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GENISTEIN INDUCED ALTERATIONS OF THE TESTICULAR BIOCHEMISTRY AND HISTOMORPHOLOGY OF ADULT MALE MICE, *MUS MUSCULUS.*

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ABSTRACT

Thirty male *Mus musculus* were divided into two groups of 15 animals each, where the controls received vehicle only and the experimental animals had 10mg/kg body weight genistein /day orally (dissolved in 1:4 DMSO:PBS-vehicle) for 30, 60 and 90 days . Biochemical analyses of testicular phosphatase enzymes (ACP and ALP), protein, cholesterol, glucose and fructose as well as hormone estimations (LH, FSH and Testosterone) reported less steroidogenic activities and low sperm maturity ($p<0.001$, via two-way ANOVA analysis). Along with these the histo-pathological investigations of testes illustrated that there had been low spermatogenic activities in the treated groups with more severity at the later part of the experiments which suggests that genistein may lead to reproductive insufficiency in adult male mice, in terms of lesser availability of mature spermatozoa. The alterations observed may be under the direct influence of testosterone and unavailability of the gonadotropins or may be via hypothalamo-hypophysial testicular axis.

KEYWORDS: **Genistein, *Mus musculus*, testes, spermatozoa, histopathology.**

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INTRODUCTION

There is currently great uncertainty whether some synthetic chemicals, acting as 'hormone-mimics' and released into the environment, might have the potential to disrupt the endocrine system. Even though exogenous endocrine modulators can involve any hormonal system, the principle focus of this work is on soy isoflavone genistein-mediated effects on the male reproductive system of adult *Mus musculus*. The primary emphasis here for potential adverse effects resulting from exposure to environmental estrogens is due to oral administration because such exposures can occur through dietary intake during adulthood[1]. Substantial evidence indicates that diets high in plant-based foods may explain the epidemiologic variance of many hormone-dependent diseases that are a major cause of mortality and morbidity in Western as well as Eastern populations[2&3]. On the contrary, the consumption of soy-rich diets has been associated epidemiologically with reduction in risk of several diseases such as endometrial cancer and prostate cancer[4,5&6]. There is also evidence indicating a role for the phyto-estrogens present in soy beans and some soy-based nutritional products in mediating these effects[7]. Diverse profile of male reproductive tract anomalies has been attributed to phytoestrogens in studies of laboratory animals and nonhuman primates. For example, neonatal rats sustained on a diet containing 5 and 300 ppm genistein exhibited decreased testis size and serum testosterone (T) level[8]. Sub-cutaneous (sc) administration of genistein at a daily dose of 2.5 mg/kg/day for 9 days resulted in reduced serum and testicular T concentrations and decreased prostate gland weight in adult mice[9]. Neonatal marmosets fed with soy-based formula [1.6–3.5 mg isoflavones /kg/day from 5th day to 45th day postpartum] subsequently had low serum T levels ranging from 1.2 to 2.6 ng/ml compared to cow milk formula fed paired animals [range 2.8-3.1 ng/ml][10]. In contrast to inhibitory effects, testis weights were increased in mice treated neonatally with genistein (1 mg/pup) [11], and sc administration of genistein at 4 mg/kg/day to neonatal rats stimulated germ cell development relative to control animals[12].Similarly, infant marmoset monkeys fed with soy-based formula resulted in increased testis size, serum T concentrations, and Leydig cell numbers in adult animals[13]. However, other reports have suggested that phytoestrogens cause only modest effects on male reproductive activity[14]. Despite inconsistency in results from different laboratories, the bulk of the data demonstrates that phytoestrogens possibly regulate male reproduction. Leydig cells express Estrogen receptors (ER) and thus their functioning are under regulation of estrogens. Therefore, administration of Estrogen (E_2) suppresses Leydig cell regeneration in rats treated with the cytotoxin ethane dimethylsulfonate[15]. The levels of T in the blood and testis are dependent on two factors: the numbers of Leydig cells and T production rate per Leydig cell. It is not clear whether phytoestrogen-induced changes in serum T levels are related to differences in T production rates and/or Leydig cell numbers. Moreover, the perinatal period of reproductive tract development is a particularly sensitive window of exposure to exogenous estrogens or xenoestrogens[16]. The present study was designed to propose a possible relationship between long term oral exposure to genistein and male reproductive abnormalities in adults. This study was carried out to determine whether long term oral administration of genistein resulted in adverse reproductive health consequences in adult male *Mus musculus*.

MATERIALS AND METHODS

Commercially available 98% pure HPLC grade Genistein powder (Sigma Aldrich) was dissolved in 1: 4 Dimethyl sulfoxide (DMSO): Phosphate buffered saline (PBS) and was administered orally to mice at a dose of 10 mg Genistein/kg body weight/day. Approximately 7-8 week old thirty adult male mice weighing 30±5 g were housed and acclimatized at approximately

24±2ºC temperature with 10h: 14h light-dark cycle in the animal house. Maintenance and experimentations carried out were as per the guidelines of Animal Ethical Committee for the use of laboratory animals in biological research approved by Institutional Animal Ethical Committee (IAEC), Bhopal. All mice were divided into two groups of 15 each, where group first was vehicle treated control and the second (experimental group) were given 10 mg Genistein/kg body weight/day for 30, 60 and 90 days and colorimetric analyses of different enzymes, biomolecules, hormonal assays and histo-morphological studies were carried out by opting appropriate techniques.

A) Biochemical estimations:- Testes were used for colorimetric estimations of acid and alkaline phosphatase enzymes[17], total protein[18], total cholesterol[19], glucose[20] and fructose[21].

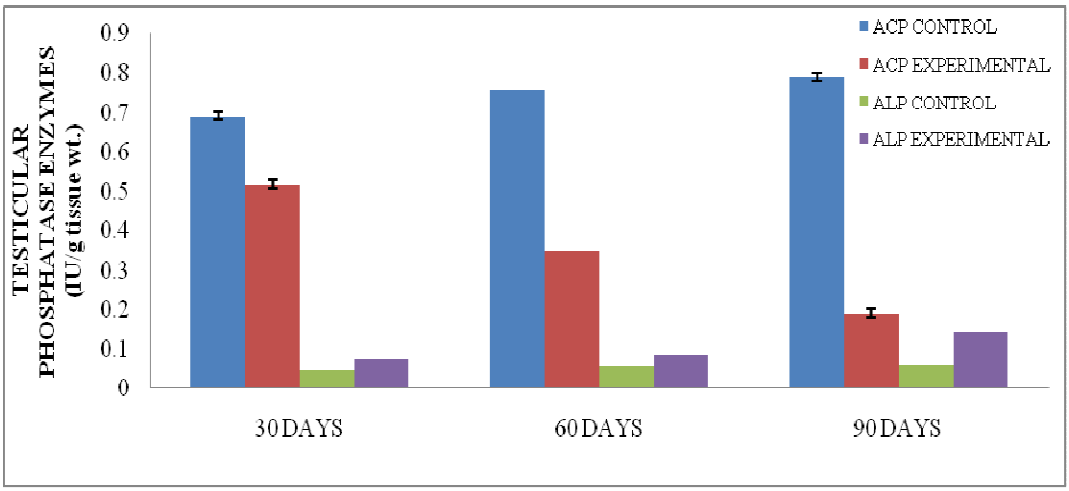
B) Hormone estimations:- Blood serum collected via cardiac puncture method was used to assay luteinizing hormone (LH)[22], follicle stimulating hormone (FSH)[23] and testosterone hormone[24].

C) Histopathology:- Bouin's fixed testes were used to prepare 5-7 µ (micron) sections, H&E stained[25]. Photomicrography was done at 100X and 400X magnifications with the help of a photomicrography unit (Motic-DMB1-B microscope fitted with digital camera).

D) Statistical analyses:- Two-way ANOVA analysis[26] was performed to compare and assess any/at all differences at $p<0.05$ to $p<0.001$ between control and experimental groups.

RESULTS

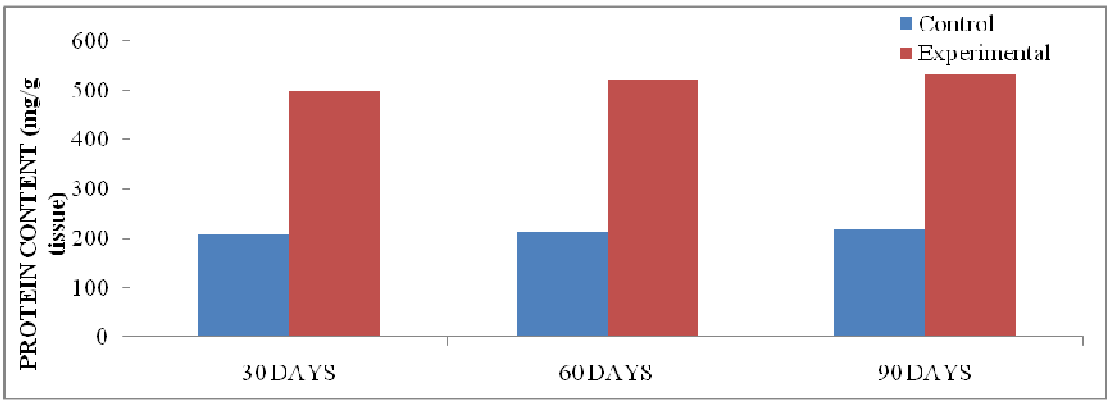
1. Biochemical analyses



Hist.1

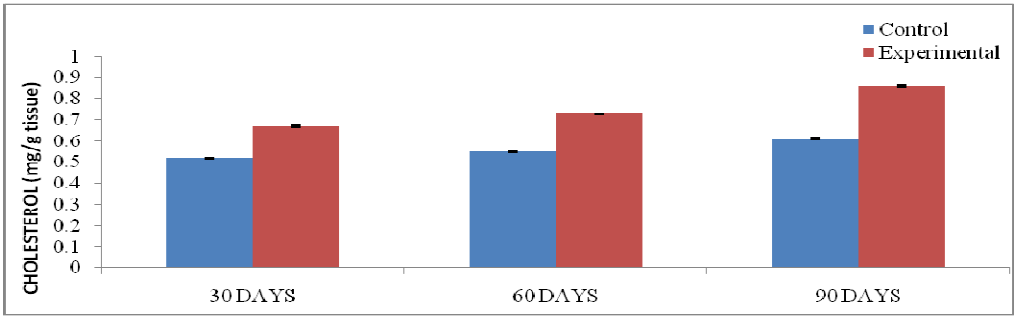
Comparison of testicular phosphatase enzymes (ACP & ALP) between Control and Genistein treated Mus musculus after different intervals. Values are expressed in Mean ± SEM. Significance of difference: p<0.05 to 0.001-Two way ANOVA.

There was significant decline in testicular ACP enzyme level and a sequestered increase in testicular ALP enzyme level along the three treated groups. In both cases the alterations were more prominent at the later part of the experiments *i.e.* after 60 and 90 days of treatment in *Mus musculus*.



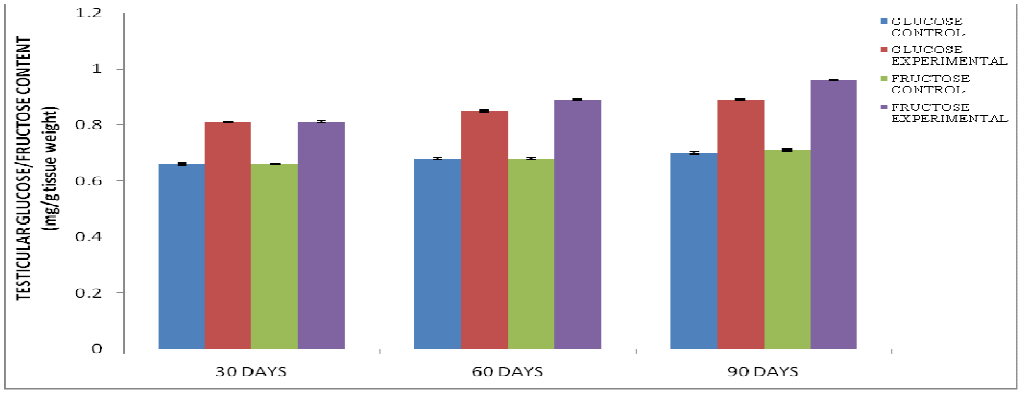
Hist. 2

Comparison of Testicular Protein content (mg/g tissue) of Control and Genistein treated Mus musculus. Values are expressed in Mean ± SEM. Significance of difference: p<0.05 to 0.001-Two way ANOVA. There was significant increase in protein content after 30 days of treatment and the increase was somewhat maintained along the treatment groups.



Hist. 3

Comparison of Testicular Cholesterol content (mg/g tissue) of Control and Genistein treated Mus musculus. Values are expressed in Mean± SEM. Significance of difference: p<0.05 to 0.001-Two way ANOVA. There was also a significant chronological increase in testicular cholesterol content in the treated groups.



Hist. 4

Comparison of Testicular Glucose and Fructose content (mg/g tissue) of Control and Genistein treated Mus musculus. Values are expressed in Mean ± SEM. Significance of difference: p<0.05 to 0.001-Two way ANOVA.

There was significant increase in testicular Glucose as well as Fructose content in a chronological manner along the three treated groups. In both the cases there had been considerable increase in the contents after 30 days of treatment as well as after 60 and 90 days of treatment also.

2. Hormone estimations

PARAMETERS	BATCHES	DURATION		
		30 DAYS	60 DAYS	90 DAYS
LH(mIU/ml)	CONTROL	3.18±0.006	3.22±0.004	3.28±0.004
	EXPERIMENTAL	2.18±0.002***	1.86±0.016***	1.57±0.012***
FSH(mIU/ml)	CONTROL	4.55±0.016	4.69±0.004	4.98±0.031
	EXPERIMENTAL	3.54±0.021***	2.98±0..013***	2.56±0.016***
TESTOSTERONE (ng/ml)	CONTROL	6.56±0.027	6.69±0.023	6.84±0.029
	EXPERIMENTAL	5.47±0.015***	4.87±0.021***	3.87±0.015***

Table 1

Comparison of circulating Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Testosterone hormone level, within Control and Genistein treated male mice, Mus musculus, after 30, 60 and 90 days. Values are expressed in Mean± SEM. Significance of difference: p<0.05 to 0.001-Two way ANOVA (= p<0.001).***

LH, FSH and testosterone hormone levels chronologically declined after treatment with Genistein with more prominent changes were after 90 days treatment.

3. Histopathological studies

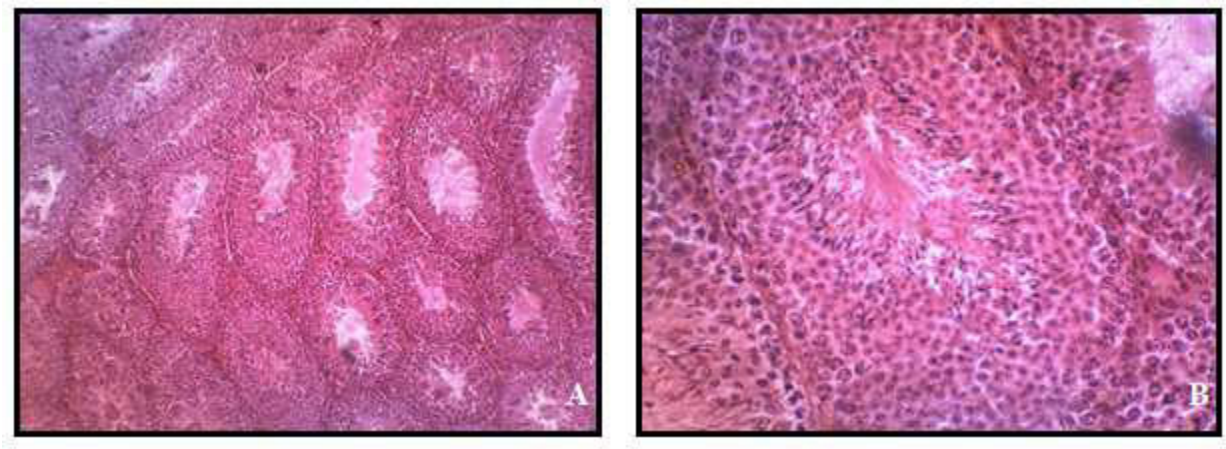


Figure 1

Transverse Section (TS) (H & E) of testes of Control Mus musculus, showing normal testicular histo-architecture, well defined seminiferous tubules having proper lamina propria and Sertoli cells along with different stages of spermatogenesis. Lumen filled with spermatozoa and well defined interstitial cells of Leydig are visible [A=100X and B=400X].

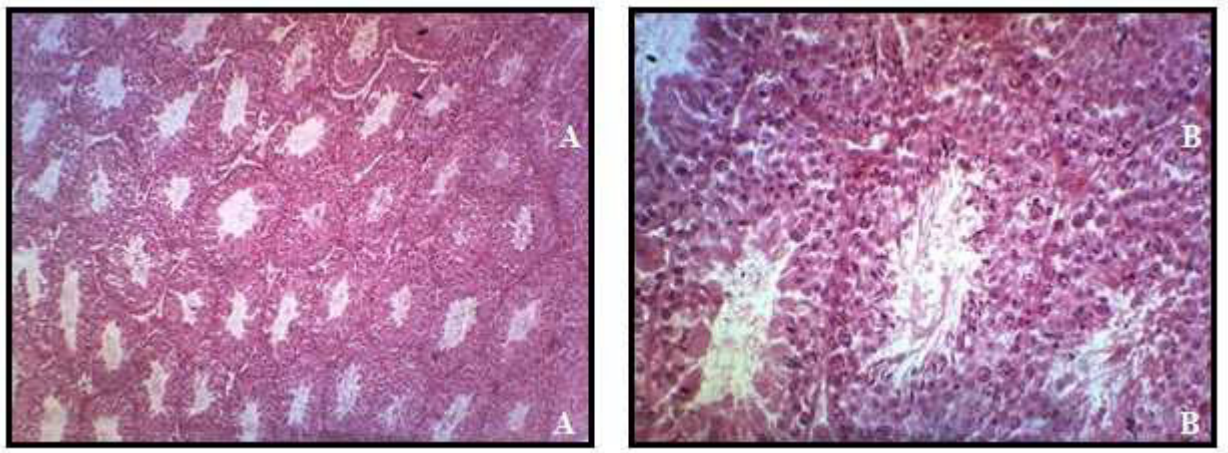


Figure 2

TS (H & E) of testes of 30 days Genistein treated mice, showing somewhat altered testicular histo-architecture, with atrophied seminiferous tubules and hypertrophied Sertoli cells along with different stages of spermatogenesis. Lumina are almost devoid of spermatozoa and diminished numbers of interstitial cells of Leydig are visible [A=100X and B=400X].

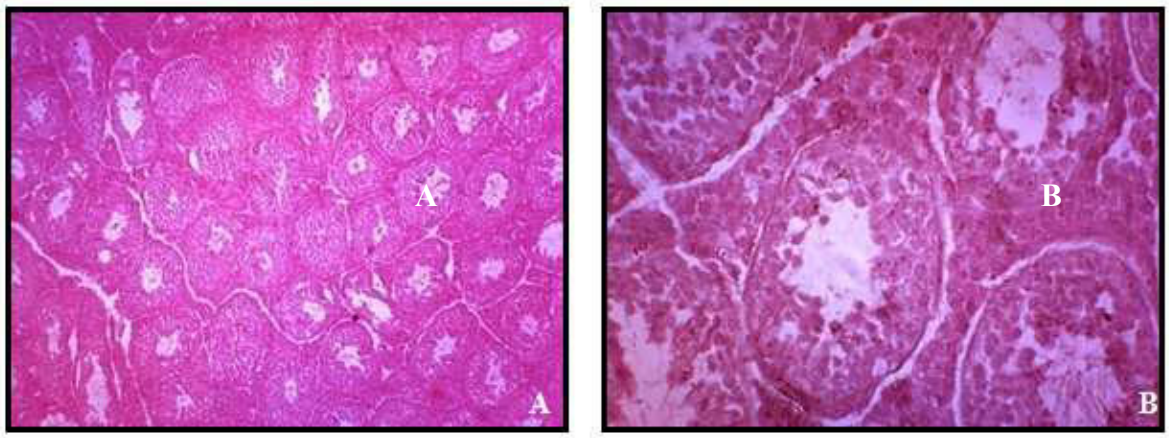


Figure 3

TS (H & E) of 60 days Genistein treated testes showing abnormal testicular histo-architecture, showing somewhat altered testicular histo-architecture with hypertrophied seminiferous tubules Sertoli cells. Lumina are almost empty and distorted interstitial cells of Leydig are visible [A=100X and B=400X].

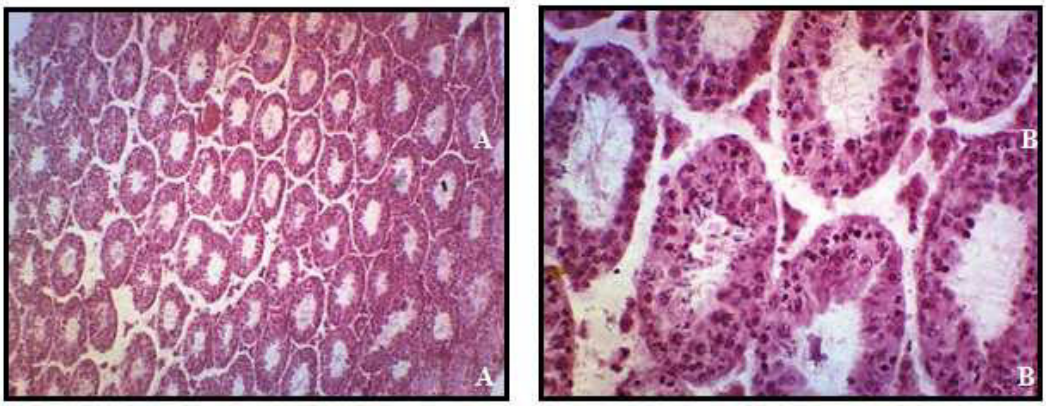


Figure 4

TS (H & E) of testes after 90 days Genistein treatment in Mus musculus, showing abnormal testicular histo-architecture with distorted seminiferous tubules, improper lamina propria and Sertoli cells. Lumina are almost devoid of spermatozoa. Interstitial cells of Leydig are diminished in size of Leydig [A=100X and B=400X].

DISCUSSION

Genistein is a compound with estrogen-like activities[27] and is also categorized as the second most abundant phytoestrogen to be derived from soy-based products, after Diadzein [28]. After oral ingestion, it leads to the highest blood concentrations as most of the ingested genistein shows entero-hepatic circulation[29]. However, we slightly modified the administration method from that used in studies documenting the various after effects of oral administration of genistein via gavages or laboratory made artificial diets[30, 31]. Testicular ACP or ALP level relates with any kind of physiological impairment caused by any exogenous chemical. Alterations in these enzymes may inhibit the testicular function of *Mus musculus* which was due to histopathological changes in testes due to treatment. The degenerative activity in testicular spermatogenic cells, as evidenced from the histological studies of the testes may be considered as one inevitable cause behind the increase in the phosphatase activities in the testicular tissues. To the contrary there has been reduction in the ACP enzyme level (Hist. 1) in the treated groups. This contradiction is inexplicable under the light of the above facts, but is evident that exposure of genistein may have caused the alterations in the testicular enzymatic levels.

After 90 days of treatment there were more severe effects on reproduction in terms of gonadotropin and testosterone hormone production as well as certain biochemical parameters. The decreased concentrations of the gonadotropins (LH and FSH) (Table: 1) can be due to the negative feedback of testosterone mediated via the hypothalamo-hypophysial testicular axis or may be due to direct action of testosterone on hypothalamus[32]. Nevertheless, there had been significant decrease in the testosterone hormone concentration in a chronological manner along the treatment groups. A possible argument for such changes may be that, genistein being a potent estrogen mimic[33] may have increased the total androgenic concentrations in circulation of the experimental animals, which could have produced the negative feedback signal for the lowering of gonadotropins. Although, what rules behind such phenomena cannot be suggested unless proved by feedback control studies, but agonistic actions of the estrogen mimic genistein, can certainly be liable to. Lesser availability of gonadotropins leads to the stall in maturation of the spermatogenic and Interstitial cells, which, in

turn may lead to the lesser production of testosterone hormone, necessary for gamete production and maturation, thus, proceeding towards temporary recess in reproduction in terms of gamete production. In this study, the testicular total protein (Hist.:2), cholesterol (Hist.:3), glucose and fructose (Hist.:4) levels showed chronological increase along the three treatment groups. These results are in accord with the phenomena of lowering sperm maturity. Increase in protein in the testicular tissues confers that there had been lowering in the rate of gamete maturation, otherwise the protein content would never have been increased instead of being utilized in the spermatogenesis process.

Glucose requirement for successful fertilization by epididymal mouse sperm[34, 35, 36, 37, & 38] during a short period of time at the end of capacitation and at later stages of fertilization is behind hyperactivated motility[35] and sperm-oocyte fusion[37]. Glucose is also required to support significant levels of hyperactivated motility, but could be substituted by fructose and both the sugars are equally important to promote progressive motility during fertilization[39]. Significant increase in the glucose and fructose content (Hist.:4) can therefore be due to decrease in the production and maturation of spermatozoa. Both biomolecules were not utilized for sperm motility, capacitation or fertilization, *i.e.*, the reproduction process proper. Total, LDL- and VLDL-cholesterol levels significantly increases in rabbits when treated with 50% Soy protein extract containing feed[40]. Isoflavones (genistein and diadzein) can reduce the plasma concentrations of total and LDL without affecting concentrations of triglycerides or HDL in mildly hypercholesterolemic humans consuming a specific formulated diet[41]. Significant reduction in LDL (about 6.5%) concentration in the high isoflavone diet (132mg/d) and an almost significant decrease in the low isoflavone diet (65 mg/d) compared with the control group in normo and mildly hypercholesterolemic postmenopausal women[42] have been reported. It has been showed that higher doses of subcutaneously injected soy isoflavones genistein and diadzein to decrease serum cholesterol levels in male middle-aged rats and to bring about an unfavorable increase of serum triglycerides[43]. High levels of testicular cholesterol content (Hist. 3) validate reduced steroidogenesis along with decreased spermatogenesis.

This study has also reported that there had been considerable degenerations in the testes tissues. Severities have also shown to increase in a sequential manner (Fig.:2-4) in the genistein treated groups. Sections showed hypertrophied Leydig cells, Sertoli cells, degenerated lamina propria and less number of mature spermatogenic cells compared to control (Fig.:1). The studies of Akingbemi[30] et al. provides evidence that exposure to phytoestrogens in the perinatal period affects Leydig cell function in adult rats, causing a decrease in testicular T secretion at a low dose and *vice versa*. Androgen insufficiency during the period of reproductive tract development is thought to be associated with congenital malformations of the male urogenital tract, *e.g*. hypospadias and cryptorchidism, collectively described as testicular dysgenesis syndrome[44] Wisniewski[7] and others observed persistent de-masculinization of the male reproductive system associated with decreased testosterone concentrations due to impaired testosterone biosynthesis by the testes, alterations in sensitivity to androgens at the level of the androgen receptor or changes in sex hormone binding globulin that would affect the bioavailability of testosterone. 40 Genistein acts as an anti-androgen in normal male subjects whereas if administered at a dose of 10mg/kg body weight (for 5 days) to castrated males it showed agonistic effects against circulating androgens[45]. They also confirmed that this effect of genistein was mediated via androgen receptors and the actions were tissue specific. The significant lowering of T concentration in circulation due to genistein administration, can validate that it must have happened due to the competitive binding of genistein to the androgen receptors in the adult subjects and have caused the negative feedback inhibition of androgen synthesis in them to result in loss of reproductive ability.

CONCLUSION

Genistein administration in adult male mice has been shown to depict altered reproductive outcomes such as decreased male reproductive hormones and spermatozoa quantity having a direct correlation with reproductive fecundity. Very low amount of genistein when available in the body for longer durations, may pose temporary reproductive interruption in male mice. These synergistic effects that were revealed from the studies may also conclude that long term exposure may lead to temporary/permanent reproductive stall, as evidenced from hormone estimations, biochemical analysis and histo-morphological investigations.

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