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Isoflavone maternal-supplementation during periconception period: Influence on the reproductive organs of the first generation (F1) murine weanling-stage offspring

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ABSTRACT

Objective: To determine the influence of maternal Isoflavone-supplementation during periconception period on the reproductive organs of the first generation (F1) murine weanling-stage offspring. **Methods:** Sexually-mature female mice were randomly distributed into the Control group (C) that was fed with food pellets only, treatment groups that were designated as High Dose (HD), Medium Dose (MD) and Low dose (LD) that were fed with food pellets + Isoflavone Genistein dosages of 150.0, 100.0, and 50.0 mg/kg body weight, respectively. Supplement was given two weeks prior to mating and throughout a 21-day gestation period. The pups that were delivered on the 21st day were allowed to mature up to the 28th-day postnatal age then sacrificed by cervical dislocation. The testis and epididymis of males and ovaries and oviducts of females were obtained for histological analyses. **Results:** Histological analysis of the testes showed clogged luminal spaces and indistinct population of spermatogenic cells in the HD group while the development of the ovaries from all test groups appeared intact. The luminal and tubular diameter of epididymis were unaffected in the isoflavone-supplemented groups. The epididymis of HD group exhibited poorly developed cauda segment and atrophy of the caput segment but not for MD and LD groups. The oviducts of the HD and MD groups exhibited collapsed muscularis layer and indistinguishable mucosa layer with HD being the more severe. The LD and Control groups showed well-developed mucosa layer. **Conclusion:** Results showed that maternal isoflavone-supplementation when administered at a high dose of 150 mg/kg body weight during periconception period can induce adverse effects on the reproductive organs of first generation (F1) murine weanling-stage offspring.

1. Introduction

Isoflavones belong to a class of naturally occurring plant-derived chemicals called phytoestrogens. Legumes are almost the exclusive source of isoflavones, with the soybean being the most abundant carrier[1-4]. Soy is frequently consumed in Asian diets as a traditional food, while it still only plays a small role in Western diets despite its reputation of being a reliable dietary fiber and protein source[5]. A considerable amount of evidence shows that diets rich in plant-based foods may explain the epidemiological variance of many hormone-dependent diseases that primarily cause mortality and morbidity in Western populations[6].

The two main classes of phytoestrogens that are of

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current interest in clinical nutrition are the lignans and isoflavones[6]. Isoflavones are known to have a structure quite similar to mammalian estrogens[7] and behave as estrogen mimics[1]. As a phytoestrogen, soy isoflavones bind to estrogen receptors (ER) and affect estrogen-mediated processes[8]. Soy isoflavones such as genistein, daidzein, glycitein and their respective glycosidic conjugates have varying affinity to estrogen receptors due to their different structures. Of these, genistein was shown to have a higher ER-binding affinity than the other derivatives[9]. Estrogen receptors can be found in many non-reproductive tissues, including the bones, liver, heart, and brain (National Cancer Institute, 2005). The tissue selective activities of phytoestrogens are of interest to scientists since anti-estrogenic effects in reproductive tissue may help reduce hormone-related cancer risk in the breast, uterus, and prostate, while estrogenic effects in other tissues may help to improve cholesterol levels and maintain bone density. The extent of the estrogenic and anti-estrogenic effects of soy isoflavones in humans has been the focus in much scientific research[10].

Although soy or soy-containing rich products seem safe and of potential benefit, the safety of consuming soy isoflavone supplements in high doses over a long period of time is still unknown. Presently, no strong convincing evidences exist that infants fed soy-based formula (SBIF) have higher risk of adverse effects than of those infants fed with cow milk-based formula. There is still much debate on the presence of anatomical or physiological defects due to infants' soy isoflavone intake, or whether positive or negative impacts exist on the reproductive health of both male and female adults[11].

Reports on the effects of soy isoflavones on health and development are filling-up[11-13]. In one recent study[14], isoflavone maternal supplementation during pre- and gestation stages given at a high dose of 150 mg/kg body weight brought about masculinization of 21-day old pups as made evident by significant increase of anogenital distance (AGD). Given this finding, there are no follow-up studies yet to further investigate any longer-term influence on the reproductive organs of pups towards the later stages of development.

While there is increasing number of reports on the impact of isoflavones on the reproductive health due to their endocrine-disruption property, there are calls for studies on trans-generational effects. Future perspectives are addressing questions such as whether maternal supplementation could have adverse effects on the first generation offspring (F1) and whether the affected first generation offspring could manifest the effects into their offspring as well (F2).

To address some of the above-mentioned gaps, this study aims to determine the influence of maternal isoflavone-supplementation during peri-conception period, on the reproductive organs of first generation (F1) weanling offspring in mice. This study could add up to the strengthening of any claim on the impact of soy-based infant formula (SBIF) on the development and/or formation of the reproductive organs. Further, the results obtained from this study could provide further insight into exploring the role of isoflavone compounds in disease treatment and prevention.

2. Materials and methods

2.1. Test animals and set-up

High-quality seven-week-old ICR female mice and fifteen-to-eighteen-week-old male mice, weighing an average of about 25 grams were obtained from the Bureau of Animal Industry, Diliman, Quezon City, Philippines. These were kept in the animal house of De La Salle University-Manila. The cages were sanitized on a weekly basis and filled with autoclaved wood chips, feeding plates were washed and dried twice a week, and drinking bottles were washed and filled every other day. For approximately one week, the mice were allowed to adjust to the light-dark cycle that they were exposed to, which includes 12 h light: 12 h dark for inducing ovulation at 28-30 ℃. Food pellets and mineral water were supplied *ad libitum* during this period.

Proper handling and maintenance of the test animals were guided by the Guiding Principles on the Proper Use of Laboratory Animals advocated by the Philippine Association for Laboratory Animal Science.

2.2. Chemicals and reagents

Genistein Soy Complex was obtained from Source Naturals USA. This was made from isoflavone-rich soybean powder yielding approximately 42.5 mg of Daidzein, 25 mg of Glycitein and 10 mg of Genistein, with a total isoflavone amount of 77.5 mg per 2.5 g.

2.3. The experimental design

After the acclimatization period, the female mice were randomly assigned into four (4) test groups, based on their designated diet-regimens. At this time, these were then 8-week old and sexually-matured. The control group (C1) was fed with food pellets and drinking water. The treatment groups were designated as High Dose (HD), Medium Dose (MD) and Low dose (LD). These were fed with food pellets

mixed with varying dosages of Isoflavone Genistein adapted from Dinsdale & Ward (2010). The dosages of Isoflavone Genistein for the supplemented groups were as follows: LD Group, 50.0 mg/kg body weight, MD Group, 100.0 mg/kg body weight, and HD Group,150.0 mg/kg body weight. To make sure that the mice would consume the genistein, it was initially mixed into 0.5 grams of food pellets and given at 0800H. After the initial food supply was consumed, the remaining 3.5 grams food pellet of an ideal daily consumption was given. Isoflavone-supplementation started on the 8th week period lasting for two weeks. This period was considered as pregestation exposure period.

At the end of the two-week isoflavone-supplementation, mating was allowed to take place. A ratio of 1:2 of male to female mice was joined together per cage at 1800H. Female mice were monitored for the presence of copulatory plug anytime between 0700-0800H the following day. Female mice that were plug-positive were considered to have embryos aged at 0.5 day-post coitus (dpc.). Pregnant mice were returned to their respective cages to undergo gestation period of 21 days. The diet-regimens were continuous all throughout the 21-day gestation period.

The pups delivered on the end of gestation period were allowed to wean by the dams for the next 21 days. On the 22nd day, the offspring were separated from the dams into their respective cages up to the 28th day. On the 28th-day, the weanlings were sacrificed by cervical dislocation and the reproductive organs were obtained.

2.4. Gonadal tissue preparation

The testis and epididymis of males and ovaries and oviducts of females were immediately fixed in 10% buffered formalin. The fixed tissues were processed by standard histological methods using hematoxylin and eosin stain at the Histopathology Research Laboratory of the College of Medicine University of the Philippines - Manila.

2.5. Histological observations of tissue samples

Histological analyses of the processed samples and documentation of the qualitative data were performed using Nikon Eclipse TS100. Control samples were compared with those of treated groups (LD, MD, HD). Overall tubular wall diameter and lumen diameter of the cauda epididymis only were measured to the nearest micrometer (μm) using an installed software of the Nikon Eclipse TS100.

2.6. Statistical analysis

The data on the Epididymis tubular and luminal diameter expressed as means were subjected to One-way Analysis of Variance (ANOVA). The level of significance in all cases was set at $P< 0.05$.

3. Results

3.1. General observations

Accounts of the male offspring were five for Control Group, and six for all treated groups, LD, MD, and HD while that of the female offspring was five for the Control Group, five, six and four for the LD, MD and for HD respectively.

3.2. Histological analyses of sex organs

3.2.1. Testes

The testes of the Control mice (Figure 1a and Figure 2) exhibited highly convoluted and intact seminiferous tubules. The integrity of the epithelial lining of the tubules, which consist of columnar cells, is very distinct. A rich population of distinct spermatogenic cells surrounds the wide and clear luminal spaces. These histological features were observed in all the control samples. Interstitial cells, known as Leydig cells, can be observed between the seminiferous tubules of the Control group.

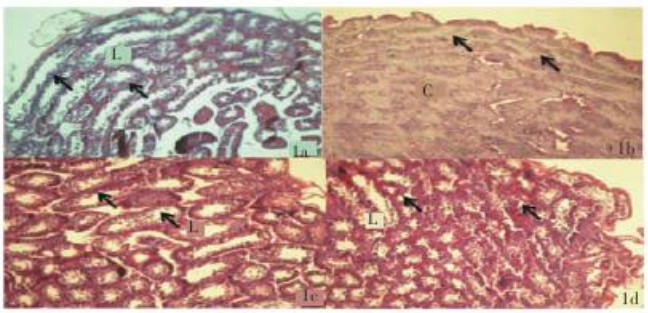

Figure 1. Histology of the testis (peripheral portion) of all experimental groups (10×).
Control (1a); High Dose (1b); Medium Dose (1c); Low Dose (1d). Seminiferous tubules indicated by arrows, luminal spaces (L); Clogged luminal spaces (C).

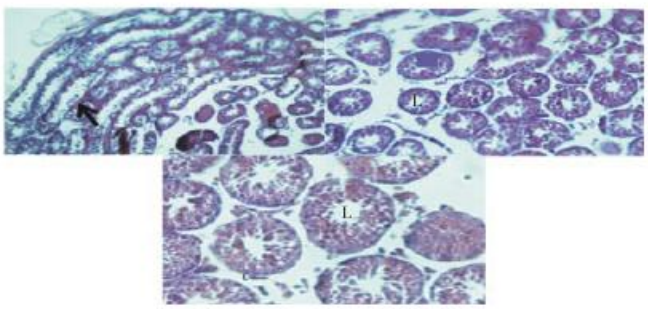

Figure 2. Photomicrograph of testis of control group at different magnifications 10×, 20×, 40× respectively.
L (lumen); arrows (seminiferous tubules).

Among the three isoflavone–treated groups, HD group (Figure 3) showed the most contrasting histologic appearance from the Control. The luminal spaces are clogged with materials which could be suspected as remnants of the cytoplasmic bridges of the spermatogenic cells. Spermatogenic cells appear poorly–developed and indistinct. The seminiferous tubules, however, appear similar to the convoluted and compact features of the Control group. These features were observed among the five of the six samples.

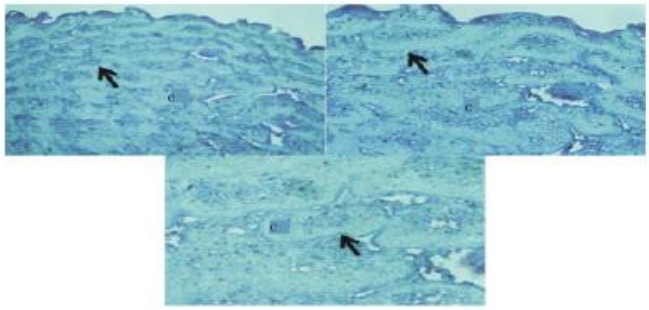

Figure 3. Photomicrograph of testis of high dose group showing clogged (C) and indistinct luminal spaces at different maginifications 10×, 20× & 40× respectively.

The MD group (Figure 4) exhibited histologic features most comparable with the Control group. The integrity of the seminiferous tubules, the distinct populations of the spermatogenic cells and the clear, wide luminal spaces (Figure 1c) are also evident. These characteristics were exhibited among the five of the six male samples. There is one, however, that was more closely akin to the features of the LD group.

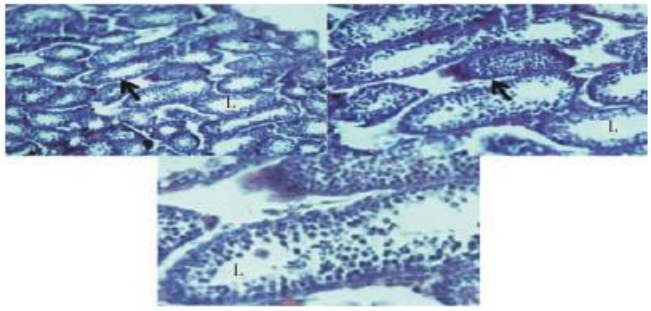

Figure 4. Photomicrograph of testis of medium dose group at different maginifications 10×, 20×, 40× respectively.
L (lumen); arrows (seminiferous tubules).

The LD group (Figure 5) likewise exhibited compact, convoluted seminiferous tubules but most of these are slightly shrunken as made evident by some indistinct luminal spaces (Figure 1d). The distinct populations of spermatogenic cells were observed to be similar with those of the MD and Control groups. These characteristic features were observed among all the samples.

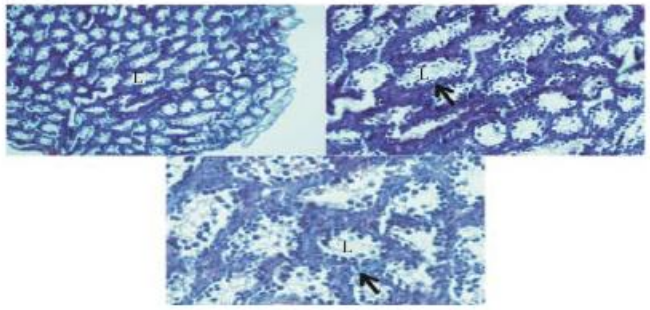

Figure 5. Photomicrograph of testis of low dose group showing shrunken appearance of seminiferous cells at different magnifications 10×, 20×, 40× respectively.
L (lumen); arrows (seminiferous tubules)

3.2.2. *Ovary*

The general ovarian histology of all groups shows the characteristic presence of a surrounding mesentery, the mesovarium, and the intact connective tissue capsule. The dense connective tissue called tunica albuginea also appeared normal underneath the surface epithelium of the ovary. Cortex, located in the peripheral region, and medulla, located in the central region appeared intact in the control mice. The connective tissue stroma appears homogenous albeit some technical nicks incurred in the treated samples. All ovaries were observed to contain oogenic cells at different stages of development (Figure 6, Table 1). The histological features of the Control group are similar with those of the HD, MD and LD groups (Figure 8). In Table 2, accounts of the different stages of oogenic cells at any one time are shown.

Table 1
The mean number of oogenic cells of the ovarian samples from all groups.

Groups	n	1° follicle	2° follicle	3°/Graafian follicle	Atretic follicle
Control 1	5	2.70	1.30	0.07	5.00
High Dose (HD)	5	0.07	6.70	0.07	3.70
Medium Dose (MD)	6	2.30	6.00	1.70	0.70
Low Dose (LD)	4	1.70	6.70	1.30	2.00

N= sample size

Table 2
The mean measurements of epididymal tubular and luminal diameter of male weanlings from all groups.

Groups	Tubular wall diameter(μm)	Tubular lumen diameter(μm)
Control 1	7.70	3.94
HD	10.90^{ns}	7.12^{ns}
MD	10.20^{ns}	7.93^{ns}
LD	9.58^{ns}	7.97^{ns}

ns = not significantly different from the other treated group and from the control group.

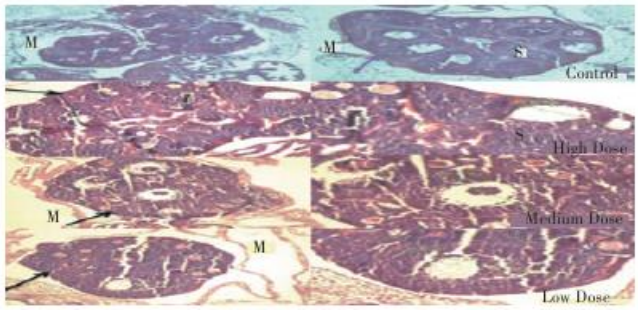

Figure 6. Histology of the ovary from all experimental groups (labeled as Control, HD, MD,LD, respectively).
Left column: 10×, Right column: 20×. Mesovarium (M); stroma (S) Arrows indicate intact ovarian capsules.

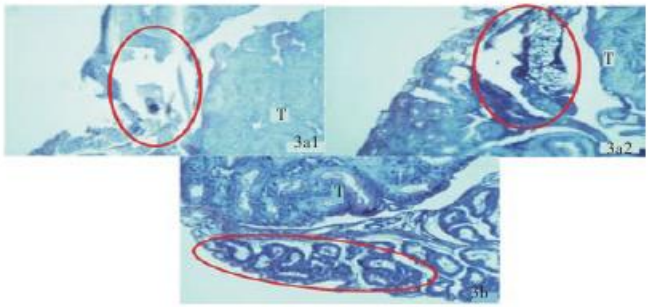

Figure 7. Epididymis of HD group (10×), Caput epididymis emphasized by red circles: 3a1 and 3a2 showing poorly developed caput epididymis. 3b taken from the only HD sample with developed caput epididymis. Testis (T).

3.3. Histological analyses of the reproductive tracts

3.3.1. Epididymis

The caput, corpus and cauda epididymis were observed to be distinctly formed in all groups except that of the HD group (Figure 7, 8) where four (4) out of the five (5) samples exhibited atrophy of the caput epididymis (Figure 8a1 & 8a2). There was only one of the 5 samples that exhibited distinctly formed caput (Figure 7b).

The epididymis of control, MD and LD groups are entirely lined with connective tissue. Smooth muscle fibers surround each individual tubules. The convoluted tubules had various forms, ranging from nearly round, oval and elongated–shaped tubules. The epithelium of the epididymis tubules was composed of pseudostratified cells.

The mean of tubular diameter and mean of tubular lumen diameter of HD, MD and LD are not significantly different from the control (Table 2). No significant differences found in the tubular wall and lumen diameter among all groups were observed ($P > 0.05$).

3.3.2. Oviduct

The oviduct is composed of a fimbriated end, an infundibulum, an ampulla, an isthmus and an intramuscular portion. In the Control group, the intramuscular segment of the oviduct is characterized by thick smooth muscle (muscularis layer) and epithelium (mucosa layer). Mucosa layer is lined with ciliated and secretory cells. Mucosal glands produce oviduct secretions essential for supporting the unfertilized oocyte, supporting the spermatozoa in the oviduct as well as for the proper development of the embryos. Peg cells and columnar cells with very distinct nuclei are tightly packed in the epithelial mucosa layer (Figure 9)

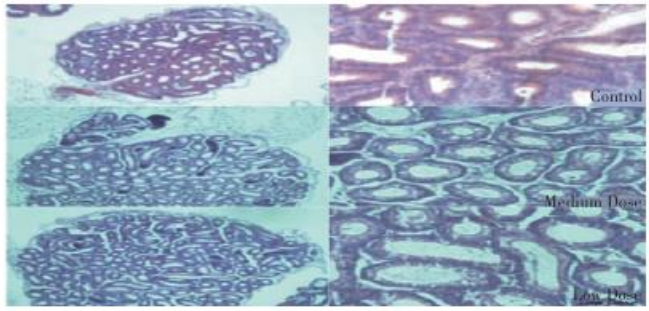

Figure 8. Epididymis from Control, MD, and LD.
Left column 10×, Right column 40×.

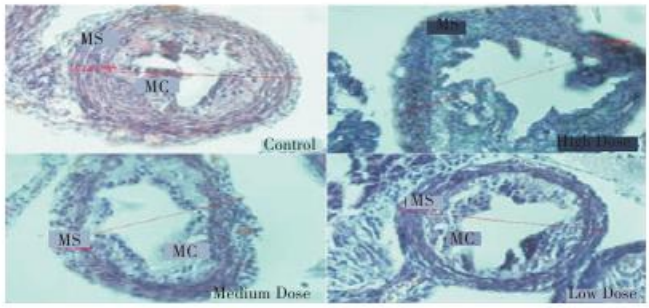

Figure 9. Photomicrograph of the intramuscular layer of Control, HD, MD and LD group.
Muscularis layer (MS); Mucosa layer (MC).

In the HD group, the intramuscular segment exhibited muscularis layer with similar thickness as that of the Control group but with mucosa layer that is poorly distinguishable. Columnar cells are not as conspicuous as that of the Control group. In the MD group, both muscularis and mucosa layers are thinner than those of the control groups and with the mucosa layer exhibiting collapsed and crumpled feature. In the LD group, the intramuscular segments have both the muscularis and the mucosa layers as intact as those of the control group but are evidently thinner.

The isthmus–to–ampulla segment of the Control group is comparable with that of the HD and that LD groups in terms of overall thickness of layers (Figure 10). The MD group, however, appears to have the thickest muscularis layer. In terms of the occurrence of mucosal epithelial folds, the Control group exhibited more prominent and longer primary

folds than those of the treatment groups, HD, MD and LD. All the treatment groups exhibited similar occurrence of primary folds that are distinctly shorter than that of the Control group. (Figure 10)

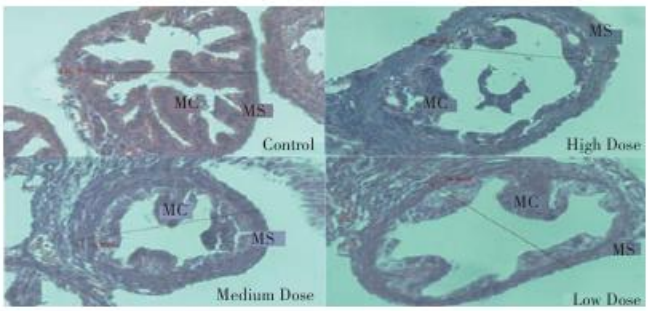

Figure 10. Isthmus-to-ampulla segment of all treatment group including control showing Control with comparable features with HD and LD while MD shows thickest muscularis layer.
Muscularis layer (MS); Mucosa (MC).

4. Discussion

Based on overall assessment and taking into consideration the sample size affected per treated group, HD is the treatment that incurred the most aberration or deviation from the normal histological features of the control followed by the LD while MD appeared to be the one most supportive of normal development.

This histological feature of the testes in HD group may be an indication of a failing process of spermatogenesis, a maturational process known to be supported by the hormone testosterone. Thus, the observed poor population of spermatogenic cells could be due to low level of testosterone. Isoflavones have the ability to decrease serum testosterone through reduction of steroidogenesis in testicular Leydig cells[15]. Reported studies state that long term exposure of male rats to isoflavone Daidzein triggers decrease in plasma levels testosterone and erectile dysfuction[16]; and that long term exposure to an isoflavone Genestein in male rats suppresses the steroidogenic response of Leydig cells to human chorionic gonadotrophin (hCG) and cyclic AMP by down regulating the expression of a microsomal enzyme cytochrome P45017 α -hydroxylase/ C17-20 lyase enzyme (P450scc)[16]. This microsomal enzyme catalyzes two distinct activities; 17 α -hydroxylase activity converts pregnenolone or progesterone to 17 α -hydroxypregnenolone or 17 α - hydroxyprogesterone (17 α -OHP) and c17-20 lyase activity breaks the C17-20 bond of C21 steroids 17 α -hydroxypregnenolone or 17 α -OHP to produce dehydroepiandrosterone (DHEA) or androstenedione (AD), respectively. P450c17 controls an important branch point in steroid hormone biosynthesis leading to the production of three classes of main steroid hormones namely glucocorticoids, mineralocorticoids and precursors of sex steroids[17]. Thus, down regulating the said enzyme will reduce serum levels of the androstenedione, the precursor for testosterone production. This may explain the lack of cell integrity in the spermatogenic cells in the testis exhibited by the HD group (Figure 3) as testosterone is necessary for normal development and maturation of the spermatogenic cells as well as the testis.

As for ovarian histology, it appears that isoflavone-supplement was supportive of ovarian development. Based on the mean number of oogenic cells, it could be inferred, though weakly, that the isoflavone Genistein treatment might have been, at the least, supportive of follicular development. This is, however, most apparent only in the bigger number of secondary and tertiary follicle stages in the treatment groups (MD and LD most specially) than the Control group. Further, this could be supported by the lesser number of atretic follicles formed among all the isoflavone-treatment groups compared with those of the Control groups. However, the inference may still be considered weak due to the small sample size (n=4 to 6) that was histologically analyzed. It is for this reason that any statistical significance could not be strongly asserted for this matter. In a review[18], mice treated with isoflavones were found to be super-ovulated. There was a significant increase of ovulated oocyte in mice treated with high dose and medium dose. Exposure to isoflavones causes failure of oocyte nest breakdown and leads to the development of multi-oocyte follicles. However, occurrence of multi-oocyte follicles does not ensure or suggest fertility.

The observed disappearance and atrophy of caput epididymis can also be associated with disruption of estrogen levels as it is the main reproductive hormone affecting growth, development, maturation and function of reproductive tract as well as the sexual differentiation[19]. Estrogens not only play a crucial role in the development and function of the female reproductive system, it is also essential in male reproductive system development and function[20]. Estrogens act via two types of receptors, the ER α and ER β . Both receptors have direct differentiative influences on reproductive organs and have similar binding affinity to estradiol[9]. However, phytoestrogen such as Genistein have relatively weak affinities to these receptors and they can have agonist or antagonist activity depending on whether estradiol is also present[21]. ERs are present in the testis, efferent ductules and epididymis of most species. Even the primordial germ cells, the precursors of oocytes and spermatogonia, and the somatic cells of the sex indifferentiated gonadal ridges of mouse embryo express ER β or ER α , respectively. This suggests that compounds that interact with these receptors, such as isoflavones are potentially able to modulate and eventually interfere with the development of the reproductive system. Genistein, have relatively higher affinity to ER-b, which are found in the testis, efferent ductules, epididymis and prostate[9]. The efferent ductules and initial segment of the epididymis are dependent on hormones and/or testicular factors derived from the lumen for normal maintenance of epithelial function [22]. Since the development of a male-type reproductive system is dependent on multiple hormones, male fetuses are more susceptible to endocrine disruption than females. For this experiment, only the HD group (150 mg/kg body weight) showed obvious disruptive effect in terms of epididymis morphology. Because phytoestrogens have lower affinity to the estrogen receptors, only those who are administered with

high amount of phytoestrogens exhibited adverse effects. The development of the epididymis in terms of tubular and luminal diameter was not influenced by the isoflavone genistein.

As previously stated, estrogen is the main hormone responsible for growth and development of reproductive tract and act on ER α and ER β. Isoflavones are capable of binding to ER α and ER β but have more affinity to ER β. Both ER α and ER β are present in the oviduct of mouse [19]. These receptors are already present at fetal stage. With the aforementioned we can infer that altering the normal levels of estrogen during fetal development and differentiation may have caused the morphological defects that occurred in the oviduct of the HD and MD groups.

Results of the experiment showed that high dosages of isoflavones (150 mg/kg) are capable of disrupting reproductive organs of pups exposed to isoflavones (genistein and daidzein) through maternal supplementation. They are susceptible to disruptive effects because of genistein and daidzein's ability to cross the mouse placenta and its potential to alter the levels of estrogen and testosterone which are essential to the development of the reproductive system.

Maternal isoflavone-supplementation during the peri-conception period of the murine was capable of inducing adverse effects, particularly when the isoflavone supplements were given at a high dose (150 mg/kg body weight in mice). It likewise presents that high dose isoflavone supplementation results to a poorly developed cauda segment and atrophy of the caput segment in the epididymis of the exposed male murine and a collapsed muscularis layer and indistinguishable mucosa layer in the oviducts of the exposed female murine.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowlegment

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