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INFLUENCE OF HIGH PHYTOESTROGEN DIET ON RAT MALE REPRODUCTIVE SYSTEM

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ABSTRACT:

Aims and Objective: To evaluate the effects of phytoestrogen diet alone and high phytoestrogen diet with rutin and naringin on male reproductive system.

Experimental Protocol: A total of 48 adult male wistar albino rats were divided in to 8 groups, each contains 6 rats. Low phytoestrogen diet (LPD) and high phytoestrogen diet (HPD) were administered to the two different groups of rats for 35 days. Rutin and naringin, antioxidants were also given in different doses (5mg/kg, 10/kg) for a period of 35 days along with high phytoestrogen diet in different groups of rats. A sham control group animals fed on commercial pellet diet and vehicle control group animals with 0.1% sod CMC were also maintained until completion of 35 days. On 36th day bilateral orchiectomies were performed for all the animals. The testicular tissue was evaluated for various sperm parameters, biochemical estimations and histological changes.

Results: Animals fed on low and high phytoestrogen diets has shown fall in sperm parameters and lipid peroxidation and increase in testicular antioxidant enzymes. Rutin and naringin have not shown any significant effect on sperm parameters. But decrease in lipid peroxidation and increase in antioxidant enzymes was observed. No histological changes were observed.

Conclusions: High phytoestrogen diet has reduced all the sperm parameters drastically. Antioxidants have not shown any effect on sperm parameters but amplified the antioxidant enzymes and reduced MDA levels.

Key words: High phytoestrogen diet, Rutin, Naringin, male infertility

INTRODUCTION

Phytoestrogens are plant-derived compounds. Soybeans, and foods derived from soy, are a rich source of phytoestrogens and they are able to bind and activate estrogen receptors (1, 2). Estrogen is essential for maintenance and normal activity of the male reproductive tract (3, 4). Exposure to exogenous estrogen or inhibition of endogenous estrogen, either during development or adulthood, induces structural and functional changes in the male reproductive tract. Phytoestrogens were associated with the disruption of mammalian fertility (5,6), by effecting on sexual development and reproductive function.

Exposure of neonatal rats to estrogenic chemicals reduces sperm concentrations, plasma testosterone (7,8), Sertoli cell number (9), gene expression (10), rete tubule distension, and height of efferent duct epithelium (11,12,13). Similarly, distension of the rete testes, efferent ducts, and epididymides, and subsequent infertility, is seen in ERa null mice (14). Similar structural and functional abnormalities can be induced by anti estrogen treatment of adult rats (15), while exposure to low levels of the potent synthetic estrogen diethylstilbestrol (DES) reduces weights of reproductive organs, decreases epididymal sperm, and decreases fertility (7).

Apoptosis is induced by disruption of the endocrine actions of estrogen by the synthetic DES due to suppression of gonadal testosterone (16). ERb inactivation decreases apoptosis of spermatogonia in neonatal mice (17), while in adult human testes in vitro estradiol has been shown to inhibit apoptosis of spermatocytes and spermatids (18). Furthermore in aromatase-deficient mice, spermatid development is disrupted with increased germ cell apoptosis (19). This mouse model has also demonstrated that dietary phytoestrogens exposure through a soy-containing rodent feed, influences spermatogenesis independently of the hypothalamo–pituitary–gonadal axis (20).

The epididymis, a steroid-dependent organ, is responsible for the post-testicular maturation and storage of sperm. Because of the composition of the sperm plasma membrane and their lack of cytoplasm, sperm in the epididymis are susceptible to damage from reactive oxygen species (21). The epididymis protects sperm from oxidative damage by secretion of antioxidant enzymes (22) under steroid regulation (23).High phytoestrogen diet received rats has shown high levels of lipid peroxidation.(24).

While many studies have demonstrated the effects of fetal and neonatal phytoestrogen exposure on testis development and subsequent fertility of the adult male (25,26,27), very few studies have investigated the effects of adult male exposure to dietary phytoestrogens on spermatogenesis. This study aimed to evaluate high phytoestrogen diet induced effects on sperm parameters, oxidative stress, antioxidant status and histopathology of adult male rats. The diet used in this study was chosen from previous studies (28,29,30).

MATERIALS AND METHODS:

Animals

Male albino wistar rats (National Institute of Nutrition, Hyderabad, India) weighing 165 – 210 g were used in the study. They were maintained under standard laboratory conditions at 25 ± 2^0 C, relative humidity 50 ± 15% and normal photoperiod (12 h dark/ 12 h light). Low phytoestrogen diet (10% Soy meal) and High phytoestrogen diet (35% Soy meal) were prepared in the laboratory. Diet and water were provided *ad libitum.* Sham control animals received commercial pellet diet (Rayons biotechnologies Pvt Ltd, India). The experimental protocol has been approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA).

Chemicals:

Rutin and naringin were procured from sigma chemicals, St Louis, USA. Thiopentone sodium was purchased from neon labs, Mumbai, INDIA. All other chemicals and reagents were used of analytical grade.

Experimental Groups:

Total 48 adult male rats were selected for the study and divided in to 8 groups each containing 6 rats. Group 1 is the sham control group, received only water. Group 2 rats have received low phytoestrogen diet (LPD) for 35 days. Group 3 animals have received high phytoestrogen diet (HPD) for 35 days. Group 4 animals were fed on HPD and received 0.1% of sodium CMC treatment every day, which is used as vehicle for the preparation of rutin and naringin suspensions. Groups 5,6,7,8 were test groups and they were also fed on HPD and received test drugs as described below: Group 5 animals received rutin 5mg/kg/day. Group 6 animals received rutin 10mg/kg/day. Group 7 animals received naringin 5mg/kg/day. Group 8 animals received naringin 10mg/kg/day. Both rutin and naringin were given daily for 35 days continuously.

Experimental protocol:

After completion of 35 days of drug treatment, the animals were sacrificed with lethal ether anesthesia and laporatomy was conducted. Testes and epididymis were collected. The epididymis was used for the evaluation of sperm parameters. The right testis was processed for histopathological studies and left one was homogenated for biochemical estimations.

Collection of spermatozoa for evaluation of sperm count, sperm motility, sperm viability:

Epididymal spermatozoa were collected by cutting the cauda region of the epididymis in to small pieces in 2ml of normal saline pre warmed to 37°C. Sperm was forced out of the cauda

epididymis with fine forceps by putting pressure on lower region of cauda epididymis, not forcing out excess material i.e. immature cells. In this study sperm motility, count, and viability were evaluated by using conventional methods (31,32,33). Progressive sperm motility was done immediately after collection of sperm. Number of motile spermatozoa were calculated per unit area and expressed as percentage sperm motility. Sperm counts were done using haemocytometer and results were expressed as millions/ml of suspension. Sperm viability was done using Eosin and Nigrosin stain. The dead sperm took up the stain. Hundred sperm cells were counted in order to obtain the percentage of live/death ratio.

Biochemical Parameters Estimation:

Malanoldehyde (MDA) levels in the testicular tissue were measured by the method developed by Ohkawa et al. (34) This is based on measurement of absorbance of thiobarbituric acid malanoldehyde. The tissue MDA levels were expressed as nmol/g tissue. Super oxide dismutase (SOD) activity was determined by the method developed by Fridovich. (35) This method was based on inhibition of reaction of super oxide radicals with phenyl tetrazolium chloride. The specific activity was expressed in terms of units for mg of protein. Catalase activity was measured based on the method Aebi. (36) Activity of catalase was based on the disappearance of hydrogen peroxide. Activity of catalase was expressed as μmoles of H_2O_2 metabolized/mg protein/min. One unit was defined as 1pmol of H2O2 consumed per minute, and the specific activity was reported as units per milligram of protein. Protein was estimated by the method developed by Lowry. (37)

Histopathological examination:

The testis were fixed in 10% formalin and embedded in paraffin. Five-micron thick sections were prepared and stained with hematoxylin and eosin (H&E). The tissue sections were evaluated under light microscopy by a blinded pathologist.

Statistical analysis

The results are expressed as mean ± SD. Differences in tissue lipid peroxide levels, SOD and CAT were determined by factorial one-way analysis of variance. Individual groups were compared using Tukey's test. Differences with $P < 0.001$ were considered statistically significant. Statistical analysis was performed using Graph Pad Prism software (Version 5).

RESULTS:

Effects on Sperm parameters:

Sperm Count, motility and viability were significantly affected by phytoestrogens in control groups. Values of sperm count, motility and sperm vitality were given in table.There was drastic fall in the sperm count and reduction in percentages of progressively motile and viable spermatozoa in both LPD and HPD received animals. The effect was dose dependent. Rutin and naringin in both doses have not shown any significant effect on all the three parameters i.e. sperm count, motility and sperm vitality. (Figures 1, 2, 3).

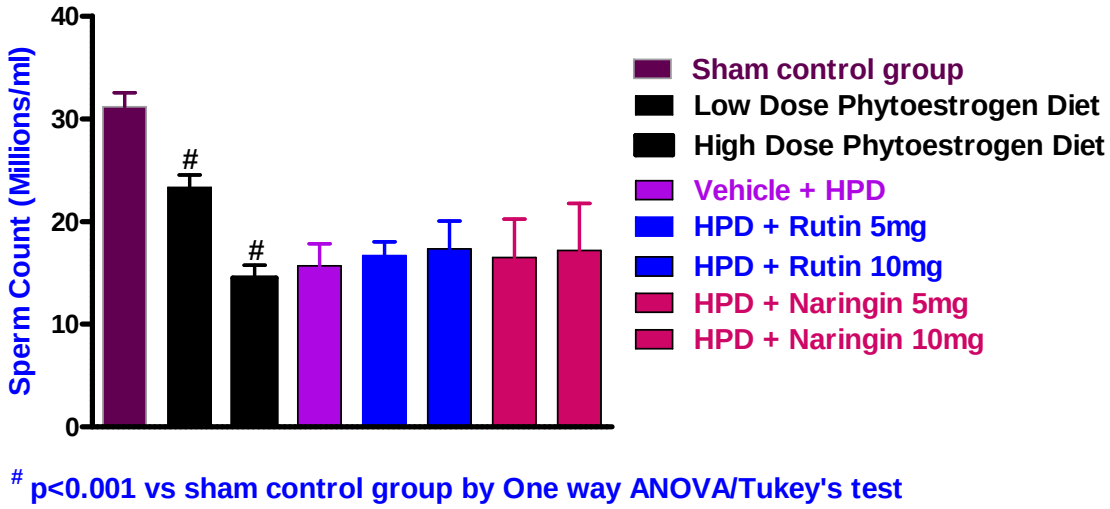
Effects on biochemical Parameters:

MDA, SOD, Catalase levels in testicular tissue were given in Table. There was significant decrease in MDA levels ($p<0.001$) and increase of antioxidant enzymes SOD and catalase in both LPD and HPD received groups when compared to sham control group. Low doses of rutin and naringin (5mg.kg/day) have not shown any effect but high dose of rutin (10mg/kg/day) significantly reduced the MDA levels and increased the SOD levels. Naringin in high dose (10mg/kg) significantly increased the SOD levels. (Figures 4, 5, 6)

Effects on histopathology of testicular tissue:

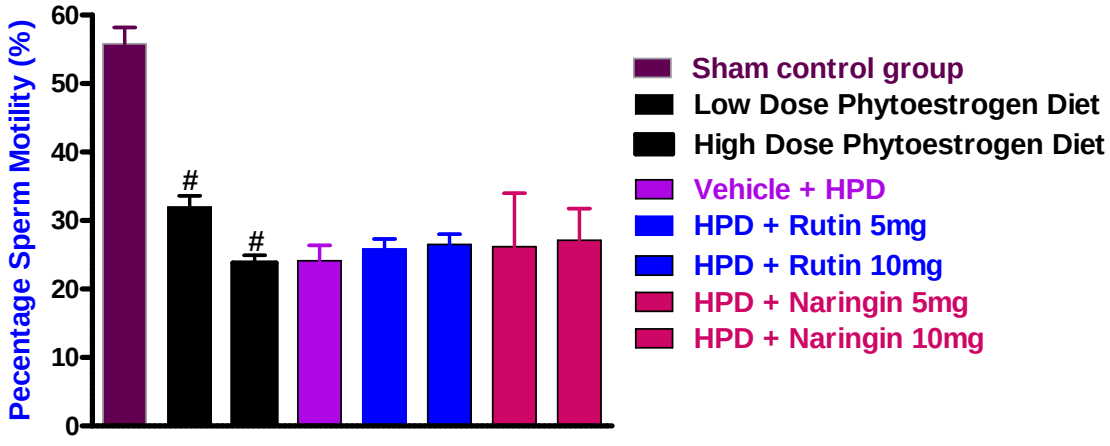
Both LPD and HPD fed control group animals have not shown any significant change in the histological observation. Rutin and naringin in both the doses also not shown any significant change.

Fig 1: Sperm Count



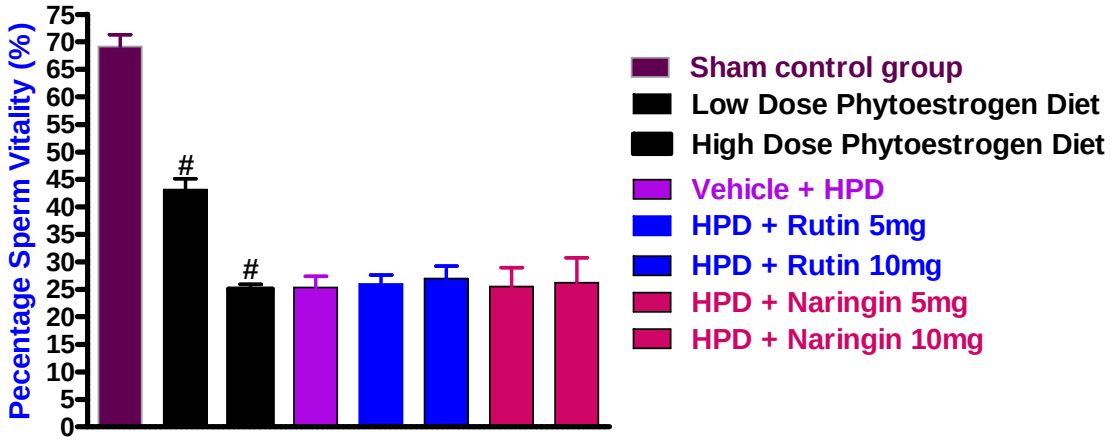
# p<0.001 vs sham control group by One way ANOVA/Tukey's test

Fig 2: Sperm Motility



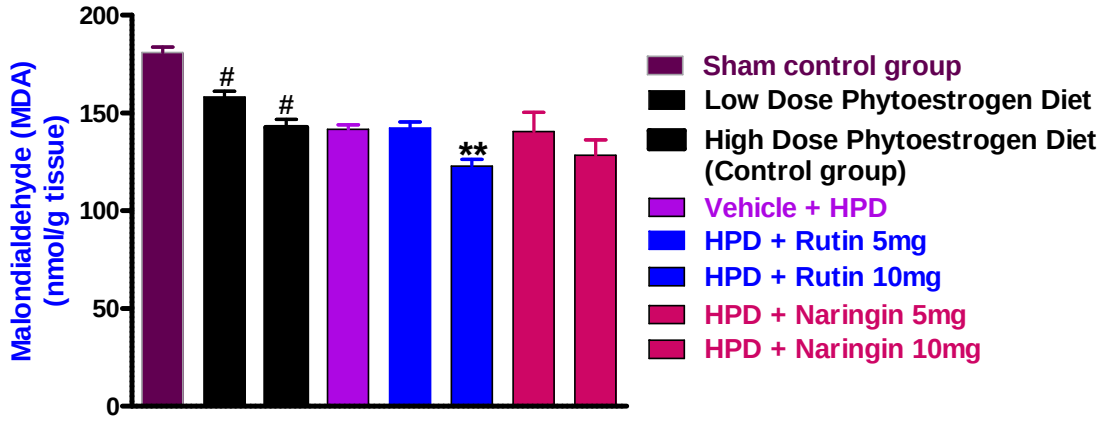
# p<0.001 vs sham control group by One way ANOVA/Tukey's test

Fig 3: Sperm Vitality



# p<0.001 vs sham control group by One way ANOVA/Tukey's test

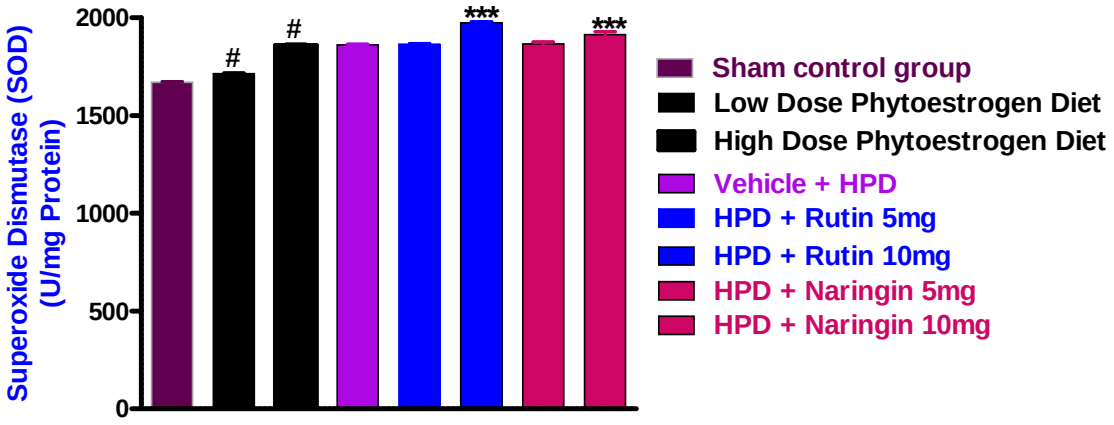
Fig 4: MDA levels



# p<0.001 vs sham control group by One way ANOVA/Tukey's test

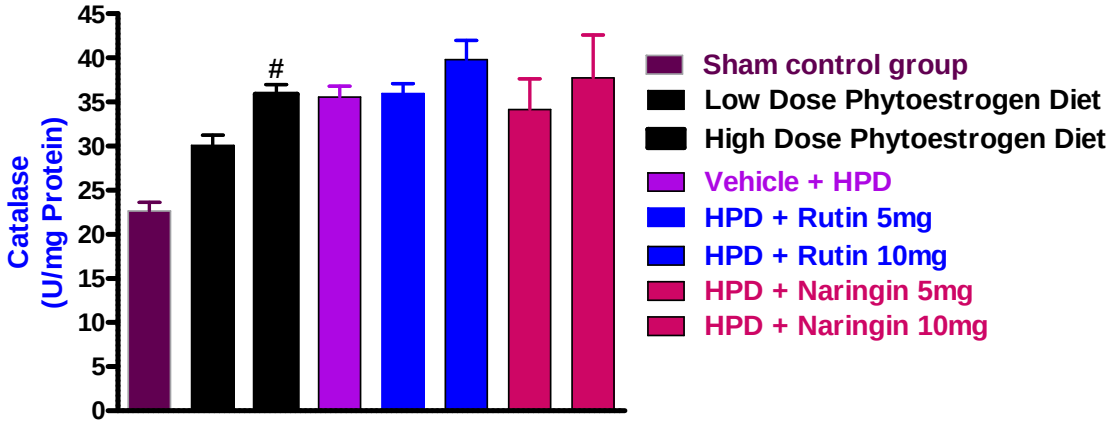
**p<0.01 vs control group by One way ANOVA/Tukey's test

Fig 5: SOD levels



# p<0.001 vs sham control group by One way ANOVA/Tukey's test
***p<0.001 vs control group by One way ANOVA/Tukey's test

Fig 6: Catalase levels



# p<0.001 vs sham control group by One way ANOVA/Tukey's test

Table: Sperm Parameters and Biochemical estimation values:

Groups	Sperm count (millions/ml)	Sperm motility (%)	Sperm viability (%)	MDA	SOD	Catalase
1	31.16±3.4	55.83±5.84	69.16±5.3	180.94±7.04	1670.67±5.43	22.63±2.43
2	23.33±2.94	32±3.89	43.16±4.79	158.21±7.17	1715.02±6.76	30.08±2.82
3	14.5±3.01	23.83±2.71	25±2.28	142.62±10.06	1858.24±13.30	35.91±2.60
4	15.66±5.35	24.16±5.45	25.33±4.92	141.65±5.44	1861.95±7.35	35.54±3.02
5	16.66±3.26	25.83±3.60	26±3.94	142.42±7.62	1865.21±6.79	35.96±2.67
6	17.33±6.62	26.5±3.61	27±5.44	122.92±8.55	1975.38±11.83	39.78±5.40
7	16.5±3.72	26.16±7.83	25.5±3.44	140.59±9.78	1866.77±10.83	34.15±3.50
8	17.16±4.57	27.16±4.57	26.16±4.53	128.44±7.89	1912.94±15.73	37.74±4.86

DISCUSSION:

Phytoestrogens occur naturally in plants. Common classes comprise isoflavones, lignins, coumestans, or resorcylic acid lactones that are structurally similar to the mammalian estrogen and have a weak estrogenic potency ranging from 1/500 to 1/1000 that of 17-estradiol. A major source of human consumption of phytoestrogens is soybean derived products, which contain the isoflavones daidzein and genistein. Because of the great differences in the consumption of such products, adults in the United Kingdom and the United States ingest approximately 1 mg of isoflavones per day; Asians ingest 50 to 100 mg/day. Infants who are fed soy-based formula as a sole source of nutrition ingest 22 to 45 mg/day. Song et al. investigated the relationship between isoflavone intake and semen quality in

a group of 48 men with abnormal semen parameters and 10 men with normal semen parameters. (38,39).

The role of perinatal exposure to phytoestrogen on male reproductive health has been thoroughly evaluated in animal models. In rodents, exposure to phytoestrogens in utero or during early post-natal life through diet or subcutaneous injection results in multiple reproductive abnormalities during adult life, including decreased testicular weight or size (40,41,42), decreased spermatogenesis (43), lower testosterone (44), DHT (45) and FSH levels (40), decreased testicular expression of steroid hormone receptors (46), decreased anogenital distance and alterations of reproductive and aggressive behavior (42,43).

However, these changes are not always consistent across studies. In marmoset monkeys, soy formula feeding starting at 3 days of age resulted in suppression of the neonatal testosterone surge and increased Leydig cell number lasting into adulthood, but no adverse effects on pubertal progression or fertility were documented (47,48). A study in rabbits found that a phytoestrogen-rich diet increased libido and improved all conventional semen quality parameters (49). A study in macaques found no changes in testicular weight or semen quality in response to different dietary doses of phytoestrogens (50).

The present study aimed to evaluate the effects of low and high phytoestrogen diet on male reproductive system. As there were reports on increased oxidative stress by the phytoestrogens, we have evaluated the role of bioflavonoids rutin and naringin also in HPD received rats.

In the present study sperm count, sperm motility and sperm vitality were significantly reduced in both LPD and HPD received rats. The effect was more with HPD. This study has demonstrated that feeding a diet of high phytoestrogen content to adult rats disrupts normal spermatogenesis by increasing apoptosis of developing germ cells. A previous study using a comparable high

phytoestrogen diet reported similar effects on sperm parameters (51). Many reports reveled that, the HPD causes alterations in gene expression of AR, ERα, and ERβ in the adult rat epididymis. (24) Regional differences in gene expressions are well documented in the rat epididymis (52) and reflect the complex changing environment that sperm experience in the epididymis in order to mature and gain their fertilizing ability. (53) HPD received rats were also treated with rutin and naringin in two different doses (5mg/kg/day and 10mg/kg/day) But there was no significant change in sperm parameters when compared with control rats (rats received only HPD).

In the current study biochemical parameters like MDA, SOD and catalase were evaluated. MDA levels were reduced and SOD, Catalase levels were increased significantly in both LPD and HPD received rats. This indicates the antioxidant role of phytoestrogens. Rutin, a bioflavonoid at 10 mg further decreased the MDA levels and increased the SOD levels and has not shown any significant change with respect to the catalase levels. But in the previous study by A. Glover et al, increase in the lipid peroxidation was observed. They have given HPD only for 3 days and study was acute. In the present study the rats were fed on HPD for 35 days. (24)

In the histological examination of the testes, the normal control group has numerous seminiferous tubules that are small and more rounded in shape. They are tight and closely packed interstitium with leydig cells and scanty spermatocytes in their lumens. No noticeable changes were observed in control and other treatment groups. They were also similar to the sham control group. Similar study conducted by Fritz and colleagues with HPD also did not determine any histological changes in the reproduction organs of the male rats. (54)

In conclusion, chronic exposure of adult male rats to the phytoestrogen diet reduced the sperm count, motility and sperm vitality. MDA levels were reduced and SOD, Catalase levels were increased in rats which received phytoestrogens. Rutin and naringin has not shown any effect on sperm

parameters, but they amplified the effects of phytoestrogens with respect to biochemical parameters.

No significant histological changes were observed. Further research on the potential tissue-specific effects of phytoestrogens is needed to know the real risks associated with phytoestrogen-containing diets. The sperm parameters only are decreasing with the effect of phytoestrogen diet .No histological changes were observed. Probably temporary reversible infertility may be possible with the excess intake of phytoestrogens by males.

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