Biomedical Research 2014; 25 (3): 391-400 *ISSN 0970-938X*
http://www.biomedres.info

Effects of genistein on male sprague dawley rats reproductive development.

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Abstract

Genistein (Gen), is commonly consumed phytoestrogen among Asian and known to exert weak estrogenic effects. To account for potential reproductive effects in male rats, Control, Gen1, Gen10, Gen100 mg/kg body weight and Estradiol were administered to gestational day 10 (GD10) female Sprague Dawley for 5 weeks. At postnatal day, P50, the rats were sacrificed. Blood was taken and reproductive tissues were processed. At birth, body weight (BW) and anogenital distance (AGD) in Gen10 and Gen100 decreased significantly from Control. Throughout experiment, BW and AGD of Gen10 decreased significantly. Preputial separation (PPS) was significantly longer in Gen100 and one rat from Gen10 exhibited unilateral testis descent at P50. Testicular weight and serum testosterone level were reduced in a dose-dependent manner. Histopathological analysis of the seminiferous tubules in Gen1 group is comparable to the Control group. However, the seminiferous tubules of the Gen10 and Gen100 groups showed evidence of overstimulated spermatogenesis. From the immunohistochemical (IHC) analysis, there was higher staining intensity indicating increased expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) and Connexin43 (Cx43) in Gen10 and Gen100 groups compared to Control. Thus, administration of genistein during the critical period of early development could cause antiestrogenic or/and estrogenic influence on the development and functions of the male reproductive system.

Keywords: Soy, testis, estrogens , testosterone, nutrition

Accepted May 08 2014

Introduction

Genistein is a plant isoflavone with polyphenolic compounds derived from a common class of phytoestrogens. Phytoestrogens are classified into groups according to their chemical structures. The greatest estrogenic activity is found in flavones, flavonols, flavonones, lignans, chalcones and isoflavones [1]. These compounds share structural similarities to steroidal estrogens, hence, its binding to estrogen receptors therefore capable of exerting weak anti- and/or estrogenic effects mediated by ER-α and also ER-β through alternative signaling pathways [2-6]. Thus, it is known as an endocrine disrupting chemical (EDC) [4-6]. The relative affinity of phytoestrogens to the ER-β is higher than ER-α [7-9]. Isoflavone was first found to increase plasma concentrations of endogenous estrogens associated with infertility in ewes [10]. Genistein and daidzein form the most predominant isoflavones in soybean which contribute to the most important dietary source of phytoestrogens for mammals [11]. In human, exposure to genistein is mainly from consumption of soy-based food products such as soymilk, tempeh, tofu, miso, soy flour and soy sauce [12]. It was reported that Asian people consumed 1.5 mg genistein or other isoflavones daily higher than most Europeans and North Americans [13, 14].

Many EDCs in the environment are identified as environmental estrogens. These environmental estrogens may endanger fetuses as they are at the stage of highly susceptible to minor endocrine disturbances that may give rise to developmental abnormalities including testicular dysfunction [15] and thus lead to infertility.

The morphological alteration is associated with lack of gap junction protein, Connexin43 (Cx43) which controls cell growth and differentiation of the germ cells [16-20]. Thus, Cx43 expression can be used as an indicator for the well-being of the intercellular communication in the seminiferous epithelium where it was normally expressed in rat testis [21], and was down-regulated in mice testis with disrupted spermatogenesis [22-24]. Failure of spermatogenesis is also associated with low testosterone levels due to disturbance in testicular steroidogenesis which depends on 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme for the synthesis and secretions of testosterone [25]. This is in agreement with analysis of contralateral testis of men with testis anomaly due to

immature Sertoli cells is lacking in germ cells and Leydig cells (3β-HSD positive) hyperplasia [26, 27].

For centuries, soy-based products have become the preferred alternative diet among vegetarians. Besides, soy-based supplements have a growing popularity because it is claimed to have many health benefits. Thus, the public is at risk of being exposed to high genistein content in soy. Therefore, it is necessary to investigate the potential adverse effects of genistein on human health especially the effects on developing fetuses from maternal exposure.

The present study aimed to investigate the safe dose of genistein from soymilk on early development of the male reproductive system. We hypothesized that genistein exposure during the critical period of perinatal development can have detrimental effects on early development of the male reproductive system that may cause disruption of the hormonal functions leading to infertility.

Materials and Methods

Chemicals

Genistein of at least 99 % purity were obtained from Indofine (Indofine Chemical Company, Hillsborough, New Jersey, USA),17-β Estradiol was obtained from Sigma (Sigma Aldrich Chemical, St. Louis, MO, USA). A testosterone enzyme-linked immunosorbent assay (ELISA) kit was purchased from IBL International GMBH (Germany). For immunohistochemical (IHC) study, specific goat polyclonal antibodies against steroidogenic enzyme 3β-HSD and Cx43 were used with the aid of ImmunoCruz™ goat ABC staining system (Santa Cruz, CA, USA).

Animals

A total of 10 time-mated (gestational day, GD10) female Sprague Dawley (SD) rats weighing 203.5 ± 4.87 g were obtained from Faculty of Medicine Animal Facilities, University of Malaya.This study has been approved by the Animal Care and Use Committee (ACUC) of University of Malaya. Animals were housed in individual cages in an air-conditioned room at room temperature of 25 ± 1 ˚C with a 12 h light: 12 h dark period. Rats were given free access to standard rat chow (RainTree, Australia) and water *ad libitum* in glass bottles. All experiments were carried out according to the institutionally approved protocols according to University of Malaya Guidelines for the care and use of laboratory animals (Ethic Number ANA/01/10/2007/0810/NMK(R))

Treatment scheme and dosing

The presence of vaginal plug was designated as gestational day 1 of pregnancy (GD1). Pregnant dams were divided into five groups and received the following treatment: a) Control: received the vehicle only (Tween-80 (1:9, v/v)) (Sigma Aldrich, St. Louis, MO, USA), b) Gen1: received genistein 1 mg/kg BW, c) Gen10: received genistein 10 mg/kg BW, d) Gen100: received genistein 100 mg/kg BW, and e) Estradiol group: received 7.5 µg/kg BW of 17-β Estradiol. Rats were treated daily with genistein dissolved in Tween-80 using oral gavage tube from GD10 to GD21.

Dams were allowed to litter spontaneously and the day after birth was designated as postnatal day 1 (P1). Only male pups were chosen for this study. Male pups continued to receive genistein treatment by subcutaneous injection until P21. Body weight and anogenital distance of pups were recorded weekly till P21. Testis descent were monitored. Study has been carried out without treatment from P22 until P50 by observing the evidence of testis descent and sign of puberty. Testis descent in rat normally occurs on P21 while the onset of puberty is when they attain preputial separation (PPS) which normally occurs between P40-P45 according to Korenbrot *et. al.* [28].

Necropsy and sample collection

At P50, rats were fasted overnight prior to sacrifice and were sedated followed by intraperitoneal injection of chloral hydrate (0.1 ml/100 g of BW). Blood samples (5 ml) were obtained from transcardiac puncture, centrifuged at 1000 rpm for 5 minutes, and serum was stored at -20 ˚C until assayed for testosterone. Gross morphology of the external genitalia was examined. Testes were weighed and fixed in 10 % formalin fixative before being processed.

Histological analysis and microscopy

Testes were processed using an automated tissue processor (Thermo Scientific) and embedded in paraffin (ParaPlast Plus, USA). Tissue sections of 5 µm thickness were mounted onto labeled glass slides and stained with haematoxylin and eosin (H&E). Diameters of 100 seminiferous tubules in transverse section per testes were analyzed under light microscope (Olympus CH-B145-2) and representative areas were measured using NIS-Elements Software (NIS-Elements Advanced Reasearch, Nikon, Japan). Briefly, the round or approximately round seminiferous tubules (the shortest to the longest axis ratio greater than 0.8) were chosen based on the previous study [29].

Serum testosterone level in male pups

Serum testosterone level was measured using an ELISA kit reader (IBL International GMBH, Germany) according to the manufacturer's guideline.

Immunohistochemical localization of Cx43 and 3β-HSD

Paraffin sections of 5 µm were cut and placed onto polysine-treated glass slides. Sections were dewaxed and rehydrated through decreasing concentration of ethanol

solutions. The sections were then autoclaved at 30 ˚C for 15 min in 10 mM in citrate buffer (pH 6.0) for inactivation of endogenous peroxidase activity with 0.1 % H_2O_2 in methanol (room temperature, 30 min), and were pre-incubated with normal donkey serum in phosphate buffer saline (PBS) for 1 h to block non-specific reaction. Following that, the sections were incubated overnight with goat polyclonal antibody against 3β-HSD and Cx43 respectively, at 4 ˚C (Santa Cruz, CA, USA; at dilution of 1:100), followed by three rinses in PBS. After that, the sections were incubated with secondary antibody for 1 h followed by the ImmunoCruz^{TM} goat ABC staining system. Finally, the sections were counterstained with Mayer's haematoxylin. For the negative control slides, normal donkey serum was used instead of primary antibody.

Qualitative analysis of 3β-HSD and Cx43 staining expression were carried out under a light microscope (Olympus CH-B145-2). Representative areas were photographed with a Nikon Eclipse 80i upright microscope equipped with a digital color camera controller (DS-5Mc-U2).

Statistical analysis

All data were tested for normality followed by *Levene static test* for homogeneity of variances. All the parameters were compared using *One-way ANOVA* and *Tukey post hoc test* for the determination of differences among the groups. The significant difference between the treatment group was considered at $P<0.05$. We performed the calculations using PASW18 student's program for ANOVA.

Results

Body weight

Exposure of male rats to genistein during prenatal life from GD10 to GD21 continued through neonatal life from P1 to P21 has resulted in a significant decrease in the mean BW of Gen10 and Gen100 groups compared to Control and Estradiol groups. A significant reduction of the mean BW was observed at birth and during subsequent weeks in all genistein-treated rats compared to the those of the Control and Estradiol (P1 to P21). However , the mean BW was normalized in the final week (P50) with no significant difference observed (Table 1).

Anogenital distance

At P1, the AGD of male rats were significantly shorter in all genistein-treated rats compared to those of the Control and Estradiol rats. After a week (P8) only the AGD of Gen10 rats were significantly shorter than those of the Control and Estradiol group, while at P15, it was significantly shorter in Gen10 and Gen100. During weaning (at P21), the AGD of Gen1 rats were significantly shorter compared to the Control while the AGD of Gen100 rats were longer compared to the Estradiol group. There was no significant difference of AGD in rats during adulthood (at P50) (Table 1).

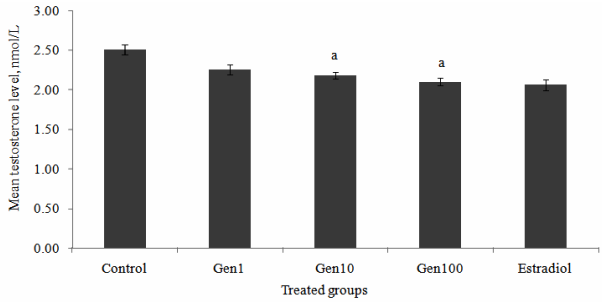

Figure 1. *Graph showing a dose-dependent decrease in circulating serum testosterone level at P50 of genistein- and Estradiol-treated rats. The data are expressed as Mean ± S.E.M. Means with different superscripts are significantly different.* $^{a}P<0.05$ *versus normal Control group and* $^{b}P<0.05$ *versus Estradiol group*

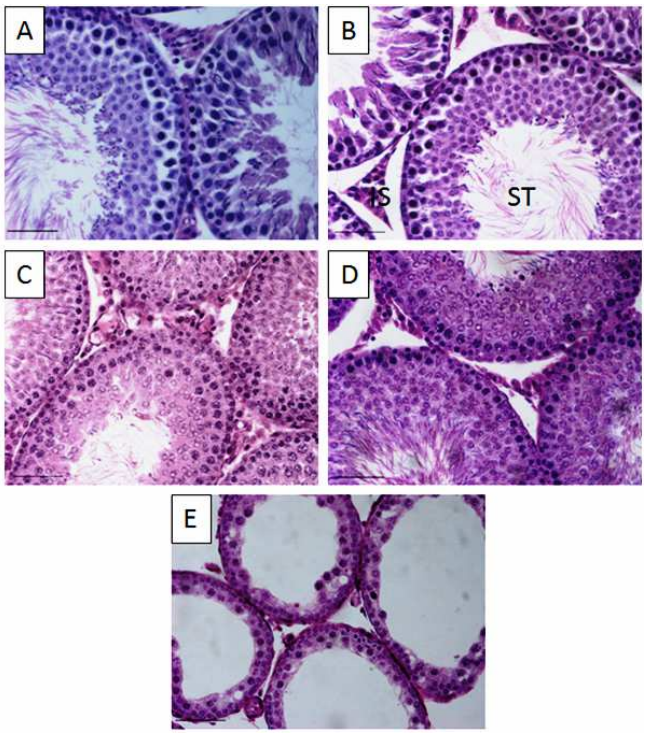

Figure 2. *A histopathological changes of transverse section of rat testes were observed at P50. (A) Control, (B) Gen1, (C) Gen10, (D) Gen100, and (E) Estradiol. High activity of spermatogenesis were observed in Gen10 and Gen100; no evidence of spermatogenesis in Estradiol group. H&E staining. Scale bar= 50μm. IS= Interstitial space, ST= Seminiferous tubule*

Table 1. *BW (g) and ADG (mm) of all experimental animals*

	Control	Gen 1	Gen 10	Gen 100	Estradiol
BW (g)					
P1	7.76 ± 0.10	7.74 ± 0.21[a,b]	6.38 ± 0.20[a,b]	6.53 ± 0.10[a,b]	10.18 ± 0.18[a]
P8	18.04 ± 0.40	16.92 ±0.56[b]	10.89 ± 0.69[a,b]	14.06 ± 0.11[a,b]	20.01 ± 0.42
P15	29.60 ± 1.0	27.88 ± 0.79[b]	21.52 ± 0.28[a,b]	23.88 ± 0.92[a,b]	31.28 ± 0.63
P21	46.75 ± 1.31	38.54 ± 2.55[a,b]	36.94 ± 0.38[a,b]	43.77 ± 0.48	47.83 ± 1.14
P50	172.67 ± 12.12	207.43 ± 15.44	138.67 ± 10.53	182 ± 13.70	173.83 ± 5.33
AGD (mm)					
P1	7.57 ± 0.21	6.27 ± 0.21[a]	6.06 ± 0.17[a]	6.20 ± 0.27[a,b]	6.77 ± 0.32
P8	9.87 ± 0.37	9.59 ± 0.38	7.94 ± 0.11[a,b]	9.32 ± 0.9	9.57 ± 0.54
P15	13.28 ± 0.14	12.83 ± 0.62	11.48 ± 0.27[a,b]	11.62 ± 0.14[a,b]	14.07 ± 0.63
P21	19.34 ± 0.52	17.12 ± 0.55[a]	17.64 ± 0.47	20.76 ± 0.44[b]	16.71 ± 0.73
P50	33.57 ± 1.02	37.53 ± 1.25	32.19 ± 0.92	35.86 ± 2.38	33.98 ± 0.87

The data were expressed as Mean ± S.E.M. Means with different superscripts are significantly different. [a]P<0.05 versus Control group and [b]P<0.05 versus Estradiol group.

Table 2. *Effects of perinatal exposure to genistein on testis descent and preputial separation*

Group	Control	Gen1	Gen10	Gen100	Estradiol
Descent at P21					
(i) Right	6/6	6/6	5/6	5/6	0/6
(ii) Left	6/6	6/6	6/6	6/6	0/6
Descent at P50					
(i) Right	6/6	6/6	5/6	6/6	4/6
(ii) Left	6/6	6/6	6/6	6/6	2/6
PPS, day	42.5±0.55	41.7±0.52	43.7±1.51	44.3±1.03[a]	NA

The data were expressed as Mean ± S.E.M. Means with different superscripts are significantly different. [a]P<0.05 versus normal Control group and [b]P<0.05 versus Estradiol group. Incidence of undescended testes: Gen10 (1/6, Inguinal), Estradiol (Left,1/4: Abdominal, 3/4: Inguinal; Right, 2/2: Inguinal). NA: Not available

Table 3. *Effects of perinatal exposure to genistein and Estradiol on testicular weight and seminiferous tubule measurement*

	Control	Gen 1	Gen 10	Gen 100	Estradiol
Testis	1.22±0.18	1.25±0.3[b]	0.83±0.07[a,b]	0.82±0.1[a,b]	0.42±0.1
Tubular diameter	202.50±1.596	229.94±1.416[b]	191.91±1.162[b]	187.58±0.876[b]	134.26±0.891
Luminal diameter	81.11±1.274	80.59±1.303[b]	83.78±1.284[b]	52.72±0.915[a,b]	34.37±0.984
Epithelial thickness	65.31±0.677	70.08±0.485[b]	69.79±0.619[a,b]	66.56±0.479[b]	34.37±0.593

The data are expressed as Mean ± S.E.M. Means with different superscripts are significantly different. [a]P<0.05 versus normal Control group and [b]P<0.05 versus Estradiol group.

Preputial separation (PPS) and testes descent

The mean day of PPS of rats in the Gen1 group was slightly earlier compared to the Control group while those of the Gen10 and Gen100 were delayed. However, only the mean PPS of the Gen100 group was significantly delayed compared to the Control group, while no rat in the Estradiol group exhibited PPS (Table 2).

As for influence of phytoestrogens on testis descent, it was noted that rats in Gen1 group exhibited normal testis descent similar to the Control group (Table 2). However at P50, one rat from Gen10 group exhibited unilateral undescended testis, while those of the Estradiol group, 4/ 6 testes on the right side and only 2/ 6 testes on the left side descended into the scrotum.

Testicular weight and seminiferous tubule measurement

The mean testis weight in the Control group was 1.22±0.18 g while that of the Estradiol group was 0.42±0.1 g. In the experimental groups, the mean testis weight in Gen1 group was comparable with the Control group, while rats in the Gen10 and Gen100 groups exhibited significantly reduced testis weight compared to the Control rats but not as low as that of the Estradiol group (Table 3).

Quantification of morphological changes showed that the diameters of seminiferous tubules were smaller in Gen10 and Gen100 groups compared to Control. However, it was not significant. The spermatogenic cells were found normal without cell loss evidenced by increased epithelial thickness in genistein-treated groups (not significant) compared to Control. The population of Sertoli cells were indistinguishable between genisten-treated and Control animals. The interstitial tubular space contained many Leydig cells compared to Control. However, we did not quantify the number of Sertoli and Leydig cells in this study.

Serum testosterone

The mean serum testosterone levels of all the treatment groups decreased in a dose-dependent manner compared to the Control group. However, only the Gen10 and Gen100 groups showed a significant decrease testosterone levels compared to the Control (Fig. 1).

Microscopic evaluation of the testes

The Control testes comprised of seminiferous tubules closely arranged exhibiting the various stages of normal spermatogenesis with normal Sertoli cells and germ cells. The testes of Gen1 rats also exhibited normal spermatogenesis at various stages comparable to those of the Control testes. There were no apparent changes in the cells of interstitial spaces including Leydig cells. In contrast, the testes of Gen10 and Gen100 rats exhibited an overstimulated spermatogenesis with increased in germ cell population thus the germinal epithelium appeared thicker than that of the Control testes and some of the tubular lumina were completely filled with sperm tails. In the Estradiol group, the seminiferous tubular diameters as well as their epithelial height were very much reduced. There was no evidence of spermatogenesis and the number of Leydig cells in the intertubular spaces was also diminished (Fig. 2).

Immunohistochemical detection of 3B-HSD and Cx43 expression in rat testes

3β-HSD expression in the testis was localized in the Leydig cells with less intense staining in the seminiferous epithelium especially in the late stage spermatids in the control testis. The intensity of 3β-HSD expression in Gen1 was comparable to Control. The expression of *3β-HSD* was more intense in Gen10 and Gen100 testes

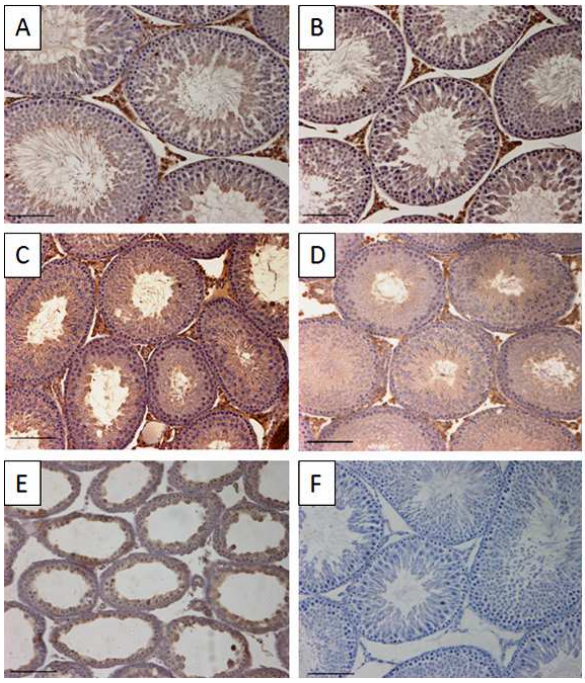

Figure 3. *Expression of 3β-HSD in (A) Control, (B) Gen1, (C) Gen10, (D) Gen100, (E) Estradiol, and (F) Negative control. The staining intensity was increased in Gen10 and Gen100. The staining intensity of the intertubular spaces was reduced in Estradiol group. Scale bar= 50μm*

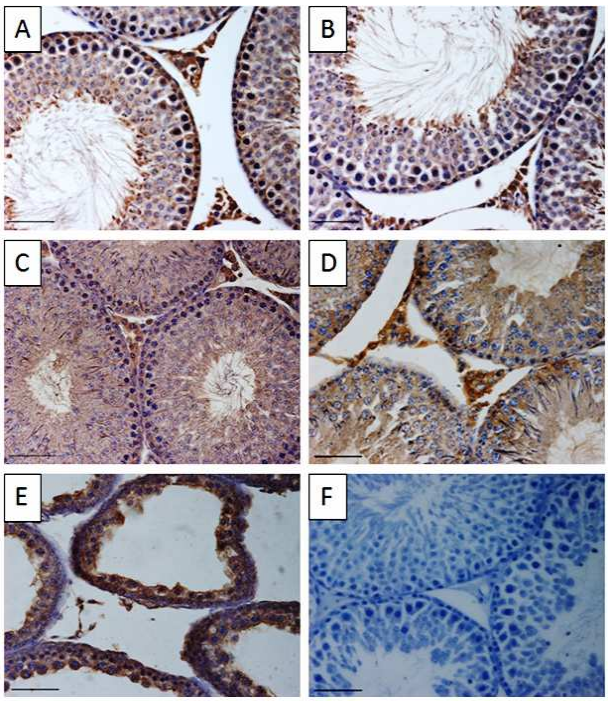

Figure 4. *Expression of Cx43 between Sertoli cells and germ cells in seminiferous tubules and Leydig cells. (A) Control, (B) Gen1, (C) Gen10, (D) Gen100, (E) Estradiol, and (F) Negative control. More intense expression was noted in Gen10 and Gen100 groups. Scale bar= 50μm*

compared to Control and Gen 1, while there was almost no immunostaining of the intertubular spaces of the Estradiol testis. However, there were some staining of the seminiferous epithelium in the Estradiol testes (Fig. 3).

Similarly, the staining intensity for Cx43 was also localized in the Leydig cells as well as the seminiferous epithelium especially the spermatogonia and spermatocytes and late stage spermatids. There was no apparent difference in staining intensity between Gen1 compared to Control. Cx43 expression in Gen10 and Gen100 appeared more intense especially of the spermatogonia and spermatocytes. While in the Estradiol testes, the staining intensity was much less compared to Gen10 (Fig. 4).

Discussion

Estrogens are important in reproductive development and function. But, excess exposure to estrogens during critical period of development may exert detrimental consequences on development of the reproductive organs [30, 31]. For centuries, soy has been widely consumed and its health benefit has been documented [32, 33]. In the present study, we used low (Gen1), moderate (Gen10) and high (Gen100) doses of genistein to compare with the recommended level in the diet with genistein (6 to 9 mg/kg kg BW) [34]. Our study showed that perinatal exposure to genistein resulted in reduction in the mean BWof all genistein-treated rats at P21 except for the Gen100 rats where their mean BW increased at P21. It was hypothesized that fat deposition does not occur until the onset of suckling probably due to their habitual huddling with littermates in order to maintain body temperature [35, 36]. The increased of mean BW in Gen100 rats at P21 is probably due to stimulation of adipogenesis at higher doses as reported by Heim *et. al*. [37] and might be the estrogenic effect of genistein. During adulthood (at P50), we noticed that the mean BW of all genistein-treated rats normalized and comparable to the Control.

Our study also showed that there was impairment of male reproductive system development as evidenced by reduction in testicular weight and AGD. In the present study, the AGD of Gen10 rats was significantly reduced from P1 to P21 but not significant at P50. This observation suggested that genistein interfered with the early development of external genitalia in males similar to the previous findings that showed endocrine disrupting chemicals intereference on the reproductive parameters that were identified as potential risk to the reproductive system development [38-40]. We also noted that all the AGD measurements of the rats normalized at P50. This observation has lead us to hypothesized that the development of the male external genitalia proceeds normally after weaning (at P21) once genistein treatment is discontinued.

Studies have shown that factors capable of interfering sexual development can also affect the development of the external genitalia thus, is commonly assessed by determining the anogenital distance [41, 42]. In the present study, the reduction of AGD in genistein-treated rats could be due to reduction in androgen production. Since 17-β Estradiol is a potent estrogenic compound, it was used in our study to test the estrogenic activity *in vivo* as was employed by Lee et. al. [43]. Similarly, PPS is also used as an indicator for male puberty which also depends on androgens. Our findings showed that PPS was significantly delayed in Gen100 rats compared to the Control. This is in contrast to previous findings, which showed no significant effect on PPS of rats treated perinatally with genistein at 50 µg/d [44].

In rats, the testis descent is accomplished by P28 [45, 46]. Our findings showed that testis descent was disturbed in some of the Gen10 and Gen100 rats and in all Estradiol rats at P50. This might be due to the chemical property of phytoestrogen, which acts as anti-androgen that interfered with testis descent in these groups or it might just be a transient effect. These findings can be correlated with the significant dose-dependent decrease of serum testosterone level in these groups and in studies using isoflavones in different doses [47-50]. However, this notion is not in agreement with the findings by Piotrowska and partners [51] who reported no significant difference in serum testosterone levels, but found significant reduction in testicular testosterone levels in rats treated with genistein.

Hormonal imbalance in experimental rats from perinatal exposure to genistein may also cause detrimental effects on the morphology of the testis in adulthood. The present study, showed that diet containing Gen10 and Gen100 effectively stimulate spermatogenesis in rat testes as evidenced by a significant increase in germinal epithelial thickness. Activation of cell proliferation by genistein on fetal testis is also seen in previous study (52). Additionally, genistein also increases the proliferation rate of gonocytes in vitro isolated from neonatal rats (53). Interestingly, in another study using cytotoxic drug that caused damage to rat testes, genistein was reported to suppress the drug cytotoxicity, as well as testosterone levels but stimulated spermatogenesis in rats [54]. Therefore, from our study, we can deduce that suppression of testosterone level was caused by the increase expression of stem cell factors (SCFs) of the Sertoli cells that are essential for spermatogenesis [54, 55]. Genistein action on target cells appears to be associated with its estrogenic activity in addition to its inhibition of the key steroidogenic enzymes by its influence on the estrogen receptors [56].

Genistein exposure during the perinatal period was shown to increase proliferative activity of Leydig cells but exerted an opposite effect on androgen concentration in pubertal male rats. Androgen is an autocrine regulator of Leydig cells and mainly responsible for androgen production [57]. Therefore, it is possible that genistein inhibits testosterone secretion by delaying Leydig cell differentiation, which contributes to the decrease androgen secretion. The present findings of reduced testosterone concentration contradicts with previous finding that showed the increment in serum steroid hormone production under the perinatal exposure [58]. Serum sex hormone concentration was paradoxically depend not only on steroidogenic capacity but also on the number of Leydig cells [59]. Albeit increased proliferation was observed under immunohistological study, we did not quantify Leydig cell numbers in the present study. The reduction of serum testosterone levels in this study could be attributed by the inhibition of the steroidogenic pathway, not due to direct effect of genistein on Leydig cell numbers as in previous study (60). Nevertheless, it is likely that the greater Leydig cell populations may result from the longer duration of perinatal exposure to isoflavones [61].

Previous studies showed that increased steroidogenic acute regulatory (StAR) protein levels with decreased LH stimulation and reduced StAR phosphorylation, which is critical for translocation of cytosolic cholesterol into mitochondria [58, 62, 63]. However, this is in contrast with general consensus that StAR protein increased in the presence of decreased testostosterone production. The increase in Leydig cell numbers in the present study could be a consequence of the compensated Leydig cell failure in the presence of supranormal LH serum level [64]. Although serum LH levels were not assayed in this study, decreased 3β-HSD expression has been attributed to reduced LH stimulation of Leydig cells as evidenced by hypogonadal testis and feminized mice testis [65]. Therefore, the markedly increased expression in 3β-HSD protein expression in Gen10 and Gen100 in the present study were probably due to homeostatic adjustments provoked by diminished LH stimulation of cholesterol availibility and/or utilization in Leydig cells.

Several studies have reported on the influence of isoflavones on testicular morphology and Leydig cell development. In one study, genistein was found to induce hyperplasia of Leydig cells in mice [43], and in another study with marmoset developed large testes and increased number of Leydig and Sertoli cells [66]. Lower concentrations of serum testosterone associated with an increased number of Leydig cells have been observed in neonatal marmosets fed with soy milk formula when compared with animals fed with cow milk formula [67]. It has also been reported that long-term dietary administration of genistein reduces serum levels of testosterone (50) and also suppresses both basal and LH-stimulated androgen production by rooster Leydig cells *in vitro* [68]. The present results confirmed that genistein is capable of regulating Leydig cells function and support the direct action of genistein on Leydig cells as suggested previously (58) as well as incubation with isoflavones induced proliferative activity and supressed steroidogenic capacity in the Leydig cells [61].

The seminiferous epithelial cells are adjoined by different types of gap junctions that are linked with each other via their common adaptors or signaling processes [69, 70]. Gap junction protein, Cx43, participate in germ cell development; any alteration to it can lead to loss of germ cells in either males or females [71-73]. In patients with low testosterone level (eg; carcinoma-in-situ or testicular seminoma), the Cx43 expression was reported to be down regulated indicating reduction of this gap junction protein [74-76]. Suprisingly, based on our findings, there was high expression of Cx43 in Gen10 and Gen100 testes. Thus, we hypothesized that there is no effect of weakening the signaling pathways following high dose genistein treatment and the germinal cells in the seminiferous epithelium were intact with no sign of cell loss observed.

To date, the effects of genistein to reproductive health are still unclear. Although many studies reported some deleterious effects of isoflavone exposure to infants at different doses and routes, the results are still inconsistent. It is worthwhile to highlight the agents that may induce toxicity on critical stages of development of the reproductive system as well as its possibility on interfering with the later stages of development.

Conclusion

Perinatal exposure to genistein could compromise the development of the male reproductive system in rats, as evidenced by the reduction in body weight, anogenital distance, delayed preputial separation, and overstimulation of spermatogenesis and other reproductive parameters. However, there is no evidence that genistein at the recommended dosage can produce the similar effects in human. Future studies with longer duration of genistein exposure is necessary to better account for its effects and possible mechanisms as an endocrine disruptor to the reproductive system of men. Thus, despite the indiscriminate recommendation on the use of soy and its derivatives, the results of this study show that genistein is not totally free from undesirable effects.

Acknowledgement

This work was supported under grant number: RG203-10HTM. The authors also would like to thank Department of Anatomy, Faculty of Medicine, University Malaya,

Kuala Lumpur for the facilities provided in the conduct of the research.

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