Research Article

Effect of isoflavone (SOY) on histopathology of reproductive organs of rabbits (*Oryctolagus cuniculus*)

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Abstract

The main source of isoflavones is soy foods. Soy isoflavones have been extensively studied for its antiestrogenic and estrogenic effects. These exogenous chemicals have less potent effect but there mechanism of action is same as endogenous estrogen. They have adverse effects on histology of testis and ovaries. In present study we evaluate the effect of soy isoflavone on histopathological changes in testis and ovaries. Selected 84 adult rabbits were divided into 8 groups. The dose groups were (2.5 mg/kg per body weight= A group), (3.5 mg/kg per body weight= B group), (5 mg/kg per body weight = C group) and control (D) group separately for male and female. The results showed that soy isoflavone have negative effect on reproductive organs and more pronounced effect on male than female. Many histological problems were observed in testis and ovaries. This study shows that soy foods are not good for reproductive health, and use of soy foods in high amount for prolonged time should be prohibited.

Keywords: Histopathological changes; Soy isoflavone; Rabbits; Reproductive hormones; Reproduction

Introduction

Isoflavons are natural plant substances which belong to class phytoestrogens [1]. Phytoestrogens are diphenolic yet non-steroidal compounds that have structural similarity with mammalian estrogen 17- β estradiol and they have the ability to bind with estrogen receptors (α & β) [2]. Phytoestrogens consists on more than 100 molecules depending upon their chemical structures into (1) Isoflavones (daidzein, genistein, formononetin, biochanin A), (2) Stilbens (resveratrol), (3) Lignans (secoisolariciresinol- diglucoside, matairesinol), (4) Coumestans (4-methoxycoumestrol, coumestrol). Isoflavones mainly present in roots and seeds of 300 different types of plants e.g.

extracts of red wine, linseed, red clover, alfalfa. Soybeans (*Glycine max* L.) are the richest source of isoflavones, and 1g dry soybean contains 1.2-4.2mg of isoflavones. Isoflavones concentration in soybeans depends upon processing, soil, climate and age of maturity [3]. In nature isoflavones present in conjugated form which is biologically inactive form of isoflavones. After ingestion conjugated form of isoflavones change into biologically active form (genistein, daidzein and aglycon) due to hydrolyzation by β -glucosidases of intestinal bacteria [4]. These phytochemicals have many health related benefits for prevention of cardiovascular diseases, age related (osteoporosis) and hormone dependent conditions (breast and

prostate cancer). Phytoestrogens have estrogenic as well as anti-estrogenic effects and when they prevent breast cancer it demonstrate its anti-estrogenic effect [5]. In recent years the physiological effects of soy isoflavones extensively studied but public health recommendations of soy isoflavones remain controversial because they have adverse effects on reproduction [6]. Some plants produce phytoestrogen substances which act as endocrine disrupters in insects, mammals, birds and fishes. It was considered that these substances evolved as a defense against herbivores. These substances vary in potency depending upon the environmental conditions, age and species of plant. In Japanese quail testicular weight decreases due to exposure with phytoestrogens. The birds that are exposed with phytoestrogens suffered from infertility decreased reproductive success and differential breeding response. Phytoestrogens has more pronounced effects on male as compared to female reproductive system [7]. In critical developmental stage of rodents, a high dose of phytoestrogens cause temporary infertility, decline sperm quality, altered gonadotropin level and reproductive tract disorders [8]. Earlier studies reported that soy isoflavones have harmful effects on thyroid gland functions. It was reported that Soy isoflavones increase the level of thyroxine (T4) and triiodothyronine (T3) which is the cause of goiter in rodents [9]. The soy isoflavones have beneficial effects on plasma lipoprotein and lipid concentration by significantly reduction in low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in males and females and significantly increase of high density lipoprotein (HDL) in females and less in males [10]. Soy isoflavones have antioxidant properties and genistein is a good and active scavenger of glutathione peroxidase, catalase and superoxide dismutase because in C-5 position it has third hydroxyl group. Genistein has significant effect on protein tyrosine kinase

inhibition (PTK). In some brain regions (hippocampus), stomach, prostate and breast cancers PTK are highly expressed. A high dose of isoflavones inhibit PTK and prevent cancer in these tissues. Moreover inhibition of PTK also improves cardiovascular health. Due to the estrogen like properties genistein suppress the arthritis, autoimmune diseases, delayed of type II hypersensitivity and antigen induced asthma [11]. In premenopausal women Soy proteins regulate the menstrual cycle; it delayed the menstruation and increases the length of follicular phase [12]. Isoflavones improve bone health by regulating the B-lymphopoiesis and it also reduces bone loss in estrogen deficient females [13]. Due to soy isoflavone the total number of sperm cells and their mobility decreased very significantly. The sperm cells morphology of soymilk treated groups become abnormal and decreased number of leydig and sertoli cells [14]. The lumen of uterine become highly branched and increased the thickness of uterine wall due to glandular epithelium hyperplasia, increase blood supply and connective tissue edema as well as formed new blood vessels [15]. Soy isoflavone decreased in number of corpus luteum and increased atretic follicular count [16].

The main objectives of this research is to determine the effects of Soy isoflavone on histopathological changes in testis and ovaries of rabbit.

Materials and methods

Chemical

Soybeans were obtained from grain market, Railway road, Gujrat, Pakistan.

Selection and handling of rabbits

This study was conducted on healthy rabbits which were used as experimental animals weighing about 1kg-1.5kg. Rabbits were allowed to acclimatize the environment for one week before starting the experiment. In this research equal ratio of both male and female rabbits were obtained from Veterinary Research Institute Ghazi Road Lahore. Rabbits were kept in a large room and each group was

separated by a brick wall. The waste was removed every day to prevent infection and stress. Rabbits had *ad libitum* access to food and water. Different types of animal colors were put on both sexes for identification in case of any mishap. On the floor of each portion 1 foot soil was spread for healthy environment.

Groups and dosage

Selected 84 adult rabbits were divided into 8 groups. Three treatment (A,B,C) and one control group (D) for female, similarly three treatment (A,B,C) and one control group (D) for male. All dose groups of both sexes have three replicates in each group e.g. (A= A1, A2, A3), (B= B1, B2, B3) and (C= C1, C2, C3). All treatment (A,B,C) groups of male and female containing 12 rabbits in each group while control (D) groups of both sexes have 6 rabbits in each group (Table 1). Group name was written on the paper and stick on the wall of each group. Soybeans crushed in powder form and all doses were mixed in soaked bread so that soybean powder stick to bread pieces. Rabbits were given fresh bread, vegetables, Barseem hay (*Trifolium alexandrinum*) and water.

Table 1. Treatment groups

Female	Male
Group F(A) Low: 2.5mg/kg B.W (Low)	Group M(A): 2.5mg/kg B.W (Low)
Group F(B): 3.5mg/kg B.W (Medium)	Group M(B): 3.5mg/kg B.W (Medium)
Group F(C): 5mg/kg B.W (High)	Group M(C) 5mg/kg B.W (High)
Group F(D): Untreated (Control)	Group M(D): Untreated (Control)

Histopathology procedure

To study the histological alteration in ovaries and testis; tissue processing and microtomy was done in Zoology laboratory.

Dissection

After every 2 weeks pithing of 3 rabbits were occurred from each control group and dose groups of male and female. Rabbits

were dissected by following [17] method. After dissection testis and ovaries were removed and washed with water then soaked in 0.085% saline solution to remove dust, grim and impurities. Then these organs were cut into 3-4mm pieces with the help of sterile dissection box for further histopathological studies.

Fixation

Tissue fixation was the first step of histopathology. Tissues were kept in Bouin's fixative for 7-8 hours; it prevents the decomposition of tissues. Fixation of tissue thickened the cross links between proteins and made impossible to dissolve the protein.

Dehydration of tissue

This was the second step of histopathology. In this step increasing series of alcohol was used for dehydration of tissues. Tissue were kept in 70% alcohol for 1-2 hours and then in 95% alcohol for same time. After that tissues were kept in absolute alcohol for 1 hour.

Washing/Clearing

Pure xylene was used as a clearing reagent. Xylene has the compatibility for alcohol and paraffin wax. Tissues were kept in Xylene I for 3 hours and then kept in xylene II for same time [18].

Infiltration and tissue embedding

Paraffin wax was melted at 56° in wax dispenser. Then wax was poured in to glass tubes and tissues were kept in molten paraffin wax and placed in incubator for overnight.

Paper blocks were made with the help of wooden chuck. Blocks were filled with molten paraffin wax and tissue was placed in center of wax with the help of forceps. When paraffin wax was solidified in blocks then blocks were transferred to cool bowl of water and then placed in refrigerator for 12 hours.

Wax block trimming and mounting

Trimmed the excess paraffin wax from all sides of block with the help of sharp knife that only 5-6mm wax was left on each side of block. For mounting the block on wooden chuck trimmed wax block was

melted from one side with the help of flame on attach with chuck then chuck was placed in chuck holder of rotary microtome machine [19].

Turn on water bath

Water bath was turned on and set temperature at 37-39°C [20].

Sectioning

Tissue sectioning and slide preparation was done by microtome. knife was placed into knife holder; set proper cutting angle and securely tightened the knife. Wheel of microtome was in locked position. Then cut the sections carefully having diameter 5-7microns. Grasp the beginning of section with the help of camel hair brush and gently lift up from knife and slowly lay the tissue section on tissue floating bath. It removes the wrinkles in ribbon of tissue section.

Mounting the sections on glass slides

Use cleaned glass slides and labeled the slide with accession number as was on the cutting block. Mayer's albumen was applied on glass slide for adhesion of tissue section then inserts the glass slide in water bath perpendicularly and centrally orients the sections on slide and then carefully lifts the slide from water. Glass slides were dried at room temperature for 8-10 hours.

Staining

Deparaffinized the tissues

The first step of staining was dewaxing of tissues by kept the slides in pure xylene for 2-3 min.

Rehydration

The slides were passed through downward series of alcohol and then rehydrate by passing through alcohol. In each downward series of alcohol slides were kept for 5 min.

Hematoxylin and eosin staining

For hematoxylin staining slides were kept in hematoxylin stain for 2 min and after that washed with running water for 5 min. For discoloration of excessive stain passed it through 70% alcohol for 2 min and 90% alcohol for 3 min.

For eosin staining slides were kept in eosin stain for 2 min. For removing excess stain slides were passed thorough 90% alcohol

for 2 min and then absolute alcohol for 2 min.

Lubrication of tissues

For lubrication of tissues; slides were dipped in cedar wood oil for 1 min.

Clearing of tissues

For clearing the cedar wood oil, slides were dipped in Xylene for 2-3 sec.

Mounting the cover slips

Put the Canada balsam on tissues and then carefully placed the coverslips. Slides were allowed to dry before observing in the microscope.

Slides observation

Slides were kept in germ free environment and carefully observe the alterations in tissues of testis and ovaries thorough the light microscope attached with digital camera [18].

Results

The findings of the present study revealed that soy isoflavone cause multiple reproductive abnormalities in adult male and female rabbits. Male rabbits were more affected as compared to female rabbits. When soy isoflavone was gave to experimental rabbits it was observed that ovaries weight significantly increased in females and testis weight was significantly decreased in male rabbits. The FCR was also decreased in soy treated groups as compared to control groups. The histological alterations were also observed in ovaries and testis in this study. The reproductive hormones (Estrogen, progesterone & testosterone) in dose groups were also compared to control group. There was significant difference was observed in sex hormonal level of dose and control groups. According to [21] lower level of soy isoflavone have not adverse effect on reproductive parameters but high dose of soy isoflavone adversely affect the reproductive organs and sex hormonal level.

Histopathology results

Histological changes in testis and ovaries were confirmed by the comparison of soy treated and control groups at 15th, 30th and 45th days. Histological studies were showed

the tissue necrosis, sertoli cell damaged, hemorrhage, cysts and tumors in tissue of ovaries and testis (Figs. 1-7).

Figure 1. Shows comparison of effects of soy isoflavone on ovaries between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 15th day. There were no histological alterations seen in these groups

Figure 2. Shows comparison of effects of soy isoflavone on ovaries between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 30th day. The histological studies showed that there was tissue damaged in low dose group, haemorrhage in medium dose group and cysts and tissue necrosis in high dose groups at day 30th

Figure 3. Shows comparison of effects of soy isoflavone on ovaries between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 45th day. The histological studies showed that there was tissue disruption, cyst formation and tissue necrosis in low dose group (A), tissue damaged in medium dose group (B) and increased number of small follicles in high dose group (C) at day 45th

Figure 4. Shows comparison of effects of soy isoflavone on ovaries between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 60th day. The histological studies showed that there was tumor formation and epithelial thickness in low dose group (A), formed new blood vessels in medium dose group (B) and cysts formation in high dose group (C) at day 60th

Figure 5. Shows comparison of effects of soy isoflavone on testis between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 15th day. The histological studies showed that there was normal histology in low dose group (A), sertoli cells damage and tumor formation in medium dose group (B) sertoli cells damage and tumor formation was more formation in high dose group (C) at day 15th

Figure 6. Shows comparison of effects of soy isoflavone on testis between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 30th day. The histological studies showed that there was tissue necrosis, cysts formation and cellular coagulation in low dose group (A), there was tumor sign and necrosis in medium dose group (B) sertoli cell damage, cysts and tumor formation was more in high dose group (C) at day 30th

Figure 7. Shows comparison of effects of soy isoflavone on testis between dose group A (Low), B (medium), C (High) and control D group at 10X magnification power under light microscope at 45th day. The histological studies showed that there was tissue damage, in low dose group (A), there was sertoli cells damage medium dose group (B), and severe tissue and sertoli cells damage sertoli cell damage in high dose group (C) at day 45th

Discussion

In mammals the reproductive organs are very sensitive to exogenous chemicals. In recent years there was a decrease in quality and quantity of male sex cells. There are many types of exogenous compounds like pesticides, drugs, xenohormones, phytoestrogens etc. In current years large number of population exposed to phytoestrogen and soybeans are main source of phytoestrogens. In soybeans, isoflavones are the most studied component; but their effect on reproductive organs is not fully understood. Their effects on non rodent species is remain to be determined. In this study we used equal ratio of male and female rabbits to determine the effect of soy isoflavone on testicular and ovarian tissues. The results showed that the low dose concentration of soy isoflavone have not serious effects, while high dose concentraion have severe damaging effects on tissues of testis and ovaries. Our results were consistant with the findings of [14]. In this study 4 weeks

old 24 male Swiss mice were used to determine the effect of soymilk on male fertility. Duration of this experiment was 90 days, and animals were divided into four groups. Group A containing 6 mice from a nursing mother who fed soy and received milk for 90 days., Group B containing 6 mice from a nursing mother who fed soy and receiving plugs and water for 90 days., Group C containing 6 mice from a female witness and who received soybeans for 90 days, Group D containing 6 mice received plugs and water during the entire experimental period. After every one week blood was taken before sacrifice the mice and blood test were performed for testosterone. Then epididymis, testis and seminal vesicles were separated from body and weighed. Sperm cells morphology and mobility were also studied. The total number of sperm cells and their mobility were decreased significantly. The sperm cells morphology of soymilk treated groups was abnormal [14].

In 2008 one study was performed on man, 99 males were selected who consumed soy based diet for three months. The results showed that sperm concentration was inversally related to soy diet. It was concluded that soy foods have deleterious effects on male fertility [22]. Our results were also according to this study.

In another study male Sprague-Dawley rats were feeding for three weeks with 17 β -estradiol, bisphenol A and soy diet to determine their effects on hormones level and reproductive organs. After three weeks the histological differences were recognized in testis of all treated groups. The soy extract and bisphenol A treated groups showed damage, absence of mature spermatids, absence of lumen, sloughing of the germ cells and cellular debris in the seminiferous tubules [23]. The results of our study consistant with the results of Norazit *et al.* [23].

In another study 30, 60 and 90gm cooked soybeans were given to male and female albino rats for three months and determine their effect on fertility. The histology studies showed that soybean diet decreased the ovarian follicles, cause cystic hyperplasia and endometrial thickness. Spermatogenesis arrest and leydig cells decreased in number [24]. The results of our study were also in favor of this study.

Our results were consistent with Zin *et al.* [16] who determine the effect of genistein on reproductive system of female Sprague Dawley rats, 18 rats divided into three groups, each group consist of 6 rats. One is the control group and other two groups were treated with 10mg/kg/BW and 100mg/kg/BW from 22-42 postnatal day. In 10mg/kg BW there were decreased in uterus, ovary and body weight and reverse in 100mg/kg BW. This study showed that there was decreased in number of corpus luteum and increased atretic follicular count [16].

The results are also in conformity with Modaresi *et al.* [25] who reported that high dose of soybeans elevate the testosterone level and decrease the sperm count in male

mice. Three types of soy doses (20, 30 and 50%) were given to male mice for 8 weeks. Results showed that sperm count decreased in all soy treated groups.

According to Adesanya *et al.* [26] three diets (0%, 10% and 20% W: W) of raw soy flour were given to male Wister rats for 8 weeks. Results showed that there was a significant decrease in seminal vesicle, prostate gland and testis weight as compared to control group. In all treated groups testosterone level and sperm count were also significantly decreased. The results were accordance with this study.

Conclusion and recommendations

This study shows that soy isoflavone have damaging effects on testis and ovaries of rabbits. It cause many histological problems as well as change the hormonal level in soy treated groups. We can use the soy isoflavone in case of low level of natural estrogen in our body but the use of these exogenous compounds should be concerned as they may cause many problems. Soy isoflavones have more pronounced effects on male rabbits. This study also shows that soy isoflavone have cancer promoting effect on reproductive tissues. The specific dose of soy isoflavone which enhance fertility yet not identified. Soy products have seriously negative effects on reproduction when they use for prolong time in high amount.

Soy beans and soy products have adverse effects on male and female reproductive system. So food and agriculture organization should be reevaluating its benefits in our daily life. Prolonged use of soy products in high amount should be discouraged. Further studies should be needed by changing the model organism as well as dose concentration because soy isoflavone effects vary according to animal species and dose concentration.

Author's contributions

Conceived and designed the experiments: S Batool & R Iqbal, Performed the experiments: S Batool, Analyzed the data: S Batool & R Iqbal, Contributed reagents/

materials/ analysis tools: S Batool & R Iqbal, Wrote the paper: S Batool.

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