Dietary soybean sauce impact testicular tissue of rats in dose and duration dependent

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ABSTRACT

Soy sauce has been traditionally used as both a condiment and health food with antioxidant, anti-mutagenic, and antitumor activities. The present work aims to study the effect of soya sauce supplementation on the testis of male rats. Twenty-four male albino rats were divided into 3 equal groups. Group I; control males, group II; received low doses for consecutive 30 days, and group III; received a high dose of soy sauce for consecutive 15 days then recovered for another 15 days. Soy sauce in low doses decreases the amount of total white blood cells, red blood cells, hemoglobin, lymphocytes, and platelets levels than high doses while increasing neutrophils, and monocytes in high doses of soya. Histopathological examination showed that the testis of the low dose group had marked irregularity of seminiferous tubules periphery and degeneration of Leydig cells. Spermatogenesis arrest at the spermatid stage and degenerated spermatozoa were noticed in the center of tubules. Also, a significant increase in collagen fiber content of the testis was found in comparison with controls. However, rats treated with high doses showed restoration of the
normal testis architecture. Furthermore, free testosterone hormones, lipid peroxidation, and nitric oxide level were decreased in the low-dose group than in the high-dose group. In contrast, Glutathione level was increased in rats supplemented with a high-dose than a low-dose of soya sauce. Supplementation of rats with Soya sauce is impact testicular tissue in dose and duration time-dependent.

**INTRODUCTION**

Soy sauce is commonly used throughout East Asia to enhance the flavor of a wide range of prepared dishes and aid in digestion. Bacteria called *Aspergillus oryzae* are used to ferment the paste of roasted soybeans and brine [1]. Soybean is one of the main sources of bioactive phytoestrogenic compounds (plant estrogens) called isoflavones [2,3]. The major isoflavones in soybean are genistein, daidzein, and glycitein. Isoflavones mimic the structure and/or function of the mammalian steroidal estrogens [4]. Their structural and functional similarity to estrogens allows the isoflavones to elicit estrogenic or anti-estrogenic effects and affect a number of the estrogen-regulated systems including the reproductive system [5,6]. Soy sauce and isoflavones improved the antioxidants properties and decreased the LPO [7–9]. Isoflavones were reported to have no significant changes in hematological parameters [7,10]. However, others reported a significant increase in lymphocytes, a slight increase in RBCs [11]. Genistein showed no significant change in body weight [12,13], but others showed weight loss in numerous studies on animals and humans [14]. Soy dietary intake may have a profound physiological impact on the growth and function of male reproductive tissues [15]. Seminiferous tubule lumen and testis diameter were significantly larger in the high phytoestrogen-fed adult male rats [16], but in other studies, the tubular diameter and interstitial spaces were reduced in size [17]. Soya caused a presence of cellular debris in the seminiferous tubules, sloughing of the germ cells and absence of maturing spermatids in rodents [18–21], and hyperplasia of Leydig cells [22,23]. Genistein and soy in vivo study decreased sperm counts in testes [17,22,24]. However, isoflavones had no effects on histology and sperm numbers or morphology in the postpubertal [25]. Soy isoflavones (SIF) promote spermatogenesis in diet-induced obese male rats [26] and decreased serum-free testosterone levels in rodents.
Phytoestrogens are also caused hormonal imbalances in men [29-31]. However, soy raised serum testosterone levels [25,33]. Others reported that consuming soy protein or isoflavones had no impact on men's serum levels of total and free testosterone in men [29,32,33] and rats [16,34]. So, due to contradictory results obtained in the previous work, the primary goal of this study was to evaluate the effect of soybean sauce supplementation in a low for 4 weeks and the height dose for 2 weeks then on serological, oxidative stress, and histopathological changes in the testis of rats.

**MATERIALS AND METHODS**

2.1. Animal care conditions

Twenty-four mature male Wistar albino rats, with a body weight (170±20g), were purchased from Assuit University’s animal house, in Egypt. The rats were transferred to the animal house, Zoology Department, Faculty of Science, Assiut University where the experiment proceeded. They were kept in cages under controlled conditions of temperature (28±3°C), humidity (55-60%), and normal photoperiod cycle. The animals were acclimated to the laboratory conditions for two weeks. The human care of animals was according to regulations set by the National Institutes of Health guidelines.

2.2. Experimental design

Rats were categorized randomly into three groups (8 rats each). Group I was the control males; Groups II was received 1.3 mg/kg.b.w (orally by gavage) soybean sauce (local market, Assuit, Egypt), for 30 consecutive days. Groups III was received orally 2.6 mg/kg.b.w (orally) soy sauce, for consecutive 15 days then recovery for another 15 days (36,37). Every day the total body weight for every rat in previous groups were measured [35-37]. On the 31\textsuperscript{st} day of the experimental onset, all the animals were sacrificed.

2.3. Complete Blood Count (CBC)

The evaluation of CBC was done by Automated Hematology Analyzer (Diff3) Mek-6410/Mek-6420 in the veterinary Exigo Hematology Analyzer at the Clinical Pathology
Laboratory in the Pathology Department, Faculty of Veterinary Medicine, Assiut University.

2.4. Histological preparations

The testes were taken out and washed in saline solution. One testis from each rat was fixed in neutral buffered formalin (10%) formalin pH 7.2, dried, cleaned in xylene, and embedded in paraffin for histological and histopathological investigations. Sections were cut at 5 μm and stained with hematoxylin and eosin, Picrosirius Red Staining Protocol [38]. Photographs of the stained sections were captured by an industrial digital camera (LCMOS05100KPA, China) at Zoology Department Central Lab, Faculty of Science, Assuit University. Image J software (version 1.8) was used for morphometric analysis to measure the collagenous fiber in the testis, Fibrosis percentage % quantification is as follows: =Total positive area / Total section area X 100 [39,40].

2.5 Preparation of testis homogenate

For preparing 10% w/v homogenate of testis; 500 mg of each testis was homogenized using a homogenizer (IKA Yellow line DI 18 Disperser, Germany), in 5 ml (0.1 M) phosphate buffer (pH 7.4). The homogenates were centrifuged at 5000 rpm for 30 min at 4 °C and the supernatant cytosols were kept frozen at -20 °C for the subsequent biochemical assays.

2.6. Biochemical measurement

Hormonal assay:

Testosterone (free and total) in serum samples was assayed by enzyme-linked immunosorbent assay (ELISA) according to the method of [41] for free testosterone and [42] for total testosterone.
Estimation of some oxidative stress markers:

Malondialdehyde as thiobarbituric acid was used to measure the lipid peroxidation (LPO) in the testis, as described by [43]. To prevent additional oxidation, 1 % v/v DMSO was added after homogenization. The reaction buffer was added to 0.2 ml aliquots of tissue homogenates and subjected to spectrophotometric measurement. Nitric oxide was calculated as the concentration of nitrite in the tissue cytosols using the method of [44].

Estimation reduced glutathione

The concentration of GSH was estimated as described by [45]. Aliquots of 50 μl of tissue homogenate were added to 14.5 mg EDTA at 4000 rpm for 10 minutes. A mixture of 1 ml PBS at pH 8 and 100 μl DTNB to 100 μl was added to the supernatant, which was kept at room temperature for 5 minutes, before being used for the estimation of reduced glutathione at 412 nm.

2.7. Statistical Analysis:

One-way analysis of variance (ANOVA) was used, followed by the student Newman-Keuls T test, using the software Graph Pad Prism 3 (Graph Pad Software Inc., USA). The results are reported as mean ± SE. Statistical significance was accepted at p < 0.05.

RESULTS

3.1. Bodyweight

Supplementation of rats with low and high dose soybean sauce resulted in decreased in body weight by 7.92% and 5.3%, respectively, versus those of control. While rats supplemented with high dose and then left for recovery showed an increased (2.94%) in body weight versus those of the low dose group (Fig. 1).
Fig. 1. The effects of low and high dose of soybean supplementation on body weight. Results presented as mean ± SEM. Values with similar superscript signs are non-significantly different at P > 0.05.

3.2. Complete Blood Count (CBC)

Supplementation of rats with a low dose of sauce caused a significant decrease in platelets and total WBCs counts (P < 0.05), but does not affect RBCs, hematocrit, HB, neutrophils, eosinophils, monocytes, lymphocytes, MCV, MCH and MCHC levels compared to the control rats. However, supplementation of rats with high doses caused non-significants in all the CBC parameters (Table 1).
Table 1. Complete blood count (CBC) values, expressed as mean ± SE, and the % of changes between the treated and control group.
3.3. Histopathological Examination:

Observation of the testis of the control rats showed the normal histological structure of the seminiferous tubules and normal spermatogenesis Fig. 2 (A&B). The supplementation of rats with a low dose of sauce caused marked irregularity of the seminiferous tubule periphery and atrophy in seminiferous tubules with edema. There was degeneration and detachment of germ cells from basement membrane of seminiferous tubules, loss of regular distribution of spermatogenic cells, primary spermatogonia are vacuolated, spermatogenesis arrest at spermatid stage, degenerated spermatozoa in the center of tubules, widening of the intertubular spaces with decreased number and degeneration of Leydig cells when compared with control Fig. 2 (C&D). Supplementation of rats with a high dose of sauce with recovery caused high restoration of the normal architecture of the testis and the progressing spermatogenesis with its regular stages appeared. Another finding in this group was the remarkable abundance of interstitial cells between the seminiferous tubules. Also, mitotic figures were seen in the germ cells which form a continuous layer Fig. 2 (E&F).

Collagen staining of picrosirius red of testis sections from control showed normal content and distribution of collagen fibers, which take the red color, the seminiferous tubules lined with a definite layer of connective tissues fibers Fig2. (G). Testis of rats supplemented with a low dose of sauce revealed a mild increase in the collagen fibers content and distribution than control and high dose with the recovery of sauce groups Fig. 2 (H). Also a high dose with the recovery of sauce group showed a slight increase of connective tissue fibers (collagenous) through the testicular tissue vs. the control group Fig. 2 (I).
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Fig. 2. Photomicrograph of H&E and Picrosirius Red Staining of rat testis sections of the different groups photographed at (HE, Bar =100 um & Bar = 50 um / PSR, Bar = 200 um). (A&B) control group, showing seminiferous tubules (ST) with regular thickness germ cell layer (double head arrows). Interstitial cells of normal population (white arrow). (C&D), low dose group showing ST larger than control with disorganization of germ cell layer. Germ cell layer is less in thickness (double heads arrow). Mature sperms are absent from most tubules (Black star). Interstitial cells looked decreased compared to control. (E&F), of high dose group showing nearly normal ST with full thickness germ cell layers (double head arrow) and mature sperms in the lumen (star). Interstitial cells of normal population (white arrow). (G), control showing normal collagenous fibers in the basement membrane of seminiferous tubules (black arrows). (H) low dose group showing
collagen was increased (black arrows). (I) high dose group showed slight increase in collagen deposition (black arrows).

3.4. Morphometric analysis

**Tubule perimeter:** Quantitative results by image analysis of H&E-stained sections of testes showed that the tubule perimeter in the low-dose supplemented group with sauce significantly increased by 18.1%, however, in the high-dose group non-significantly increased by 1.4% vs. control. Statistically, it was a significant decrease (14.2%) in the tubules perimeter of the high-dose group versus these of the low-dose group ($P < 0.01$) (Fig. 3A).

**Leydig cells:** The number of Leydig cells in the low-dose group non-significantly decreased by 14.5% and the high-dose group non-significantly increased 9.7%, respectively vs. the control group (C). Also, in high-dose group revealed non-significant increase of 28.3% vs. L dose group (Fig. 3B).

**Tubules contained mature sperms:** The number of tubules containing mature sperms in the low-dose group significantly decreased by 26.8% vs. the control group, ($P < 0.05$). While this number was in the high dose group, non-significantly increased by 7.3% vs. the control group (C). When comparing this number in both high dose group and low dose group, it showed a significant increase of 46.7%, ($P < 0.05$) (Fig. 3C).

**Collagen fibers content:** Quantitative results by image analysis of collagen-stained sections of testes showed that collagen content in the testis of low dose group showed a significant increase by 34.46% vs. control. Statistically, the collagen fiber content in the high-dose group revealed a non-significant increase 12.7% vs. the control group. At the same time, high dose group was decreased by 16.2% vs. the low dose group (Fig. 3D).
Figs. 3 (A-D). Effect of low and high dose supplementation with sauce on seminiferous tubule perimeter, Leydig cells number, number of seminiferous tubules contained mature sperms, and the % of testis fibrosis. The data are expressed as Mean ± S.E.M. Means with different superscripts are significantly different.

3.5. Analysis of hormones:

The level of serum-free testosterone was significantly decreased (21.65%) for the rats group that received a low dose of sauce (P < 0.05), and non-significantly decreased (4.62%) for the high dose group compared with the control group. While free testosterone level in high dose group was significantly increased (21.82%) compared with low dose
group (P < 0.05) Fig. 4A. Serum levels of total testosterone non-significantly increased (7.55% and 3.7%) in rats who received a low and high dose of sauce, respectively, compared with the control group. While in high dose group, total testosterone non-significantly decreased (3.57%) compared with low dose group (Fig. 4B).

Figs. 4. Effect of low and high dose supplementation with sauce on free and total testosterone in blood serum of male rats. The data are expressed as Mean ± S.E.M. Means with different superscripts are significantly different at P < 0.05.

3.6. Oxidative stress biomarkers:

Lipid peroxidation (LPO) in a low dose of sauce supplemented group significantly declined (46.4%), while in rats supplemented with high dose group; it was non-significantly decline (19.9%) vs. control group. Also, there was a significant increase (49.4%) in high dose (P < 0.05), when compared with the low dose group (Fig. 5A). Nitric oxide as nitrite levels significantly decreased (45.1%) (P < 0.001), while the
Dietary soybean sauce impact testicular tissue of rats in dose decrement of NO non-significantly (15%) in the high dose group vs. control. Also, there is a significant increase (54.8%) in high dose group vs. low dose group (P < 0.01) (Fig. 5B). Glutathione content significantly was increased (48.78% and 30.5%) in low and high-dose supplemented groups vs. control (P < 0.01 and P < 0.05), respectively. Also, the changes non-significantly decreased in high dose group (12.28%) vs. low dose group (Fig. 5C).

![Fig. 5. Effect of L and H dose of SBS (1.3 and 2.6 mg/kg B.W.) in testicular activities of LPO, NO, and GSH level in male albino rats. The data are expressed as Mean ± S.E.M. Means with different superscripts are significantly different.](image)

**DISCUSSION**

Despite the various stated pharmacological benefits soy sauce has been linked to high blood pressure and an increased risk of cardiovascular illnesses and stroke when ingested at levels beyond the daily recommended dose (46). In the present experimental groups, no significant change in body weight occurred.
Akhlاغhi et al. (2017) confirmed numerous animal and human studies have shown the beneficial effect of soy on weight reduction (12). The anti-obesity effect of soy is partly attributed to its high protein content. High-protein diets have been effective in ad libitum food consumption in weight maintenance and reduction (13). Changes in leucocytes composition of low dose group may be due to the proliferation of some leukocytes that are indicative of immune response to foreign antigens. In this aspect, RBC, WBC, and HB decreased after xenobiotic oral administration that induce oxidative stress and the interaction between the xenobiotic and erythrocytes membrane proteins (47,48).

The present histological study of the testis showed, supplementation of rats with a low dose of sauce for 30 days caused an increase in testis perimeter. Also, it decreased in tubules containing mature sperms, a mild decrease in Leydig cells number, and degeneration in seminiferous tubules. These findings supported previous observations on rodents (16,18–22). It is well established that testosterone is an important for germ cells and spermatid development. The decrease of this hormone increase germ cells and spermatids apoptosis and thereby sperms apoptosis (49–51), which attributed to the disruption of the hypogonadal–pituitary–testicular axis (18). Also, it decreased tubules containing mature sperm may be associated with the anti-estrogenic effect of soy isoflavones. Earlier studies revealed that estrogen is responsible for the proliferation and differentiation of Leydig cells, therefore the decrease in the number of Leydig cells may be due to the anti-estrogenic and or weak estrogenic effect of isoflavones (18,20,52). Moreover, increased testis perimeter is due to the anti-estrogenic properties isoflavones due to estrogen receptors involved in the regulation of fluid reabsorption in efferent ductless (53,54).

Consequently, any disruption in estrogen action, by the administration of an anti-estrogen to adult rats, reduced fluid absorption in the ex-current duct system, thus increasing water retention and its accumulation in the lumen and the flattening of epithelial cells (18,55). Also, the present work observed, no toxic effect of sauce oral administration with a high dose for 15 days and then left for recovery but the result was near to control. a high restoration of the
normal architecture of the testis and the progressing spermatogenesis with its regular stages appeared. Our morphometric analysis of collagen had a significant increase in testis fibrosis in both two treated groups, and this support an earlier study that reported that soy increased collagen type I (56,57). The antioxidant properties of soya sauce polyphenols may be reflected in the current histopathological data. Phenols, a compound found in soybeans, help to significantly offset the negative effects of xenobiotic that cause the generation of free radicals in the liver (58).

In male rats who received a low dose of sauce, a reduction in serum-free testosterone levels occurred which matched with earlier studies on rodents (17, 59–66). While rats received a high dose of sauce showed, a non-remarkable difference in serum testosterone level occurred. Total testosterone had not to be affected in all experimental groups, and this finding agrees with the study (34). Reduction in serum testosterone level related to estrogenic activity of isoflavones and an inhibition of the steroidogenic enzymes (27). Our results supported that isoflavones suppressed steroidogenic capacity in the Leydig cells as suggested previously (28). Isoflavones may also interfere with the metabolism of steroid hormones, thus inhibited the enzyme activity involved in the steroidogenic pathway (67). Isoflavones were reported to elevate sex hormone-binding globulin production in the liver which binds to biologically active testosterone and thus declines the levels of free testosterone and its bioavailability to the target cells, consequently elevating total testosterone (29,68).

In the present study, soy sauce supplementation reduced LPO, NO and increased GSH in rats which confirmed previous studies (7–9). Isoflavones may repress free radical generation by enhancing endogenous antioxidants, such as superoxide dismutase and GSH (69). These effects are associated with hormonal and physiological activity (70). Isoflavones and genistein were reported to reduce concentrations of free radicals and activate antioxidant enzymes in various organs (48,71). The antioxidant potencies of isoflavones are structurally associated with the presence of hydroxyl groups at positions 4’ and 5’ and the
position of the aromatic ring (13,72, 73). Soybeans compounds, such as isoflavones, play a significant role in neutralizing the harmful effects of xenobiotics in the liver (74) due to physiological changes induced by multiple constituents (75) and enhancement of antioxidants isoflavones (76).

**CONCLUSION**

The present histopathological and biochemical observations in rats supplemented with a high dose of soya sauce; may reflect the estrogenic properties of soy isoflavones caused by hormonal imbalances.

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