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Subacute oral toxicity study of soy isoflavones on organ weights and sperm parameters in male Wistar rats

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Abstract
Soybean is rich source of protein consumed by human and animals but it elicits phytoestrogenic properties. The present study was conducted to evaluate subacute toxicity of soy isoflavones on organ weight and sperm parameters in adult male Wistar rat. Soy isoflavone (SI) extract was prepared, quantified and utilized for present study. Animals were equally divided into three groups (n=8). Group I served as control and given normal saline through oral gavage for 28 days, while group II and group III were given SI @ 250, @ 500 mg/kg bwt/day daily orally for 28 days period. After completion of 28 days animals were sacrificed and relative organ weight and sperm parameter were analyzed. The organ weight of liver and thymus were significantly decreased in both treatment groups when compared with control group. Kidney, brain and epididymis weight revealed non-significant differences. The organ weight of adrenal was significantly increased in both treatment groups compared to control group. However, relative weight of spleen was significantly higher in group II as compared to group III and control group. Testis and seminal vesicle revealed non significant dose dependent decreased values. Epididymal sperm count and sperm motility was found to be significantly decreased in both the treatment groups as compared to control group. Sperm head and total sperm abnormalities were significantly increased in group II and group III when compared with the control group, while mid piece abnormalities were significantly elevated in group III compared to group I and II. Soy isoflavone subacute toxicity @ 250 mg/kg bwt. and 500 mg/kg bwt. leads to alterations in organ weight of liver and thymus and showed decreased epididymal sperm count and elevated sperm abnormalities in adult male rats.

Keywords: Soy isoflavones, organ weights, sperm, rat

1. Introduction
Soy bean (*Glycine max*) belong to family Leguminosae which is abundantly used as a food ingredient in the diet of domestic animals, fish and human being from long time to fulfill the nutritional need of protein and fat and now a days popular for nutraceutical soy products [1, 2]. Soybean is an excellent source of quality protein, fat, and fiber. Soybean-rich diet offered to the animals also leads to a high serum concentration of isoflavones in the animals [3].
Phytoestrogens are polyphenolic, nonsteroidal compounds of plant and have importance in female reproductive physiology and can prevent breast cancer, heart disease, prostate cancer and osteoporosis [4-6].
It is well known that principal isoflavones in soybeans are genistein, daidzein, and glycitein having a chemical structure alike to that of 17-b-oestradiol and mimic estrogenic activity. The structural likeness to estradiol, soy isoflavones contribute an affinity towards estrogen receptors and produce action like estrogenic or antiestrogenic [7, 8]. Soy isoflavones also can display their effect on estrogen-regulated systems including the central nervous, skeletal, cardiovascular, and reproductive systems [9].
Common studies have focused on good effect of isoflavones during menopause and suggested that soy products do not affect to male reproductive system of male. However, rodent studies shown that isoflavones can produce negative impact on male reproductive organs and cause apoptosis in germ cells lead to reduction in fertility [10].
Therefore, the present study was planned to evaluate the subacute toxicity of soy isoflavones on organ weight and sperm parameters of adult Wistar rats.

2. Materials and Methods
2.1 Preparation of Soy Ethanolic Extract
Soy seed sample was collected from a local market. The fine flour was prepared and extraction

was done with 80% ethanol (5 mL/g flour) as per the standard method [11].

2.2 Quantification of Soy Isoflavones by HPLC
After extraction, sample was quantified by using high-performance liquid chromatography (HPLC) and extracted soy isoflavones was used for oral dosing in the present experiment.

2.3 Experimental Animals and Experimental Design
The present experiment was conducted on 24 adults male Wistar rats weighing around 150-200 gm. After approval from Institutional Animal Ethics Committee (IAEC) NO.312/04/2000/21, Date: 06/08/2021 the experiment was carried out under standard hygienic and good managemental conditions as per Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA), New Delhi guidelines. 24 Male rats were equally divided into three groups thus containing 8 animals in each group and all the group animals were given adlib soybean-free pelleted diet during 28 days of experiment. Group I was served as a control group and given normal saline, group II was given soy isoflavones (SI) @ 250 mg/kg bwt./day and group III was given the soy isoflavones @ 500 mg/kg bwt/day through oral gavaging. At the end of the experimental period of 28 days, all male animals were sacrificed by administration of intraperitoneal injection of thiopentone sodium @ 150 mg/kg bwt.

2.4 Relative organ weights
After detailed necropsy examination of each animals from all the groups, liver, kidney, spleen, thymus, testes, epididymis, seminal vesicles, and brain were separated out and weighed and data was analyzed for relative organ weights.

2.5 Sperm Parameter Evaluation
To assess sperm parameters a fresh cauda epididymis was collected from sacrificed male rat, minced, and finally diluted into the prewarmed 1ml 7.2 PH phosphate buffer solution (PBS) and kept for 15 minutes at 37 °C. From this fluid total sperm count, sperm motility, abnormal sperm count, live and dead sperm counts was carried out by using Eosin Y and Nigrosin stain as per the standard methodology [12, 13].

2.6 Statistical analysis
SPSS (version 22) software was used and data analyzed by one-way analysis of variance (ANOVA) for different groups followed by Duncan's test. Collected data calculated and values are expressed as mean±SE. For the comparison purpose of mean values, $p<0.05$ is considered as statistically significant.

3. Results and Discussion
3.1 Quantification of Soy Isoflavones
Soy bean extract sample used for dosing of male animals during present experiment revealed presence of isoflavones daidzin (244.60 ug/g), genistin (447.47 ug/g), and glycitin (52.63 ug/g) by HPLC analysis. Soy isoflavones HPLC quantification values of genistin, daidzin and glycitin in the present study correlates with the findings of Alnokari *et al.* [11] who used 80% ethanolic and 80% acetonitrile method for extraction of soybean seed.

3.2 Effect of soy bean extract on relative organ weights
After sacrifice of male rat organs were collected, weighed and relative organ weights are presented in Table 1. In the present experiment liver and thymus weights were significantly ($p<0.01$) decreased in both treatment groups as compared to control. The relative weight of adrenal gland was significantly ($p<0.01$) increased in both treatment groups as compared to control group. Spleen relative weight was significantly ($p<0.05$) higher in group II as compared to group III and group I. Non significantly dose dependent decreased in weights of testes and seminal vesicles were observed in the group II and group III as compared with the control group (I). However, kidneys, brain and epididymis relative weights were revealed non-significant differences.

Table 1: Relative organ weights (gm%) in control and treatment groups rats at the end of the experiment (28-day)

Organ	Group I- (Control)	Group II – (SI @ 250 mg/kg/day)	Group III – (SI @ 500 mg/kg/day)	P-Value
Liver	4.28±0.22^{b}	3.48±0.16^{a}	3.32±0.10^{a}	0.001
Kidneys	0.74±0.04	0.70±0.02	0.76±0.03	0.446
Adrenals	0.03±0.00^{a}	0.04±0.0^{b}	0.05±0.01^{b}	0.006
Thymus	0.18±0.01^{b}	0.19±0.01^{b}	0.11±0.01^{a}	0.000
Spleen	0.28±0.01^{ab}	0.32±0.03^{b}	0.23±0.01^{a}	0.010
Brain	0.49±0.02	0.48±0.04	0.51±0.04	0.863
Testes	1.06±0.08	1.05±0.07	0.95±0.02	0.353
Epididymis	0.57±0.04	0.50±0.03	0.51±0.02	0.129
Seminal Vesicles	0.50±0.07	0.39±0.04	0.35±0.02	0.082
Values are expressed as mean±SE for the groups; n=8 in each group. Mean values in similar row having different superscript differ significantly. SE=Standard error, SI=Soy isoflavones.				

The finding of significant decrease in relative liver weights corroborates the previous findings, wherein female rats were supplemented with soy isoflavone at 1000 mg/kg orally for 30 days which could be due to dysfunction of liver indicated by significantly increased liver enzymes [14]. Significantly increased relative adrenal weights in the present study is in line with earlier subchronic study in rats supplemented with genistein @ 500 mg/kg. It may be due to the non-specific type of stress to the rats which causing alteration in adrenal weights in the treatment group [15]. Contrary to the present findings of increase in the relative spleen weight in the treatment group II previous workers noticed significant decreased spleen weights in pregnant rats after administration of soy isoflavones during pregnancy [16] and it could be attributed to genistin causing down regulation of spleen T cells [17]. Genistein causes apoptosis of thymocytes through the estrogen dependent receptors and non-estrogen related mechanisms ultimately causing cell loss and decreased relative thymus weights [18] which in the agreement with the findings of significantly decreased relative thymus weights in the present study [19, 20]. However, Nishide *et al.* [21] noticed increased thymic weights after isoflavone supplementation immunomodulatory effect of daidzein [22]. Non-significant dose dependent decrease in the relative testis and seminal weights were observed in the present study. However, previous study showed significantly decreased testes weights [23] after soy isoflavone supplementation to male rats. Altered in the testis's weighs could be due to estrogenic hormonal effect of soy isoflavone genistein [15], loss of germ cells and inadequate production of testosterone by Leydig cells [24]. Seminal vesicle weights were significantly reduced after feeding of 15% genetically modified soya beans for 65 days period [25] and oral administration of genistein @ 500mg/kg in

rats for 4 weeks period in male rats [15], however relative weights of seminal vesicles does not differ significantly but numerically decreased in values were noticed. A significantly decreased absolute and relative seminal vesicle weights observed in previous studies may be due to presence of antinutritional components in soy which lead to malabsorption of amino acid and phytoestrogenic isoflavones activity further declines testosterone levels reducing the weights of male reproductive organs [25, 26].

3.3 Effect of soy bean extract on sperm parameters

The cauda epididymal sperm count, sperm motility and sperm abnormalities revealed significant differences in group II and group III compared to the group I and showed in Table 2. Alterations were observed in total sperm count [Figure 1a], sperm motility, and sperm morphology. Cauda epididymal sperm count was significantly (p<0.05) decreased in group II and group III compared to the group I (189.98±26.94 versus 167.45±20.63 and 163.15±14.31 X106 for group I, group II and group III). The total sperm motility significantly (p<0.05) declined in both group II and group III in comparison to control (63.81±5.77 versus 58.13±2.25 and 58.25±5.01% for group I, group II and group III). Total sperm abnormality (13.13±7.88, 22.63±3.50 and 23.25±9.44% for group I, group II and group III) and sperm head abnormality (3.25±2.19, 6.50±1.41 and 7.38±4.63% for group I, group II and group III) [Figure 1c and 1d] values significantly (p<0.05) increased in group II and group III when compared to group I. Mid-piece abnormality [Figure 1e] significantly (p<0.05) increased in group III (SI 500 mg/kg) treatment group males as compared with control group (2.38±1.60 versus5.00±2.07 % group I and group III) . Whereas, total viable sperm count and tail abnormalities [Figure 1b and1f] showed non-significant differences.

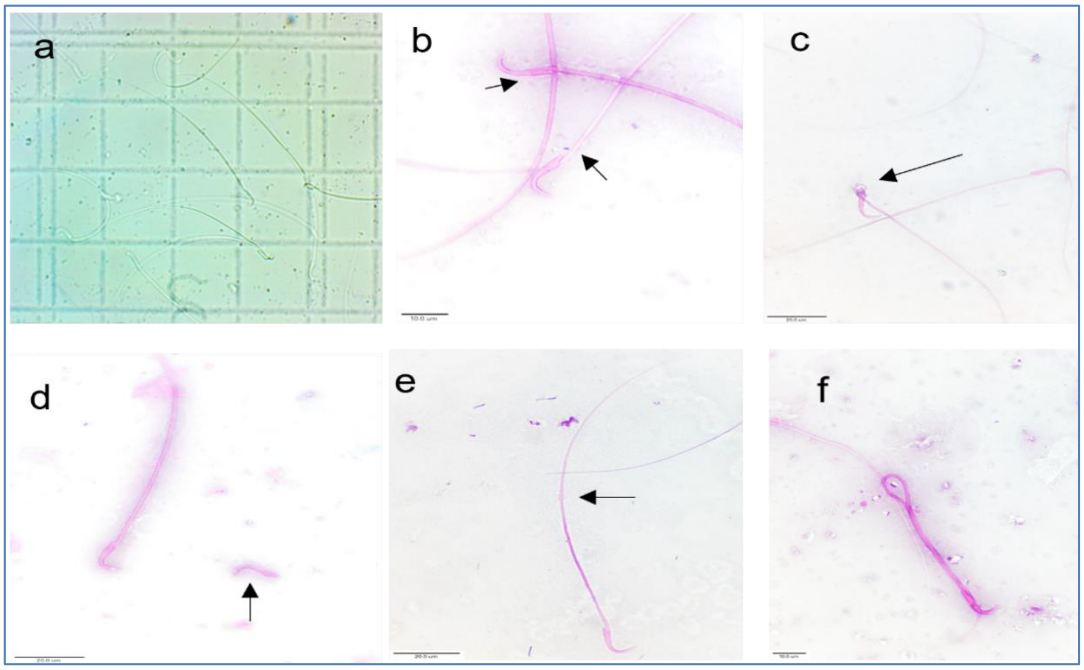

Fig 1: (a) Counting of cauda epididymal sperms over Neubauer chamber. (b) Sperm viability: Arrows showing pink colour-dead sperm and colourless-live sperm. (c) Sperm head abnormality-Bent Head (arrow). (d) Sperm head abnormality-Detached Head (arrow). (e) Sperm mid piece abnormality- Distal cytoplasmic droplet (arrow), and (f) Sperm tail abnormality-Tail bent proximally and coiled around mid-piece and neck in a dead sperm (pink colour). Fig 2(b) to Fig 2(f) stained with eosin-nigrosine stain.

Table 2: Sperm parameters in control and treatment groups at the end of experiment (28-day)

Parameter	Group I-(Control)	Group II – (SI @ 250 mg/kg/day)	Group III – (SI @ 500 mg/kg/day)	P-Value
Cauda epididymal sperm count (X106)	189.98±9.52^{b}	167.45±7.30^{a}	163.15±5.06^{a}	0.043
Sperm motility %	63.81±2.04^{b}	58.13±0.79^{a}	58.25±1.77^{a}	0.034
Total viable sperm count %	90.56±1.95	85.44±2.20	82.88±2.25	0.055
Head abnormality %	3.25±0.77^{a}	6.50±0.50^{b}	7.38±1.64^{b}	0.033
Mid-piece abnormality %	2.38±0.56^{a}	3.63±0.53^{ab}	5.00±0.73^{b}	0.023
Tail abnormality %	7.50±1.90	12.50±0.94	10.88±1.39	0.070
Total sperm abnormality %	13.13±2.79^{a}	22.63±1.24^{b}	23.25±3.34^{b}	0.020
Values are expressed as mean±SE for the groups; n=8 in each group. Mean values in similar row having different superscript differ significantly. SE=Standard error, SI=Soy isoflavones.				

Sperm count reduction after estrogen exposure in rodents [27, 28], 15% genetically modified soya beans administration for 65 days period in rats [25] and soy isoflavones administered in rodent animals [10, 23] correlates with significant declined cauda epididymal sperm count in our present study. However, studies conducted on rat and rabbit revealed no effect on epididymal sperm count after soy isoflavone administration are not in agreement with present study findings [29, 30]. Significantly decreased sperm count and sperm motility [33, 34] could be attributed to increased estrogen levels and decreased Zn levels in the epididymis after soy isoflavones administration [29]. Moreover, genistein inhibits tyrosine phosphorylation of sperm tail protein and affects capacitation and sperm activity [31] as protein phosphorylation and

dephosphorylation which is important for sperm capacitation in rodents [32]. Contrary to our present observations, previous study on genistein reveals no effects on sperm motility parameters [35].

Present study revealed significantly elevated head and mid piece sperm abnormalities, it might be due to decrease testosterone level affecting spermatogenesis and every stage of the sperm cycle in male. Studies of high phytoestrogen diet showed pre and post meiotic apoptosis germ cells of seminiferous epithelium [36] and alterations in FSH and LH levels [37]. The decreased testosterone concentration in testes causes effect on expression of androgen depended on junction proteins between Sertoli cells and germinal cells [36] as genistein and daidzein both can regulate the Leydig cells [38, 39]. However, present study results are not in agreement with a study conducted on rabbits which were exposed to the 40% soy isoflavones [30].

Conclusion

Soy bean contains isoflavones and administration of extract @ 250mg/kg and 500mg/kg dose levels in male animals causes decrease in organ weights and phytoestrogenic action on testes leads to sperm abnormalities and reduction in sperm count.

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Conflict of Interests

The authors declared no conflict of interest.

References

1. Rajcan I, Hou G, Weir AD. Advances in breeding of seed-quality traits in Soybean. J Crop Improvement. 2005;14(1-2):145-174.
2. Jayachandran M, Xu B. An insight into the health benefits of fermented soy products. Food Chem. 2019;271:362-371.
3. Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Animal models impacted by phytoestrogen Laboratory investigation. 2001;81(5):735-747.
4. Setchell KD. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr. 1998 Dec;68(6 Suppl):1333S-1346S.
5. Adlercreutz H. Phyto-oestrogens and cancer. The lancet oncology. 2002;3(6):364-373.
6. Kronenberg F, Fugh-Berman A. Complementary and alternative medicine for menopausal symptoms: a review of randomized, controlled trials. Annals of internal medicine. 2002;137(10):805-813.
7. Price KR, Fenwick GR. Naturally occurring oestrogens in foods-a review. . Food Additives & Contaminants. 1985;2(2):73-106.
8. Dixon RA. Naturally occurring oestrogens in foods: A review. Annual review of plant biology. 2004;55:225-261.
9. Thigpen JE, Setchell KD, Saunders HE, Haseman JK, Grant MG, Forsythe DB. Selecting the appropri- ate rodent diet for endocrine disruptor research and testing studies. ILAR journal. 2004;45(4):401-417.
10. Glover A, Assinder SJ. Acute exposure of adult male rats to dietary phytoestrogens reduces fecundity and alters epididymal steroid hormone receptor expression. J Endocrinol. 2006 Jun;189(3):565-573.
11. Alnokari S, ASA AA. Soy isoflavone in soy feed HPLC analysis. Int J Pharm Sci Rev Res. 2016;41(1):1-3.
12. Reynolds TR, Narang BS. Semen analysis. In: KL M, editor. Medical Laboratory Technology. New Delhi: Tata McGraw Hill. 1994;2:871-879.
13. Luthfi MJ. A simple and practical method for rat epididymal sperm count (*Rattus norvegicus*). Medicine, & Natural Product Chemistry. 2015;4:1-3.
14. Faria WC, Martinelli RC, Arcas AS, Silva D, Colodel J, Cavenaghi EM, *et al*. Acute and Subacute Toxicity Study on Dietary Supplementation with Soy Isoflavones in Wistar Rats. Current Nut & Food Sci. 2018;14:68-78.
15. Michael McClain R, Wolz E, Davidovich A, Pfannkuch F, Edwards JA, Bausch J. Acute, subchronic and chronic safety studies with genistein in rats. Food Chem Toxicol. 2006;44(1):56-80.
16. Gaffer GG, Elgawish RA, Abdelrazek HM, Ebaid HM, Tag HM. Dietary soy isoflavones during pregnancy suppressed the immune function in male offspring albino rats. Toxicology reports. 2018;5:296-301.
17. Sakai T, Kogiso M. Soy isoflavones and immunity. J Med Invest. 2008;55(3-4):167-173.
18. Yellayi S, Naaz A, Szewczykowski MA, Sato T, Woods JA, Chang J, *et al*. The phytoestrogen genistein induces thymic and immune changes: a human health concern. Proc Natl Acad Sci USA. 2002 May 28;99(11):7616-7621.
19. Tag HM, Tantawy HM, Ghoneim RM, History A. Effect of soy isoflavones on some immunological parameters in ovariectomized female rats. Egypt Acad J Biol Sci C Physiol Mol Biol. 2014;6(1):101-114.
20. Ebaid HM, Elgawish RA, Abdelrazek HM, Gaffer G, Tag HM. Prenatal Exposure to Soy Isoflavones Altered the Immunological Parameters in Female Rats. Int J Toxicol. 2016;35(3):274-83.
21. Nishide Y, Tadaishi M, Kobori M, Tousen Y, Kato M, Inada M, *et al*. Possible role of S-equol on bone loss via amelioration of inflammatory indices in ovariectomized mice. J Clin Biochem Nutr. 2013;53(1):41-48.
22. Zhang R, Li Y, Wang, W. Enhancement of immune function in mice fed high doses of soy daidzein. Nutr Cancer. 1997;29:24-28.
23. Meena R, Supriya C, Pratap Reddy K, Sreenivasula Reddy P. Altered spermatogenesis, steroidogenesis and suppressed fertility in adult male rats exposed to genistein, a non-steroidal phytoestrogen during embryonic development. Food Chem Toxicol. 2017 Jan;99:70-77.
24. Guan L, Huang Y, Chen ZY. Developmental and reproductive toxicity of soybean isoflavones to immature SD rats. Biomed Environ Sci. 2008 Jun;21(3):197-204.
25. El-Kholy TA, Al-Abbadi HA, Qahwaji D, Al-Ghamdi AK, Shelat VG, Sobhy HM, *et al*. Ameliorating effect of olive oil on fertility of male rats fed on genetically modified soya bean. Food Nutr Res. 2015 Sep 18;59:27758.
26. Liener IE. Implications of antinutritional components in soybean foods. Critical Reviews in Food Science &

Nutrition. 1994;34(1):31-67.
27. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, *et al.* Male reproductive health and environmental xenoes- trogens. Environ Health Perspect. 1996;104:741-803.
28. Sharpe RM, Rivas A, Walker M, Mckinnell C, Fisher JS. Effect of neonatal treatment of rats with potent or weak (environmental) oestrogens, or with a GnRH antagonist, on Leydig cell development and function through puberty into adulthood. Int J Androl. 2003;26:26-36.
29. Piotrowska K, Baranowska-Bosiacka I, Marchlewicz M, Gutowska I, Noceń I, Zawiślak M, *et al*. Changes in male reproductive system and mineral metabolism induced by soy isoflavones administered to rats from prenatal life until sexual maturity. Nutrition. 2011;27(3):372-381.
30. Cardoso JR, Bao SN, Mondadori RG, Bianchini E. Effects of chronic treatment with soy derived isoflavones on reproductive health of male rabbits. Bioscience Journal. 2007;23(1):75-82.
31. Mahony MC, Gwathmey T. Protein tyrosine phosphorylation during hyperactivated motility of cynomolgus monkey. Biol Reprod. 1999;60:1239-1243.
32. Furuya S, Endo Y, Oba M, Nozawa S, Suzuki S. Effects of modulators of protein kinases and phosphatases on mouse sperm capacitation. J Assist Reprod Genet. 1992;9:391-399.
33. Goyal HO, Robateau A, Braden TD, Williams CS, Srivastava KK, Ali K. Neonatal estrogen exposure of male rats alters reproductive functions at adulthood. Biol Reprod. 2003;68(6):2081-2091.
34. Eustache F, Mondon F, Canivenc-Lavier MC, Lesaffre C, Fulla Y, Berges R, *et al*. Chronic dietary exposure to a low-dose mixture of genistein and vinclozolin modifies the reproductive axis, testis tran- scriptome, and fertility. Environ Health Perspect; c2009, 117.
35. Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H. Reproductive effects in male and female rats of neonatal exposure to genistein. Reproductive Toxicology. 2001;15(4):399-411.
36. Assinder S, Davis R, Fenwick M, Glover A. Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells. Reproduction. 2007;133:11-20.
37. Cook JC, Johnson L, O'connor JC, Biegel LB, Krams CH, Frame SR, *et al*. Effects of dietary 17β-estradiol exposure on serum hormone concentrations and testicular parameters in male Crl: CD BR rats. Toxicological Sciences. 1998;44(2):155-68.
38. Sherrill JD, Sparks M, Dennis J, Mansour M, Kemppainen BW, Bartol FF, *et al.* Developmental exposures of male rats to soy isoflavones impact Leydig cell differentiation. Biol Reprod. 2010;83:488-501.
39. Napier ID, Simon L, Perry D, Cooke PS, Stocco DM, Sepehr E. Testicular development in male rats is sensitive to a soy-based diet in the neonatal period. Biol Reprod, 2014, 90(2).