at adulthood, the effects here are not due to differences in re-methylation. However, that does not preclude the possibility that there may be epigenetic effects caused by exposure *in utero* due to feeding pregnant dams a high phytoestrogen diet during embryonic days 12–15, the period of sex determination and testis development. A number of studies have been conducted on the effects of exposure to isoflavones during gestation. Kang *et al.* (2002) reported no effects on sperm count or motility in males exposed between E10 and PND21 to doses of genistein equivalent to Western diet exposure (low dose). Studies of males continually exposed to dietary genistein, from conception through to adulthood, suggest no difference in the pathology of the male tract (Fritz *et al.* 2002, You *et al.* 2002), however, no in depth studies on the effects on spermatogenesis were conducted. As yet, there have been no reports on possible epigenetic effects of exposure to isoflavones during this critical period of re-methylation.

Recent reports of the gradual decline in human semen quality have sparked much debate as to causes/risk factors. Some have suggested that spermatogenesis can be significantly affected by environmental and lifestyle factors that appear to have no other detrimental affect to

the health of the individual (reviewed by Sharpe 2000, Sharpe & Franks 2002). Two such factors associated with adult exposure are seasonality, with demonstrably reduced sperm counts in summer months (Politoff *et al.* 1989, Saint Pol *et al.* 1989, Gyllenberg *et al.* 1999) and scrotal heating (Irvine 1998, Thonneau *et al.* 1998, Bujan *et al.* 2000, Hjollund *et al.* 2000). Dietary effects in adulthood have primarily been associated with women (Sharpe & Franks 2002). Indeed, dietary phytoestrogen exposure in animals grazing estrogenic pastures has long been known to cause significant impairment of reproductive function (reviewed by Adams 1995). Clover disease, a syndrome of ewes grazing subterranean clover was first described by Bennetts *et al.* (1946). Temporary and permanent infertility is a factor only evident in females, however, with no apparent disruption of male reproductive function or general health of the non-castrate ram (Adams 1995).

Our findings also contrast previous studies in rats. However, those studies are not comparable either in the dose or in the controlled background from which animals were raised and handled prior to inclusion. A study by Ashby *et al.* (2003) reported inconsistent effects of diet, demonstrating both significant and non-significant decreases in daily sperm production. It was concluded that this inter-experimental variation was due to either inter-animal or random variation, and it was recognized by the authors that the study was confounded by non-controlled factors, such as different animal shipments and variations in animal husbandry. In this study, all animals were bred and raised in-house and all animals were fed an identical diet up until rats were randomly assigned to the high phytoestrogen diet group. In the study of Faqi *et al.* (2004), an extremely high dose of 2000 mg/kg total phytoestrogen had no effect on daily sperm production unlike the high phytoestrogen fed animals in this study where a dose of 465 mg/kg was employed. The reason for this difference is unclear. However, the length of exposure in that study was much longer with measures being made at least 12 months after subjects were transferred to the high phytoestrogen diet. It may be that there is a compensation or induced insensitivity due to such a chronic exposure. Disparity between studies is well known and many factors have been suggested in this (Akingbemi 2005).

The high phytoestrogen diet is that normally used in our animal facilities. As such no detrimental health effects are associated with its use, and Wistar rat fecundity appears normal with an average litter size of 12 pups. Indeed, feeding adult male rats for 24 days with the high phytoestrogen diet does not reduce fecundity *per se*, even though, as we report here, sperm production is reduced in these animals (Glover & Assinder 2006). However, we have shown that acute exposure to this diet causes a significantly reduced fecundity, altered expression of steroid receptors in the epididymis, and increased sperm lipid peroxidation (Glover & Assinder

2006). Anecdotally, there have been problems with breeding of some transgenic mouse lines (C57BL/6J background). These problems were rectified by transferring to the low phytoestrogen diet. It is interesting to note that in a study of endocrine disruption of the juvenile male reproductive tract, C57BL/6J mice were most sensitive to low-dose estradiol (Spearow *et al.* 1999).

It is possible that the lower proportion of fish meal in the high phytoestrogen diet could have resulted in lower levels of essential fatty acids (EFA) causing impaired spermatogenesis. Spermatogenesis is impaired in EFA-deficient rats (Evans *et al.* 1934, Alfin-Slater & Bernwick 1958) with a loss of spermatocytes. However, this is unlikely to be a factor. The essential fatty acid content of white fish meal is 2.0% (w/w), while that of soy meal is 0.4% (w/w; Food and Agricultural Organizations of the United Nations 1986). On that basis, the essential fatty acid content would be 1.7 and 1.6 mg/g of the low and high phytoestrogen diets respectively. Therefore, the estimated daily intake of EFA per day for both groups would be in excess of 20 mg/day, twice that needed to restore spermatogenesis in EFA-deficient animals (Alfin-Slater & Bernwick 1958). Furthermore, rats placed on a fat-free diet at weaning became deficient in EFAs only after 12 weeks (Alfin-Slater & Bernwick 1958), much longer than the time period here.

In conclusion, adult-only exposure of male rats to a diet of high phytoestrogen content increases germ cell apoptosis reducing testicular sperm numbers. This disruption of spermatogenesis is independent of the hypothalamo-pituitary-testicular axis and is likely to be due to disruption of paracrine and/or autocrine actions of estrogen in the testis.

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