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# The Phenotype of the Aromatase Knockout Mouse Reveals Dietary Phytoestrogens Impact Significantly on Testis Function

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**Estrogen is synthesized in the testis, both in Leydig cells and seminiferous epithelium, and its importance in spermatogenesis is highlighted by the phenotype of the aromatase knockout (ArKO) mouse. These mice are unable to synthesize endogenous estrogens. The males develop postmeiotic defects by 18 wk of age. We hypothesized that maintenance of spermatogenesis in younger animals may be mediated by exogenous estrogenic substances. Dietary soy meal, contained in almost all commercial rodent diets, provides a source of estrogenic isoflavones. We thus investigated spermatogenesis in wild-type and ArKO mice raised on a diet containing soy, compared with a soy-free diet, to elucidate the biological action of phytoestrogens on the testis. In ArKO mice, dietary phytoestrogens could partially prevent disruptions to spermatogenesis, in that they prevented the decline in germ cell numbers. They also seemed to maintain Sertoli cell function, and they blocked elevations in FSH. The impairment of spermatogenesis seen in soy-free ArKOs occurred in the absence of a decreased gonadotropic stimulus, suggesting that the effects of dietary phytoestrogens are independent of changes to the pituitary-gonadal axis. Our study highlights the importance of estrogen in spermatogenesis and shows that relatively low levels of dietary phytoestrogens have a biological effect in the testis. (*Endocrinology* 143: 2913–2921, 2002)**

ESTROGEN IS SYNTHESIZED in the male, with increasing evidence suggesting a crucial role in the development and function of male reproductive structures (for reviews, see Refs. 1 and 2). The importance of estrogen in the adult testis was highlighted by the phenotype of the aromatase knockout (ArKO) mouse, where the inhibition of estrogen biosynthesis resulted in spermatogenic abnormalities. The mice are initially fertile but, at around 18 wk of age, show a specific postmeiotic defect in early spermiogenesis coinciding with an elevation in apoptosis and a reduction in fertility (3). The ArKO mice also present with a significant reduction in copulatory behavior in adulthood, reiterating the importance of estrogen in male reproduction (4).

Because the estrogen receptors are expressed in the developing reproductive tract from fetal life through to adulthood, and ER$\beta$ is predominant in the seminiferous epithelium (5), estrogen may act directly on the seminiferous tubules to mediate spermatogenesis, such as was suggested by studies in estrogen-treated hypogonadal mice (6). The fetal development of the male reproductive tract is clearly under strict endocrine control; and thus, compounds that are potentially able to disrupt this hormonal homeostasis are of increasing concern. Included are numerous environmental compounds that are postulated to possess estrogenic-like biological activity (7), including agricultural and industrial by-products, organochlorine pesticides and insecticides, and phytoestrogens (8). Although such compounds have a low potency, it has been hypothesized that prolonged environmental exposure may result in severe malformations in reproductive systems and subsequent decreased fertility (9–11).

Phytoestrogens are estrogenic plant-derived nonsteroidal compounds comprised of three classes: isoflavonoids, coumestans, and lignans (12–14). Because many of these environmental estrogens are generally less potent than other estrogenic substances, exposure to them has been regarded as nonharmful, even beneficial (15). For instance, isoflavones, such as genistein, occur in high quantities in beans (such as the soy bean), and it is the ingestion of this soy meal, the staple diet of many Asian communities, that has been suggested by numerous epidemiological and experimental studies to cause a protective effect against hormone-dependent cancers (such as breast and prostate) in these populations (for reviews, see Refs. 13, 16, and 17).

Limited studies completed in the male have suggested a role for these compounds in male reproductive processes (15, 18, 19). The phytoestrogens, namely genistein and coumesterol, are able to bind to estrogen receptors, particularly the ER$\beta$ isoform, in an agonistic fashion with high affinity (20, 21). Given that both ER isoforms, but particularly ER$\beta$, are localized to sites important for male reproduction (2, 22), it is reasonable to speculate that these agonistic ligands may have direct effects on male reproductive function. It has been determined that virtually all natural rodent diets that include soy as a source of protein have detectable levels of estrogenic isoflavones (23, 24); and therefore, such diets may be capable of sustaining estrogen-like effects.

The ArKO mouse, which lacks aromatase products (namely, estradiol and estrone), is an ideal model to test whether the soy meal in rodent diets has agonistic estrogenic effects on the male reproductive system, particularly in terms of the maintenance of spermatogenesis. We previously hypothe-

Abbreviations: ArKO, Aromatase knockout; r, recombinant; S−, soy-free; S+, soy-containing; WT, wild-type.

sized that the late onset of the spermatogenic phenotype in male ArKO mice (3) was attributable to the fact that their diet may contain estrogen-like substances that attenuate the effect of estrogen withdrawal on spermatogenesis. This study aimed to investigate the effects of a soy free (S−) diet and a natural rodent soy-containing (S+) diet on spermatogenesis in ArKO mice, to understand whether dietary soy can have an action on the testis. We also investigated the effects of dietary soy in normal mice.

## Materials and Methods

### *Generation of ArKO mice*

The strategy used for targeted disruption of the *Cyp19* gene (25) and a description of the male phenotype have already been presented (3). Briefly, exon IX of the *Cyp19* gene was targeted for disruption by the insertion of the neomycin-resistant cassette, because of its high species homology and its role in important structural features of the enzyme (namely, the K helices and in some $\beta$ sheet regions).

### *Generation of mouse chow*

Glen Forrest Stockfeeders (Glen Forrest, Western Australia, Australia) manufactured 2 $\gamma$-irradiated mouse chows. Both are standard mouse chows containing wheat, blood meal, fish meal, meat meal, bran, pollard, vegetable oil, pure amino acids, and vitamin-mineral premix. However, one contains the standard 10% soy meal, and one does not. The S+ diet contained approximately 146 mg/g isoflavones and is identical with mouse chow that is routinely used. No isoflavones were detected in the S− diet by HPLC analysis (data not shown). Aside from 10 mating pairs kept on the regular mouse chow containing soy, the entire ArKO mouse colony was placed on the S− mouse chow approximately 2 yr before the study.

### *Animals*

ArKO and wild-type (WT) mice were housed, under a 12-h light,12-h dark cycle, in the Specific Pathogen Free Facility at Monash Medical Centre Animal House, and were provided either the S+ or S− mouse chow *ad libitum* from birth. All experiments were approved by the Animal Experimentation Ethics Committee at Monash Medical Centre. Male WT and ArKO colony mates on the S+ and S− diets were killed by $CO_2$ asphyxiation, at 14 wk of age, and their sera and testes were collected. WT and ArKO colony mates raised on the S− diet, at 1 yr of age, were compared with animals on the regular chow from the previous study (3). These animals consumed the same S+ chow used in this study.

### *Collection of tissue and histology*

Male mice at 14 wk and 1 yr of age were killed by $CO_2$ inhalation, blood was collected by cardiac puncture, and serum was frozen at –20 C. The testes were dissected out, weighed, and immersion-fixed in Bouins' fluid for 4–5 h. Both 2-$\mu$m and 25-$\mu$m methacrylate resin sections, stained with periodic acid-Schiff (26) and hematoxylin, were prepared as described previously (3).

### *Stereology*

Stereological analysis of the testes was performed as described (3). Briefly, the stereological apparatus consisted of a BX-50 microscope (Olympus Corp., Tokyo, Japan) and a TMC-6 video camera (Pulnix America, Sunnyvale, CA) coupled to an Amiga 2000 Pentium computer. The CASTGRID VI.10 software (Olympus Corp., Munich, Germany) was used to generate point grids and unbiased counting frames on the computer screen. The 2-$\mu$m sections were examined under ×10 magnification, and a point grid with a series of 4 major and 16 fine points was used to examine the volume of the seminiferous tubule, lumen, and epithelium, as well as interstitium. Cross-sections of seminiferous tubules were also measured (approximately 90 diameters per animal). To quantify germ cell numbers, the optical dissector was used as described previously (3) (reviewed in Ref. 27). Germ cells were classified into 4 major groups: spermatogonia, spermatocytes, round spermatids, and elongated spermatids. Fields to be counted were selected by a systematic uniform random sampling scheme. Cells were counted in 25-$\mu$m sections, at ×100 magnification, under oil immersion, within unbiased counting frames (2302 $\mu m^2$ and a 230 $\mu m^2$), and at least 100 cells of each type were counted per animal. Sertoli cell nuclei were also counted at ×100 magnification in at least 150 fields with a frame of 1381 $\mu m^2$.

### *Serum hormone assays*

LH and FSH were measured by RIA with the following iodinated preparations [iodinated using Iodogen reagent (Sigma, St. Louis, MO) and antisera: recombinant (r) FSH 1–8 and anti-rFSH-S-11, and rLH-1–9 and anti-rLH-S-10 (NIDDK, Bethesda, MD)]. The secondary antibody used in both RIAs was goat antirabbit IgG (GAR no.12; Monash Institute of Reproduction and Development, Monash University, Melbourne, Australia), and the assay buffer was 0.01 M PBS containing 0.5% BSA (Sigma). All samples were measured in a single assay as described previously (28). The lower detection limits of the assays were 1.05 ng/ml (FSH) and 0.08 ng/ml (LH). The within-assay coefficients of variation were 5.3% and 9.2% for FSH and LH, respectively. Because of insufficient serum, serum LH was not analyzed in 1-yr-old mice on the S+ diet.

### *Statistics*

Data were analyzed using SPSS, Inc. (Chicago, IL) 10.0 for windows. All analyses were performed using a two-way ANOVA to determine whether a significant difference existed between diet and genotype. If a significant result was achieved, least-squares difference was applied to determine differences among groups. Data were considered statistically significant when a $P$ value less than 0.05 was achieved. All data are expressed as mean ± SEM.

## Results

### *Weights*

A S− diet was associated with an increase in body weight in WT and ArKO mice at 14 wk of age (Table 1) and in ArKO mice at 1 yr of age (Table 1), compared with colony mates raised on a diet containing soy meal.

The presence or absence of soy in the diet did not change testis weight in either ArKO or WT mice at 14 wk of age (Table 1) or in WT mice at 1 yr of age (Table 1). However, ArKO mice had decreased testis weights, compared with WT mice, at 1 yr of age, regardless of diet (Table 1), and this was further decreased when ArKO mice consumed a S− diet.

### *Testicular morphology*

*Qualitative observations on testicular morphology.* Histological examination at 14 wk showed no noticeable difference in testicular morphology in WT animals consuming a S−, com-

**TABLE 1.** Weights of ArKO and WT males, at 14 wk of age, consuming either an S+ or S− diet

| | Body weight (g) | Testis weight (mg) |
|---|---|---|
| WT S+, 14 wk (n = 8) | 25.22 ± 1.2 | 119.14 ± 5.6 |
| WT S−, 14 wk (n = 12) | 33.36[a] ± 1.6 | 121.14 ± 5.2 |
| ArKO S+, 14 wk (n = 6) | 26.0 ± 2.5 | 120.88 ± 3.0 |
| ArKO S−, 14 wk (n = 11) | 31.36[a] ± 1.1 | 118.07 ± 2.8 |
| WT S+, 1 yr (n = 8) | 43.25 ± 1.2 | 134.50 ± 6.4 |
| WT S−, 1 yr (n = 12) | 49.25 ± 3.2 | 140.78 ± 6.0 |
| ArKO S+, 1 yr (n = 6) | 41.71 ± 2.1 | 101.54[b] ± 8.2 |
| ArKO S−, 1 yr (n = 11) | 52.0[a] ± 1.8 | 53.58[a] ± 7.2 |

Data expressed as mean ± SEM.

[a] $P < 0.05$, comparing the same genotype consuming either a S+ or S− diet.

[b] $P < 0.05$, comparing WT and ArKO mice on a similar diet.

pared with a S+, diet. However, some differences were apparent when comparing ArKO males raised on the S+ or S− diets. Animals raised on a S+ diet showed normal testicular morphology; however, some ArKO animals showed evidence of spermatogenic disruption. The most notable feature was the presence of occasional tubules lacking elongated spermatids (not shown).

Testes from 1-yr-old WT animals raised on either a S+ or S− diet showed no noticeable changes in morphology. As described previously (3), ArKO animals raised on a S+ diet showed a predominant phenotype of spermiogenic arrest, with most tubules showing evidence of round spermatid apoptosis and/or reductions in more mature spermatids. However, spermatogenic disruption was far more pronounced when ArKO animals were raised on a S− diet. One animal had an apparent Sertoli cell-only phenotype, with a marked increase in interstitial volume. The other three animals showed heterogeneity in tubule morphology, with some containing only Sertoli cells, other tubules with marked epithelial disruption and noticeable reductions in germ cells, and some tubules in which spermiogenic arrest was evident. The majority of tubules in two of these animals showed marked epithelial disruption (data not shown).

*Quantitative assessment of testicular histology.* At 14 wk of age, diet had no effect on the absolute volumes of the testicular lumen, epithelium, and interstitium in either WT or ArKO animals (Fig. 1). However, the tubule diameter decreased and the length increased in both genotypes when soy was absent from the diet. At this age, no significant differences were noted between WT and ArKO animals on the same diet.

By 1 yr of age, diet promoted significant changes in various morphometric parameters in WT testes. WT animals reared on a S− diet (Fig. 2) had an increase in the volume of the epithelium, a decrease in the volume of interstitium, and an increase in the length of the tubules, compared with animals raised on a diet containing soy.

The disruptions to testicular morphology in 1-yr-old ArKO males raised on a diet containing soy (3) were markedly exacerbated when ArKO animals were raised on a S− diet (Fig. 2). The absence of dietary soy promoted marked decreases in the volume of the testis taken up by seminif-

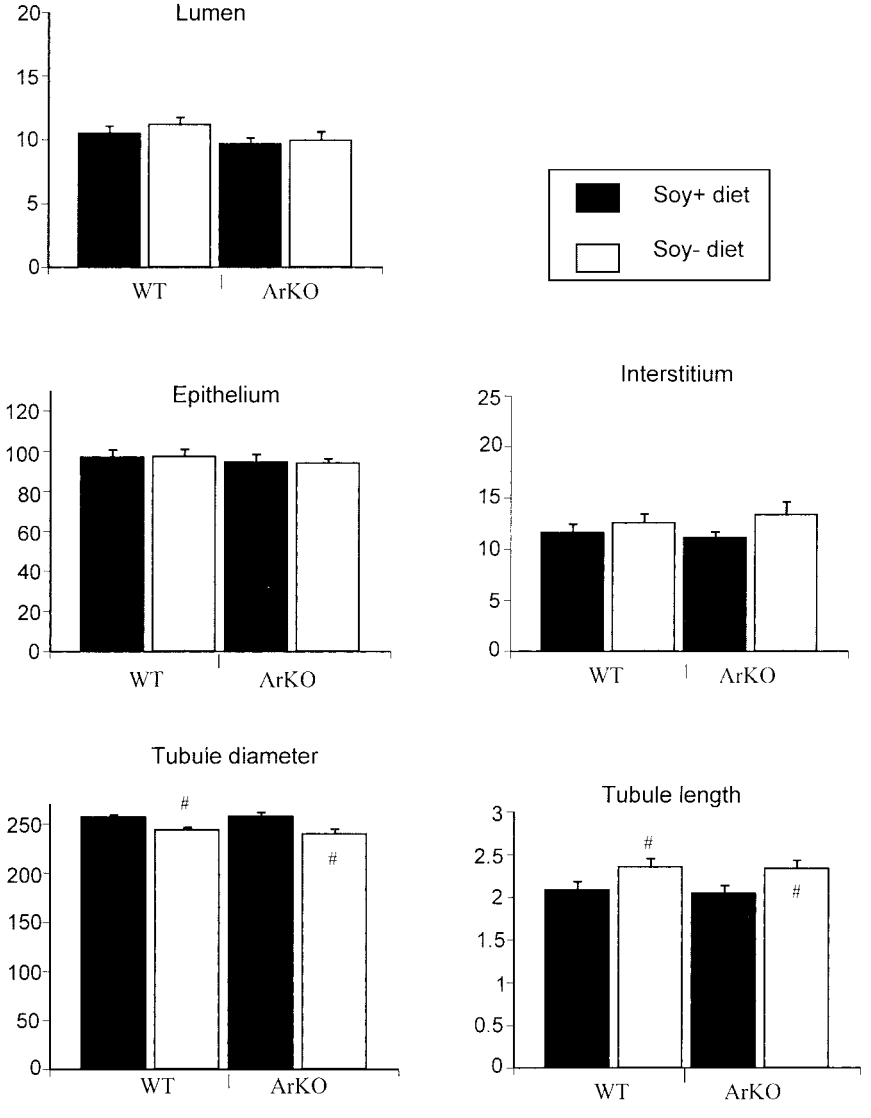


FIG. 1. Morphometric data on testicular compartments (tubule lumen, epithelium, and interstitium; $mm^3$), tubule diameter ($\mu m$), and length (m) in 14-wk-old WT and ArKO mice consuming either a S+ or S− diet. *, $P < 0.05$, comparing WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing with the same genotype consuming a S+ *vs.* a S−diet. Data are shown as mean ± SEM.

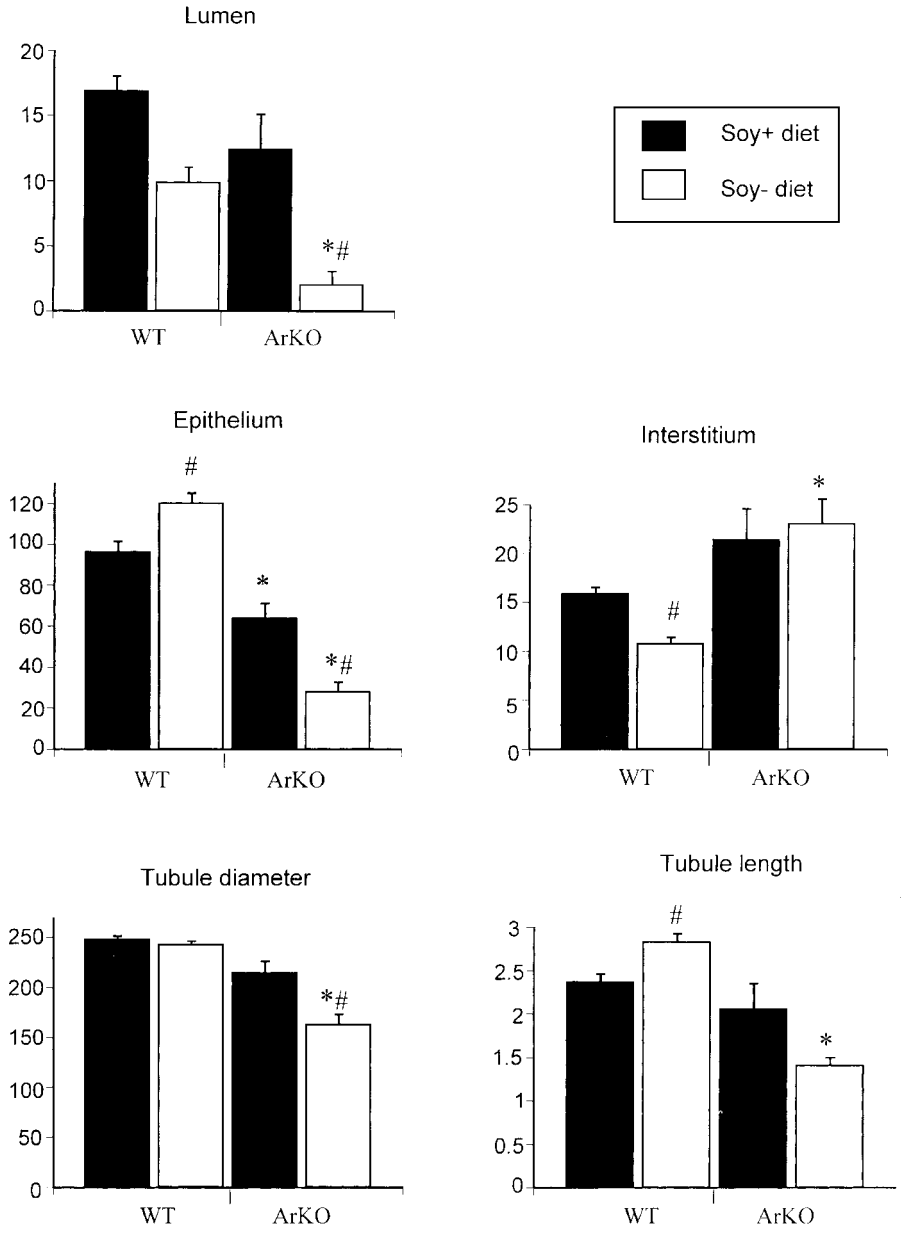


FIG. 2. Morphometric data on testicular compartments (tubule lumen, epithelium, and interstitium; $mm^3$), tubule diameter ($\mu m$), and length (m) in 1-yr-old WT and ArKO mice consuming either a S+ or S− diet. *, $P < 0.05$, comparing WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing with the same genotype consuming a S+ *vs.* a S− diet. Data are shown as mean ± SEM.

erous epithelium and lumen, being 2.3- and 6.3-fold lower, respectively, than S+ ArKO mice. Tubule diameter was also markedly decreased; however, the volume of interstitium remained unchanged.

When comparing 1-yr-old WT and ArKO animals on the S− diet, ArKO males had decreased luminal and epithelial volume, an increase in interstitial volume, and a decrease in tubule diameter and length. In contrast, the only difference between WT and ArKO animals on a S+ diet was a decreased epithelial volume in ArKO testes. Although no differences were seen in interstitial volume in these animals, we have previously shown that these ArKO mice raised on a S+ diet have an increased volume of Leydig cells (3).

*Germ cell numbers.* At 14 wk of age, germ cell numbers were not altered by diet in WT animals (Fig. 3). ArKO animals raised on a S+ diet had fewer spermatogonia than WT animals or ArKO animals raised on a S− diet (Fig. 3A). ArKO animals raised on a S− diet had significantly fewer round spermatids, compared with ArKO animals on the S+ diet (Fig. 3C).

At 1 yr of age, there was no effect of diet on germ cell numbers in WT mice (Fig. 4). As was observed previously (3), ArKO animals raised on a S+ diet had significant reductions in round and elongated spermatids, compared with WT mice (Fig. 3, C and D). These disruptions to spermatogenesis in ArKO mice were markedly enhanced when the mice were reared on a S− diet. Although there was variability in the phenotype between mice (see above), analysis of germ cell numbers per testis showed that ArKO mice on a S− diet had significant decreases in spermatocytes, round and elongated spermatids to 25, 20, and 24% of the levels in ArKO males raised on a S+ diet.

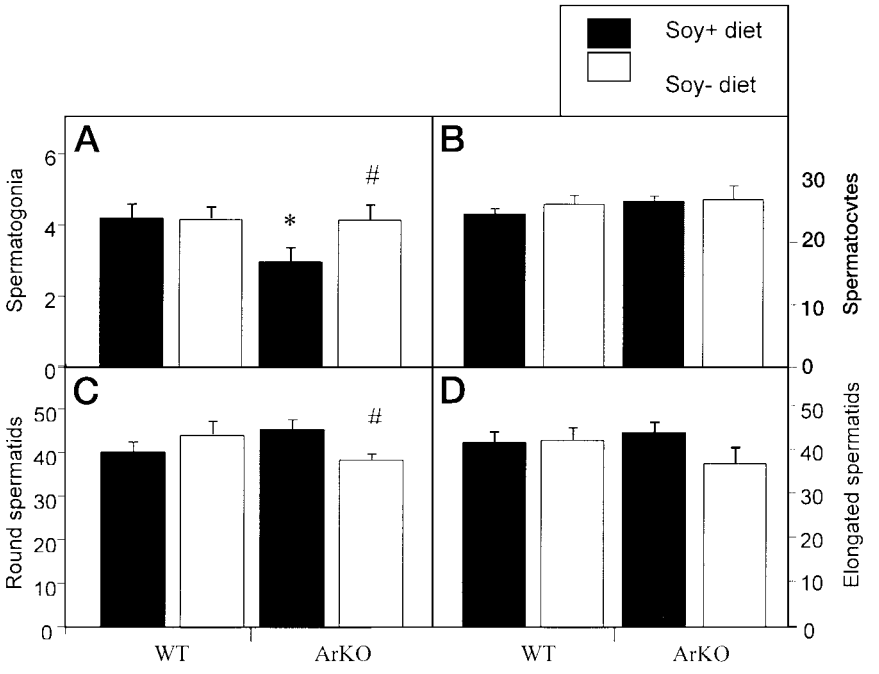


FIG. 3. Germ cell numbers (millions per testis) in ArKO and WT mice at 14 wk of age, raised on S+ and S− diet. A, Spermatogonia; B, spermatocytes, including preleptotene, leptotene, zygotene, and pachytene spermatocytes; C, round spermatids (steps 1–8); D, elongated spermatids (steps 9–16). Data are shown as mean ± SEM. *, $P < 0.05$, comparing WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing the same genotype consuming a S+ *vs.* a S−diet.

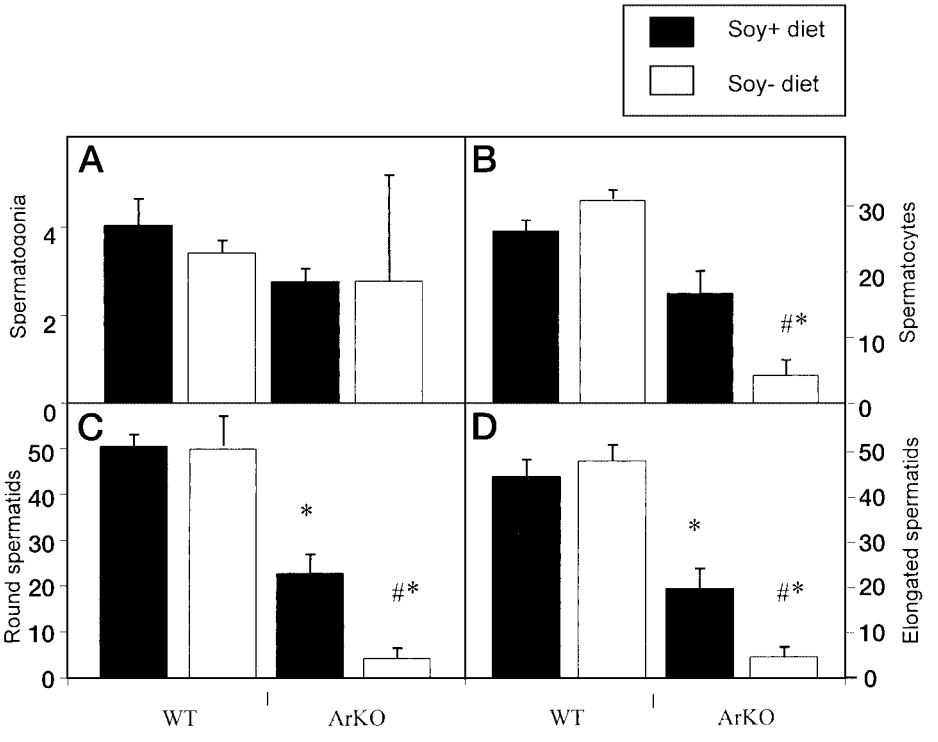


FIG. 4. Germ cell numbers (millions per testis) in ArKO and WT mice at 1 yr of age, raised on S+ and S− diet at 1 yr of age. A, Spermatogonia; B, spermatocytes, including preleptotene, leptotene, zygotene, and pachytene spermatocytes; C, round spermatids (steps 1–8); D, elongated spermatids (steps 9–16). Data are shown as mean ± SEM. *, $P < 0.05$, comparing WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing the same genotype consuming a S+ *vs.* a S− diet.

*Sertoli cell numbers.* Analysis of Sertoli cell numbers in 14-wk-old WT animals showed that mice raised on a S− diet had slightly (but significantly higher) numbers of Sertoli cells than those raised on a S+ diet (Fig. 5A), but this effect was not evident in 1-yr-old WT animals (Fig. 5B). Diet did not affect Sertoli cell number significantly in ArKO animals at either age (Fig. 5, A and B). Interestingly, a significant increase in Sertoli cell numbers in 14-wk-old ArKO mice on a S+ diet was evident when compared with WT on the same diet (Fig. 5A), with similar trends in 1-yr-old animals; however, no statistically significant differences were observed (Fig. 5B).

Because differences in Sertoli cell numbers were observed, the capacity of each Sertoli cell to support germ cells was examined between diets (Fig. 6). There were no significant differences between groups at 14 wk of age. However, at 1 yr of age, ArKO mice on both the S+ and S− diets showed less germ cells per Sertoli cell, compared with WT mice on the corresponding diet. Also, Sertoli cells in ArKO males

supported fewer germ cells when soy was absent, compared with S+ ArKO mice.

*Serum hormone levels*

At 14 wk of age, ArKO mice showed no change in serum FSH, compared with WT mice, regardless of whether the animals were consuming a S+ diet or a S− diet (not shown). In addition, diet had no significant effect on FSH levels in either WT or ArKO mice at this age. Serum FSH was also unaffected in ArKO mice at 1 yr of age, compared with WT mice consuming a S+ diet or a S− diet (not shown). Interestingly, however, diet had a significant effect on FSH in 1-yr-old ArKO animals, with an absence of soy associated with an increase in FSH (S+ *vs.* S−: 9.9 ± 0.2 *vs.* 16.6 ± 1.2 ng/ml, $P < 0.001$).

At 14 wk of age, serum LH was slightly (but not significantly) elevated in ArKO mice *vs.* WT mice, regardless of diet (not shown). At 1 yr of age, ArKO mice on the S− diet showed a trend for higher levels of LH than WT mice consuming the same diet (WT *vs.* ArKO: 0.27 ± 0.10 *vs.* 0.38 ± 0.10 ng/ml, $P = 0.38$), but this analysis could not be completed in S+ animals because of insufficient serum from WT animals. ArKO mice raised on a S− diet for 1 yr had lower (but not significantly so) levels of LH than those raised on a S+ diet (S+ *vs.* S−: 0.99 ± 0.40 *vs.* 0.38 ± 0.10 ng/ml, $P = 0.22$).

## Discussion

The ArKO mouse was used to test the hypothesis that estrogenic substances in dietary soy meal have a biological effect on the testis. Although the data indicates that dietary soy was probably not playing a role in maintaining spermatogenesis until 14 wk of age in ArKO mice, because marked disruptions to spermatogenesis were not induced at an earlier age, it does show that soy may have agonistic effects on spermatogenesis in the absence of endogenous estrogen, particularly in terms of the maintenance of germ cell development. The more marked disruptions to germ cell development and testicular morphology in S− ArKO mice cannot be explained by a decreased gonadotropic stimulus to the testis, because LH and FSH levels were not significantly lower in these animals, compared with S+ ArKO mice. Thus, the most likely explanation for the more marked spermatogenic phenotype in the S− mice is that dietary soy can have

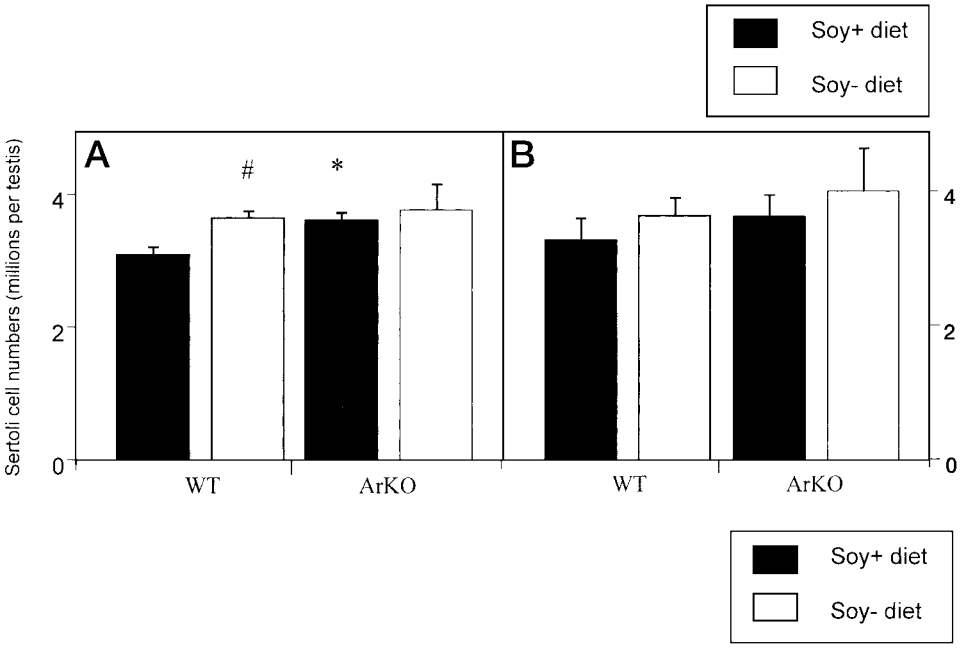


FIG. 5. Sertoli cell numbers (millions per testis) in ArKO and WT mice on the S+ and S− diet at (A) 14 wk and (B) 1 yr of age. A, Data are shown as mean ± SEM. *, $P < 0.05$, comparing WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing the same genotype consuming a S+ *vs.* a S− diet.

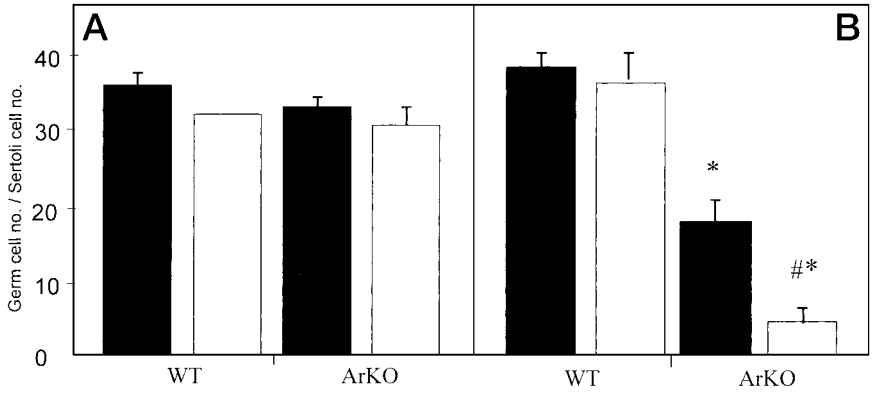


FIG. 6. Total germ cell number (millions per testis) as a proportion of Sertoli cell number in millions per testis at (A) 14 wk of age and (B) 1 yr of age. Data are shown as mean ± SEM. *, $P < 0.05$, comparing with WT and ArKO mice on a similar diet; #, $P < 0.05$, comparing with the same genotype consuming a S+ *vs.* a S− diet.

direct effects on receptors within the male reproductive tract. Dietary soy was also able to produce changes in testicular histology in WT animals, highlighting the fact that soy found in commercial rodent chow has an action on the testis of normal healthy mice.

In the absence of endogenous estrogen, as is the case in ArKO mice, soy consumption clearly had a beneficial effect on the testis by partially maintaining testis weight, germ cell development, and seminiferous tubule epithelial and luminal volume. In general, the testes were morphologically less impaired in ArKO animals consuming dietary soy, exhibiting reduced epithelial and structural damage. These agonistic effects on the testis and spermatogenesis could not be explained by increases in circulating LH and FSH and are thus likely to be mediated by direct effects on the testis. The phytoestrogens genistein and daidzein, both present in soy, are agonists at both ERs but particularly ER$\beta$ (29). Given the localization of ERs in multiple cell types and compartments in the mouse testis throughout development (30) (for reviews, see Refs. 2 and 5), including fetal and peripubertal gonocytes and spermatocytes (31), long-term agonistic effects on spermatogenesis are possible. The defects in germ cell development in ArKO mice, in the absence of decreases in gonadotrophins or androgens (3), clearly suggest a direct effect of estrogen on germ cell development. Here, we show that this agonistic effect can be mimicked, in part, by dietary estrogens, with a soy diet preventing the marked decline in spermatocyte, round, and elongated spermatid numbers caused by estrogen withdrawal. Previous studies have shown that genistein, in doses equal to those consumed in a soy-based diet (2.5 mg sc/kg body weight), is able to induce squamous epithelial metaplasia, a sign of estrogen action, in mouse accessory sexual organs, such as the prostate (32), and also elicit a high estrogenic response in the uterus of immature rats consuming a standard phytoestrogen-containing diet (210 mg/kg) (23), providing further evidence for an agonistic role of phytoestrogens in reproductive organs.

Comparison of 1-yr-old ArKO mice raised on a S− diet, with those raised on a S+ diet, not only demonstrates a biological action of dietary soy, but reveals hitherto unrecognized roles for estrogens in spermatogenesis. The demonstration of reduced numbers of spermatocytes in ArKO males on a S− diet suggests that spermatocyte development may also be dependent on estrogens. This is not unexpected, given the localization of aromatase and ER$\beta$ in certain spermatocyte populations (see Refs. 2 and 5 for reviews). Previous studies have shown that seminiferous tubules lacking ER$\alpha$ secrete less fluid (33), suggesting that estrogen action is important for the maintenance of Sertoli cell fluid secretion. This is supported by the present findings that show that 1-yr-old ArKO mice raised on a S− diet have a marked reduction in seminiferous tubule luminal volume and a significant increase in FSH levels, suggesting that Sertoli cell function, and probably inhibin B secretion, is disrupted when there is no estrogenic stimulus. Further evidence for a role for dietary phytoestrogens in Sertoli cell function is the markedly decreased seminiferous epithelium volume and tubule diameter in the ArKO males on a S− diet. Also, the decreased tubule length seen in these animals, despite no decrease in Sertoli cell numbers, would suggest decreases in Sertoli cell volume, although such a proposition is, as yet, unproven. Taken together, our observations suggest that dietary phytoestrogens can influence Sertoli cell function. Thus, both endogenous and exogenous estrogens can play a role in the maintenance of pre- and postmeiotic germ cell development and Sertoli cell function.

It is possible that the elevation in FSH in 1-yr-old ArKO mice consuming a S− diet is also a result of dietary compounds inhibiting gonadotrophin secretion from the pituitary. Endogenous estrogen has a homeostatic feedback role at the hypothalamus and pituitary, potentially through a direct action on the ERs localized specifically in these tissues (34). Because dietary soy prevents or suppresses the increase in FSH caused by the removal of estrogen, an estrogenic action of soy at the level of the hypothalamus/pituitary is suggested (35–37).

In WT animals, dietary soy had minor (but measurable) effects on the testis, as evidenced by a reduction in seminiferous epithelium volume. Our findings that dietary soy can affect WT testes is supported by previous studies in normal pubertal rats in which dietary soy influences testicular weight and histology (38). A surprising finding in this study was a slight increase in the number of Sertoli cells in ArKO animals on the S+ diet, compared with WT animals, at 14 wk, which is in contrast with the findings presented earlier (3). Similar trends were also observed in the ArKO mice on a S− diet; however, statistical differences were not observed. It is thus possible that an absence of aromatase during neonatal development could result in changes in Sertoli cell proliferation, such that more Sertoli cells are produced when aromatase is absent. This is perhaps not surprising, given that exposure to pharmacological levels of estrogen can interfere with Sertoli cell proliferation, either directly or via effects on FSH, leading to reduced Sertoli cell numbers (39). However, further studies on the effects of dietary soy during the neonatal period of Sertoli cell proliferation are required to determine the mechanism of this effect. Exogenous estrogens can also interfere with Sertoli cell maturation, as evidenced by decreases in Sertoli cell volume (39, 40) and protein secretion (41).

It is also possible that phytoestrogens may antagonize the action of endogenous estrogens (42–44) or prevent estrogen biosynthesis through the inhibition of enzymes such as aromatase (45, 46). These functions of phytoestrogens may provide an explanation for the increase in interstitial volume when a S− diet is consumed, particularly because estrogen can have inhibitory effects on Leydig cell proliferation and differentiation in rats and mice (40, 47–49). Also, the specific actions of phytoestrogens in the presence of endogenous estrogen may be concentration dependent, given that agonist actions have been observed at low doses and antagonistic actions at high concentrations, in genistein-treated MCF-7 cells (50). This proposition may explain why dietary soy seemed to have opposing effects on the volume of testicular compartments in 1-yr-old WT *vs.* ArKO mice. Thus, dietary phytoestrogens in combination with endogenous estrogens may have antagonistic effects, but agonistic effects in the absence of endogenous estrogen in ArKO mice.

ArKO mice have been reported to exhibit an increase in body weight and fat deposition (51), suggesting a role for

estrogen in lipid homeostasis. Interestingly, in ArKO mice, at both 14 wk and 1 yr of age, dietary soy prevented this increase in body weight. Consumption of a phytoestrogen-containing diet also decreased body weight in WT mice, which is in agreement with recent studies in normal adult rats (52). It is well known that exogenous estrogens can reduce body weight at birth (53, 54), weaning (55), and maturity (38, 54).

It was previously suggested that the late onset of the spermatogenic phenotype in male ArKO mice could be attributable to estrogenic substances present in their diet that are capable of agonistic effects on spermatogenesis (3). Therefore, the finding that spermatogenesis seems to progress undisrupted in the younger ArKO males, even in the absence of dietary soy, remains puzzling, however, is not unique to this ArKO mouse model. Both ArKO mice generated by Honda *et al.* (56) and Toda *et al.* (57) displayed no histological or spermatogenic abnormalities up to 16 wk of age, with active sperm present in the cauda epididymis. In addition to this, rats treated with an aromatase inhibitor presented with minimal disruptions up to 19 wk of treatment, with more pronounced disruptions after 1 yr, suggesting a time-related effect and possible compensatory mechanisms (58). Similarly, male rats treated with the antiestrogen ICI 182,780 did not exhibit testicular atrophy and infertility until treated for 100–150 d (59).

Despite this, round spermatid number did decrease significantly at 14 wk in ArKO mice raised on a S− diet, suggesting that dietary soy may have some contribution to maintaining spermatogenesis when endogenous estrogen synthesis is prevented. However, because withdrawing soy does not produce spermatogenic disruptions during the initial spermatogenic cycle and, at 14 wk, disruptions are minimal, other explanations must be invoked, such as the presence of alternative ligands. These may include growth factors, such as IGF (60) and epidermal growth factor (61), capable of activating the ER via the activation of intracellular MAPK pathways (62). Importantly, two androgen metabolites (5$\alpha$-androstane-3$\beta$,17$\beta$-diol and 5-androstene-3$\beta$,17$\beta$-diol) can also activate the ER, particularly ER$\beta$, when present in high concentrations (63, 64). Thus, nonaromatase-derived endogenous estrogenic ligands may maintain spermatogenesis in ArKO mice until approximately 14 wk of age. This issue is currently under investigation.

In conclusion, these studies reveal an action of dietary phytoestrogens on male reproduction. Measurable changes in testicular compartments are seen in WT mice; however, the effects of dietary phytoestrogens are most marked in ArKO mice, which lack a functional aromatase enzyme. The data suggest that estrogen production is important for the maintenance of germ cell development and Sertoli cell function and shows that dietary phytoestrogens can mimic the action of endogenous estrogen within the seminiferous epithelium. These findings are important because they clearly highlight the significance of these environmental estrogens in testicular development and function.

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