

Influence of prolonged flaxseed (*Linum usitatissimum*) consumption over epididymis and testicle histoarchitecture of Wistar rats¹

Lanna B.N.S. Corrêa², Ludmila F.M. de F. Cardozo³, Ilma C. de A. Ribeiro²,
Gilson T. Boaventura³ and Maurício A. Chagas^{2*}

ABSTRACT.- Corrêa L.B.N.S., Cardozo L.F.M.F., Ribeiro I.C.A., Boaventura G.T. & Chagas M.A. 2017. **Influence of prolonged flaxseed (*Linum usitatissimum*) consumption over epididymis and testicle histoarchitecture of Wistar rats.** *Pesquisa Veterinária Brasileira* 37(6):650-656. Laboratório de Biomorfologia Celular e Extracelular, Instituto Biomédico, Universidade Federal Fluminense, Rua Prof. Hernani Melo 101, São Domingos, Niterói, RJ 24210-130, Brazil. E-mail: chagas.m@gmail.com

Flaxseed is considered a functional food with several health benefits. However, because of its high phytoestrogen content, flaxseed influences hormone metabolism and affects the gonadal biomorphology. In this study, computerized histomorphometry was used to evaluate seminiferous and epididymal tubules, considering the different regions of the epididymis (head, body and tail) of rats subjected to a prolonged diet of flaxseed. Young adult male Wistar rats (n=20) were divided into 2 groups during their lactation period: Control Group (CG), fed casein-based meals and Flaxseed Group (FG), fed a 25% flaxseed meal. After 250 days of continuous ingestion, the animals were euthanized and a blood sample was collected. The testicles and epididymis were removed and fixed in buffered formalin solution. The samples were subjected to routine histological paraffin techniques and stained with hematoxylin and eosin. Immunostaining was performed using an antivimentin antibody for Sertoli cell identification. For morphometry, images of the slides were scanned and analyzed using Image J to determine the epithelial height, tubular and luminal diameter and tubular and luminal area. In the hormonal evaluation, FG had a higher serum concentration of estrogen ($P=0.001$), but no change was observed in the concentration of testosterone. The morphometric assay of seminiferous tubules and epididymal regions revealed no significant differences between the analyzed groups. Similarly, Sertoli cell quantification showed no significant differences in the FG ($P=0.98$). These results revealed that the continuous and prolonged intake of 25% flaxseed meals from gestation to 250 days of age, even with a significant increase in serum levels of estradiol, does not exert adverse effects on the testicular and epididymal structure or on the cells participating in the spermatogenesis of rats.

INDEX TERMS: Flaxseed, *Linum usitatissimum*, epididymis, testicle, Wistar rats, seminiferous tubules, Sertoli cells, phytoestrogens, histomorphometry, vimentin.

RESUMO.- [Influência do consumo prolongado de semente de linhaça (*Linum usitatissimum*) sobre a histoarquitetura dos testículo e epidídimo de ratos Wistar.] A semente de linhaça é considerada um alimento funcional com vários efeitos benéficos à saúde. Entretanto, devido ao

seu elevado teor de fitoestrógenos, esta semente pode influenciar no metabolismo hormonal e interferir na biomorfologia gonadal. Neste estudo, utilizamos a histomorfometria computadorizada para avaliar os túbulos seminíferos e epididimários, considerando as diferentes regiões do epi-

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² Laboratório de Biomorfologia Celular e Extracelular, Departamento de Morfologia, Universidade Federal Fluminense (UFF), Rua Hernani Mello 101, São Domingos, Niterói, RJ 24210-130, Brazil. E-mails: beatrizlanna09@gmail.com

ilmavetuff@gmail.com. *Autor para correspondência: chagas.m@gmail.com

³ Laboratório de Nutrição Experimental (LabNE), Departamento de Nutrição, Universidade Federal Fluminense (UFF), Niterói, RJ 24210-130. E-mail: gilsonb@gmail.com, ludmila.cardozo@gmail.com

dídimo (cabeça, corpo e cauda) de ratos submetidos a uma dieta prolongada de semente de linhaça. Foram utilizados ratos Wistar machos adultos jovens (n=20) divididos em 2 grupos, durante o período de lactação: Grupo Controle (GC) a base de caseína e Grupo Linhaça (GL) alimentados com 25% de semente de linhaça. Ao final de 250 dias de ingestão contínua, os animais foram sacrificados e amostra de sangue foi coletada. Os testículos e epidídimos foram retirados e fixados em formol tamponado. As amostras foram submetidas ao processamento histológico de rotina para parafina e coradas em hematoxilina e eosina. Foi feita a imunomarcagem com anticorpo antivimentina para identificação das células de Sertoli. Para morfometria, as imagens das lâminas foram digitalizadas e analisadas pelo software ImageJ para obtenção dos dados de altura epitelial, diâmetro e área tubular e luminal. Na avaliação hormonal o GL teve maior concentração de estrógeno sérico ($p=0,001$), mas nenhuma mudança na concentração de testosterona foi observada. Nos parâmetros morfométricos dos túbulos seminíferos e das regiões epididimárias, não houve diferenças significativas entre os grupos analisados. Da mesma forma, a quantificação das células de Sertoli não apresentaram diferenças significativas no GL ($p=0,98$). Estes resultados mostraram que o consumo contínuo e prolongado de 25% de semente de linhaça desde período gestacional até 250 dias de idade, mesmo com o aumento significativo nos níveis séricos de estradiol, não exerceram efeitos adversos sobre a estrutura testicular e epididimária, assim como nas células participantes da espermatogênese em ratos.

TERMOS DE INDEXAÇÃO: Semente, linhaça, *Linum usitatissimum*, testículo, epidídimo, ratos Wistar, túbulos seminíferos, células de Sertoli, fitoestrógenos, histomorfometria, vimentina.

INTRODUCTION

Flaxseed (*Linum usitatissimum*) is rich in lignans (phytoestrogen) and α -linolenic polyunsaturated fatty acids (omega-3) that have crucial functions in the prevention of cardiovascular diseases and the efficacy of chronic disease treatments (Kinniry et al. 2006, Kaithwas & Majumdar 2010a, 2010b). According to the United States Agriculture Department (USDA 2015), flaxseeds can be included in diets and consumed daily at 1% to 12% concentrations without any health risks.

The major phytoestrogen groups include lignans, isoflavones and coumestrol (Moutsatsou 2007). Flaxseed lignans are structurally similar to endogenous estrogen, as are the isoflavones present in soybeans, which have been described as an endocrine deregulator (Duncan et al. 2003, Wisniewski et al. 2003), depending on the administration dosage and duration. Exposure to isoflavones during prenatal life can alter testosterone levels and fertility rates (Benson et al. 2007, Eustache et al. 2009). McVey et al. (2004) reported reduced serum testosterone levels in rats that were fed soybean phytoestrogens for a long term. Tou et al. (1999) reported that a 10% flaxseed diet administered during pregnancy and lactation increased estradiol and testosterone levels in adult male offspring.

Studies have reported that it is possible to verify chan-

ges in Sertoli cell populations by specifically staining their main intermediate filament, vimentin, which is present since their development stage (Aumuller & Peter 1986, Mali et al. 1987, Russell & Griswold 1993). Sertoli cells provide primary support to the seminiferous epithelium, creating an adequate hormonal and nutritional environment for immature germ cell differentiation until spermatozoid (Allard et al. 1993). Vimentin expression is associated with complete spermatogenesis (De Miguel et al. 1997), allowing the quantification and presumption of possible alterations in germ cell populations (Clermont & Morgentaler 1955). Recently, soybean isoflavones were reported to be capable of exerting negative effects on vimentin, affecting Sertoli cell functions and possibly spermatogenesis (Yin et al. 2014).

The administration of phytoestrogen-containing diets during pregnancy and lactation can induce metabolic programming in the offspring because of its interference in the hormone system (Cardoso & Bao 2007, Wisniewski et al. 2005, Troina et al. 2010, Zhang et al. 2013). A short-term study has evaluated the effects of flaxseed phytoestrogens on the reproductive tract morphology of rats from gestation until puberty (age, 13 months old) (Oliveira et al. 2011). Sprando et al. (2000a, 2000b) analyzed lactating and developing rats that were fed 20% and 40% flaxseed diets; they did not report any alterations in the testicle structure or spermatogenesis following 70 days of consumption. No study has reported the effects of long-term exposure to 25% flaxseed diets on male reproductive systems, particularly testicular and epididymal morphometry. Current studies have focused on soybean isoflavones and their effects on reproductive health. Because of their similarity to these phytoestrogens, flaxseeds have been suggested to affect reproductive development (Boberg et al. 2013). Therefore, the present study aims at evaluating the changes in the testicular and epididymal morphology of rats whose mothers were administered flaxseed diets, from lactation to offspring adulthood (i.e., after 250 days of ingestion). We evaluated the morphometric parameters of the testis and epididymis, dosage of sex hormones and Sertoli cell immunostaining in all study groups.

MATERIALS AND METHODS

Experimental protocol. The rats used in this study were kept at the vivarium of the Laboratory of Experimental Nutrition (LabNE) of the Federal Fluminense University (UFF), Niterói, Brazil, under adequate conditions: temperature, 21°C to 23°C and 12-hour light/dark cycles with *ad libitum* water and food and a 25% flaxseed dietary supplement. The meals administered during lactation were prepared according to the diet for rodents recommended by the American Institute of Nutrition (AIN 93M) (Reeves et al. 1993). The present study was approved by the Ethics Committee on Animal Use of the UFF under the number 0583-14 and is a continuation of the study by Cardozo et al. (2012).

During lactation, the 90-day-old mothers, who were matched at a ratio of 3 female rats for every male rat, were administered a commercial meal (23% protein, Nuvilab®, Nuvital Ltda, Parana, Brazil) and were divided into Control Group (CG): rats who were fed a 17% protein casein-based meal and Flaxseed Group (FG): those who were fed a 17% protein meal with an additional 25% flaxseed. After weaning, 20 male pups were divided into 2 dietary

groups, according to their groups of origin: CG (n=10), fed a standard diet (casein-based, 10% protein) and FG (n=10), fed casein-based meals with 10% protein and an additional 25% flaxseed (AIN-93M).

After 250 days, the animals were euthanized with an intraperitoneal injection of 5% (0.15mL/100g c.p., i.p.) Thiopentax® (sodium thiopental 1G, Cristalia Produtos Químicos Farmacêuticos Ltda, Brazil) for blood collection through cardiac puncture. The blood sample was collected in tubes with and without EDTA for hormone evaluation. The right testicles and epididymis were removed for histological and immunohistochemical analyses. The left testicle was maintained in Karnovsky solution for further analysis through transmission electron microscopy.

Experimental diet. Flaxseeds were ground in blenders into flour, weighed and immediately used in meal preparations. The FG group meals had a 25% flaxseed content to administer the recommended fiber intake. The ingredients of the experimental meals (Table 1) were weighed and homogenized using an indus-

trial blender (Hobart®, São Paulo, SP, Brazil) by using boiled water for amid gelatinization. The obtained dough was transformed into pellets and dried in a ventilated oven (Fabbe-Primar® n171, São Paulo, SP, Brazil) at 60°C for 24h and was refrigerated after identification, until use. After weaning, the rats were administered a 10% protein diet in accordance with AIN-93M.

Biochemical methods. To determine hormone levels, specific commercial kits were used for each hormone. The blood samples were centrifuged (300rpm for 20 min at 4°C) to obtain the sera, which were individually stored at -20°C. Estradiol and testosterone levels were determined using the chemiluminescence immunoassay method with the sera and ELISA kits (ABCAM Cat. Numb. ab108667/ab108666) with Immulite 2000/PPC/H2967, Siemens, Los Angeles, USA analyzer.

Histological processing. Testicles and epididymis were collected following euthanasia and immediately fixed in 10% buffered formalin solution. The material was cleaved and processed using standard paraffin embedding techniques, sliced (thickness, 5µm), and stained with hematoxylin and eosin (Bancroft & Cook 1994).

Immunohistochemical processing. The sections were subjected to an automated simultaneous dewaxing and rehydration process by using PT Link DAKO PT100 equipment and washed in phosphate buffered saline (PBS) for 5 minutes. The sections were subsequently treated at room temperature with 3% hydrogen peroxide solution in methanol to block any endogenous peroxidase.

The slides were circled using a DAKO S2002 hydrophobic pen to avoid the diluted antibody solution from running. Immunostaining was performed with an antivimentin monoclonal (mouse) antibody (Code IR630, clone v9, DAKO) associated with an EnVision FLEX visualization system by using 4 µm-thick histological sections placed in silanized DAKO slides. Negative controls were incubated with PBS instead of the primary antibody (Fig.1A). Samples of a well-known tissue (palatine tonsil) with the antigen, were used as positive controls (Fig.1B). Subsequently, the peroxidase label was visualized through reaction with DAB (diaminobenzidine tetrahydrochloride) solution (Sigma-Aldrich Co, St Louis, MO, USA) at room temperature. The slides were subsequently dehydrated in decreasing concentrations of alcohol and washed 4 times with xylol. The slides were assembled using an ALLKIMIA synthetic Canada Balsam for future microscopic analyses.

For histomorphometric analyses, the sections were analyzed using an Olympus BX-51 light microscope coupled to a DP72 digital camera, where the microscope fields were transferred to a LG Flatron x17527 screen and measured using Image J software, version 1.47t.

Table 1. Control and flaxseed diets composition of each 100g of diet

Nutrients	AIN-93M	
	Casein (g)	Flaxseed (g)
Casein ^a	11.8	5.9
Flaxseed ^b	0	25
Starch ^c	61.2	54.1
Refined Sugar ^d	10	10
AIN 93G Minerals Mixa	3.50	3.50
Vitamins Mixa	1	1
Soy Oil ^e	7	0*
Cellulose ^f	5	0*
Choline Bitartrate	0.25	0.25
Cystine	0.18	0.18
Tert-Butyl hydroquinone	0.0008	0.0008
Total	100	100
Protein	11.68	11.00
Fat	19.75	22.54
Carbohydrate	68.57	66.46
Total Energy (kJ /100 g)	1485.9	1579.47

* The oil and flaxseed fiber diet are constituents to 25% of the added flaxseed. Ingredients used in diets preparation were provided by: ^a M. Casab Comércio e Indústria Ltda (São Paulo/SP, Brazil), ^b Arma Zen Produtos Naturais Ltda (Rio de Janeiro/RJ, Brazil), ^c Maisena da Unilever Bestfoods Brazil Ltda (Mogi Guaçu/SP, Brazil), ^d União (Rio de Janeiro/RJ, Brazil), ^e Liza da Cargill Agricultura Ltda (Mairinque/SP, Brazil), ^f Microcel da Blanver Ltda (Cotia/SP, Brazil).

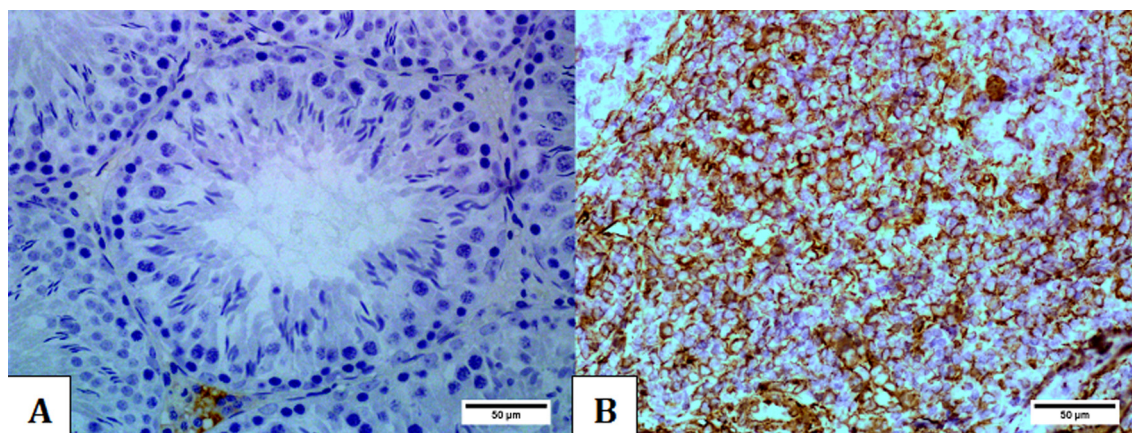


Fig.1. Photomicrographs showing the immunohistochemical staining by using antivimentin monoclonal antibody. (A) Negative control: Seminiferous tubules, not showing markings; (B) Positive control: Palatine tonsil (DAB chromogen). Magnification 400x.

Computerized histomorphometric analysis of epididymal regions and seminiferous tubules. Right testicles and epididymis were separated by the initial segment and tail region of the epididymis. The testicles were transversally cleaved, whereas the epididymis were longitudinally cleaved to for visualizing epididymal regions: head, body and tail as reported by Serre & Robaire (1998). For the morphometric analysis, 50 round seminiferous tubules, stages VIII–X of the spermatogenic cycle (Leblond & Clermont 1952), were randomly obtained from 5 sections. In epididymal tubules, 30 tubules from the head and body regions and 20 from the tail region were evaluated.

Tubular area and epithelium height quantification of seminiferous and epididymal tubules. The areas of the seminiferous and epididymal tubules were measured using the Image J *freehand tool* by demarcating the apical portion of the cells on the lumen surface. By using the Image J *straight tool*, 4 lines were drawn and evenly distributed in the epithelium to measure its height. The epithelial height and seminiferous tubules and epididymis head areas were measured using a 40× objective lens. The areas of the remaining epididymis regions were measured using a 20× objective lens.

Seminiferous and epididymal tubule diameter quantification. Tubular and luminal diameters were measured using 2 straight perpendicularly crossing lines originating from the borders of the tubules by using objective lenses that allowed visualizing the tubular structure.

Sertoli cell count. After immunostaining by using the anti-vimentin antibody, 25 tubules were randomly selected from each animal for Sertoli cell quantification. The tubules were visualized

at 40× magnification and the number of cells per tubule were counted using the plugin cell counter in Image J. The results were expressed as mean and standard deviation of the cells/tubules.

Statistical analysis. Statistical analyses were performed using GraphPad Instat 8.0 software. Data are shown as media ± standard deviation in a Microsoft Excel 2003 sheet. The normal distribution of the data was evaluated using the Kolmogorov–Smirnov test. After verifying data normality, the data were compared with independent samples by using the Student *t* test. For the results that did not show a normal distribution, the Wilcoxon nonparametric test was used. The significance level in all tests was established as *P* < 0.05.

RESULTS

Biochemical analysis

A significant increase was observed in 17β-estradiol (17%) in the FG group. Testosterone levels were not statistically significant (Table 2).

Histomorphometry of seminiferous tubules and Sertoli cell count

The height of the gametogenic epithelium, luminal area, tubular diameter and luminal diameter were not statistically significant (Fig.2A and B). Sertoli cell counts using vimentin staining (Fig.2C and D) did not show any significant differences among the studied groups, as shown in Table 3.

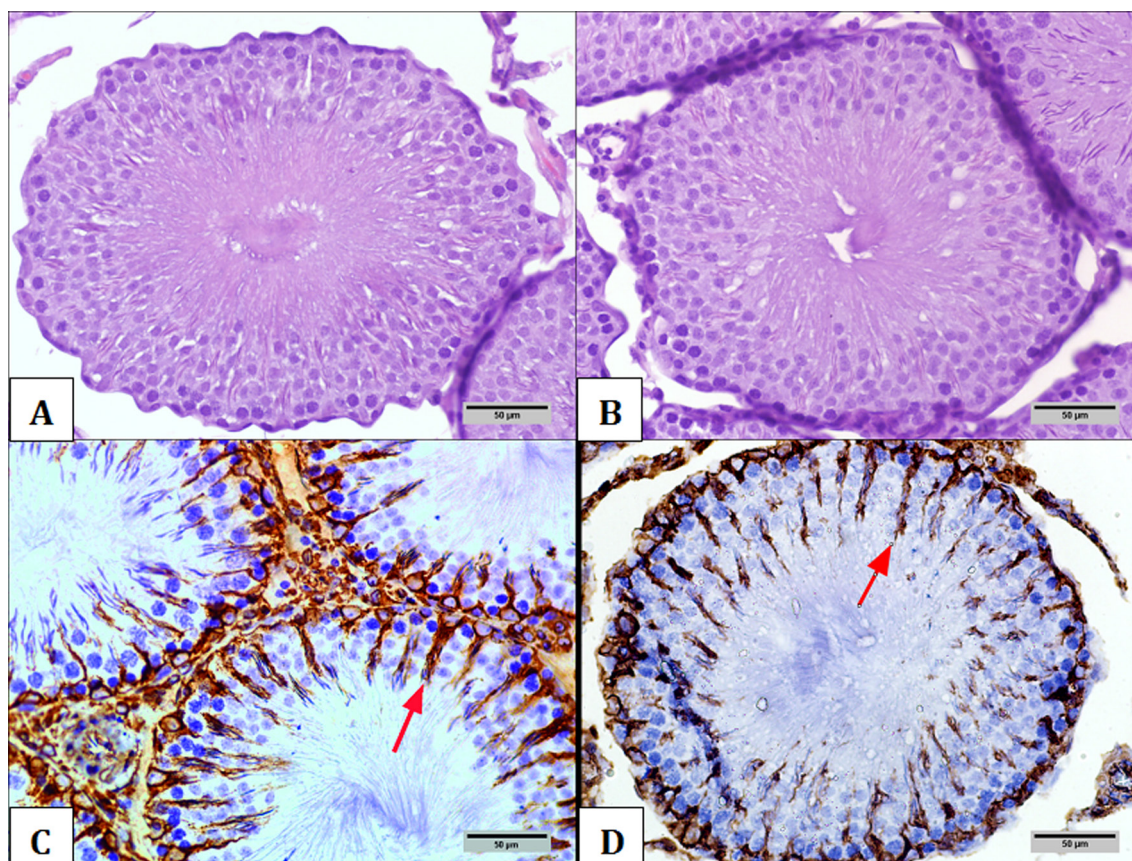


Fig.2. The seminiferous tubules. (A) Flaxseed group and (B) Control group: Morphological comparison of the Control and Flaxseed group. Hematoxylin and eosin staining; (C) Flaxseed group and (D) Control group. Immunohistochemical techniques performed for vimentin identification showed a strong immunostaining of these intermediate filaments (red arrow) in seminiferous tubule cells, enabling Sertoli cell counts. DAB chromogen. Magnification 400x.

Histomorphometry of epididymal tubules. Epididymal histological analyses of the animals administered 25% flaxseed diets did not show any tissue architecture altera-

Table 2. 17- β estradiol (pg/mL) and testosterone (ng/dL) serum concentration of 250-day-old rats

	FG (n=10)	CG (n=10)	P Value
17 β -estradiol (pg/mL)	39.50 $\pm$ 3.55	32.57 $\pm$ 3.51	p=0.001*
Testosterone (ng/dL)	303.35 $\pm$ 66.31	331.14 $\pm$ 66.92	p=0.397

FG = Flaxseed Group, CG = Control Group. *Significance p<0.05.

Table 4. Histomorphometry of tubules of epididymal regions of 250-day-old rats

	FG Head	CG Head	P Value	FG Body	GC Body	P Value	FG Tail	CG Tail	P Value
Epithelial height (μ m)	25.48 $\pm$ 3.97	25.96 $\pm$ 2.84	0.78	20.23 $\pm$ 2.83	20.86 $\pm$ 2.30	0.59	17.94 $\pm$ 5.69	17.63 $\pm$ 1.75	0.91
Tubular area (mm ²)	6.88 $\pm$ 1.35	7.21 $\pm$ 1.77	0.67	37.68 $\pm$ 9.72	38.91 $\pm$ 10.80	0.79	162.00 $\pm$ 32.94	158.63 $\pm$ 33.57	0.87
Luminal area (μ m)	91.43 $\pm$ 8.79	92.72 $\pm$ 9.49	0.78	211.49 $\pm$ 28.03	216.93 $\pm$ 32.60	0.69	413.15 $\pm$ 66.94	340.75 $\pm$ 80.88	0.16

Data show mean and standard deviation values of 10 rats per group. FG = Flaxseed Group, CG = Control Group. * Significance p<0.05.

tions compared with the control animals. Histological sections of all regions showed some sections with round tubules formed by pseudostratified columnar epithelium with stereocilia and spermatozoa in its lumen.

The mean epithelial height and tubular and luminal areas were smaller for the epididymal head and body regions of the experimental groups, unlike the tail region, which showed an increase in FG rats. However, the parameters evaluated in the different epididymal regions were not significantly different (Table 4).

DISCUSSION

Flaxseed has been used in the prevention of cardiovascular diseases (Hedelin et al. 2006) as well as in cancer risk reduction and cancer treatment because of the presence of compounds that provide health benefits (Mason & Thompson 2014). Flaxseed lignans are structurally similar to soybean isoflavones and endogenous estrogens and despite their benefits, can exert endocrine imbalances, affecting the male reproductive system (Duncan et al. 2003, Boberg et al. 2013). Therefore, the concentrations of lignans and its derivatives have gained attention because of the possibility of them affecting the gonadal morphology and hormone metabolism at certain administration doses and duration (Tou et al. 1999, 1998).

In our study, we used 25% flaxseed, attending the amount of dietary fiber recommended by the American Institute of Nutrition. After prolonged exposure to flaxseeds, serum estradiol levels significantly increased. Exposure to exogenous estrogen during gestation and lactation promotes local competition with endogenous estrogen, which can inhibit testicular androgen production (Sharpe & Skakkebaek 1993, Glover & Assinder 2006, Clark & Cochrum 2007, Wohlfahrt-Veje et al. 2009), structurally alter developing reproductive tracts and alter the functional regulation of Sertoli cells (Lucas et al. 2011), thus resulting in infertility. Troina et al. (2010) analyzed endogenous serum estrogen levels in lactating rats (age, 21 days), adult rats (age, 150 days) and their mothers who had been previously fed a 25% flaxseed diet during pregnancy. The 17- β estradiol concentrations in these mothers were transferred

Table 3. Histomorphometry of seminiferous tubules and Sertoli cell count of testes of 250-day-old rat

	FG	CG	P value
Epithelial height (μ m)	51.62 $\pm$ 4.36	53.76 $\pm$ 5.85	0.36
Luminal area (mm ²)	19.75 $\pm$ 3.08	17.76 $\pm$ 3.91	0.24
Tubular diameter (μ m)	267.26 $\pm$ 17.12	258.60 $\pm$ 28.61	0.42
Luminal diameter (μ m)	158.94 $\pm$ 11.03	148.64 $\pm$ 19.71	0.16
Sertoli cell count (Vimentin)	35.88 $\pm$ 2.44	35.91 $\pm$ 2.27	0.98

Data show mean and standard deviation values of 10 rats per group. FG = Flaxseed Group, CG = Control Group. * Significance p<0.05.

to their offspring during lactation, resulting in increased estradiol concentrations in these lactating offspring which remained high in their adulthood. Cardoso et al. (2010) observed an increase in estradiol after a prolonged intake of 25% flaxseed, without changes in the penis morphology. Although 25% flaxseed was extrapolate recommended by the USDA (2015), prolonged consumption at this concentration did not cause changes in reproductive morphology.

Androgen suppression in neonates is associated with synthetic estrogen, which results in testicular morphological alterations. Soybean phytoestrogens, in high and low dosages, have been reported to reduce testosterone serum concentrations (Weber et al. 2001, Caceres et al. 2014). Wisniewski et al. (2003) stated that reduced testosterone biosynthesis can affect the reproductive physiology and behavior of phytoestrogen-fed rats. McVey et al. (2004) evaluated rats fed with soybean isoflavones at different concentrations and exposure times and reported that during the prolonged period of 240 days, androgen levels were reduced in all the evaluated groups. In the present study, continuous exposure to flaxseed lead to a nonsignificant decrease in serum testosterone levels, without morphologically altering the reproductive system of the tested rats. These results suggest that flaxseed administered for a prolonged period is more favorably assimilated by males than is soybean and does not lead to marked alterations in serum testosterone serum levels alterations.

No significant morphological changes were observed in any of the regions of the epididymal tubules of either pre- or post-natally flaxseed-administered rats. This behavior was also observed with soybean-based diets. Piotrowska et al. (2011) subjected rats to soybean isoflavones until sexual maturation and reported an increase in estradiol, reduction in testosterone, and normal epididymal morphology.

In histomorphometry, seminiferous tubules should be analyzed because they are considered a reliable indicator of spermatogenic maturation (Schinckel et al. 1983, Tegegne et al. 1991). Moreover, tubular diameters have been used as a positive correlation parameter of testicular spermatogenic activity (Sinha Hikim et al. 1988). Sprando et al. (2000b) measured 100 seminiferous tubules per treatment group

and did not report any significant differences in the short-term results of rats fed with 20% and 40% flaxseed diets. Oliveira et al. (2011) did not observe any statistical differences in morphometric parameters among the flaxseed-fed groups and concluded that the intake of a 12% flaxseed flour diet does not exert negative effects on sperm and morphometric analyses in rats. In this study, 500 tubules were measured per group, a 25% flaxseed diet was administered and similar results were obtained. Continuous lifetime exposure of rats does not result in long-term testicular morphological alterations.

The spermatogenic capacity of adult animals depends on the adequate development of Sertoli cells. Vimentin intermediate filaments expressed during the fetal life persist in adult testicles (Frojdman et al. 1989, Stosiek et al. 1990, Rogatsch et al. 1996, Bar-Shira Maymon et al. 2000) and support germ cells. Sprando et al. (2000b) evaluated Sertoli cells in rats administered 20% flaxseed diets for 70 days and did not observe any significant differences. In our study, even after a 250-day intake of the administered flaxseed content, the number of Sertoli cells remained unchanged.

A limitation of this study is that the sperm count was not evaluated. Motility, concentration, vigor and sperm pathologies can provide additional information about the spermatogenic activity after the prolonged consumption of flaxseed.

CONCLUSION

Because of the absence of testicular and epididymal morphometric alterations in the flaxseed-fed groups, we suggest that the continuous and prolonged consumption of this seed, even if initiated during gestation led to an increase in serum estradiol levels, but did not interfere with the male gonad morphological development.

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