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Prepubertal subchronic exposure to soy milk and glyphosate leads to endocrine disruption

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ABSTRACT

Lactose intolerance is characterized by low or inexistent levels of lactase, and the main treatment consists of dietary changes, especially replacing dairy milk by soy milk. Soy contains phytoestrogens, substances with known estrogenic activity, besides, glyphosate-based herbicides are extensively used in soy crops, being frequently a residue in soy beans, bringing to a concern regarding the consumption of soy-based products, especially for children in breastfeeding period with lactose intolerance. This study evaluated the pubertal toxicity of a soy milk rich feeding (supplemented or not with glyphosate, doses of 50 and 100 mg/kg) during prepubertal period in male rats. Endocrine disruption was observed through decrease in testosterone levels, decrease in Sertoli cell number and increase in the percentage of degenerated Sertoli and Leydig cells in animals receiving soy milk supplemented with glyphosate (both doses) and in animals treated only with soy milk. Animals treated with soy milk with glyphosate (both doses) showed decrease spermatids number and increase of epididymal tail mass compared to control, and decrease in the diameter of seminiferous tubules compared to soy milk control group. Animals receiving soy milk supplemented with 100 mg/kg glyphosate showed decrease in round spermatids and increase in abnormal sperm morphology, compared to control.

1. Introduction

A large number of people have lactose intolerance in the world. Since the main treatment consists of dietary changes, it leads to an increase in the consumption of soy-based products, e.g. soy milk. The lactase (enzyme that breaks lactose into glucose and galactose) activity decreases during the human development, however, lactase deficiencies might occur in children in breastfeeding period, who cannot consume breast milk or milk from other animal sources. Thus, soy-based products are their main source of food (Mäkinen et al., 2014; Mattar and Mazo, 2010). It poses a health hazard due to the presence of phytoestrogens of soy (also known as isoflavones), compounds with well known estrogenic activity that may impair the normal endocrine development (Lund and Lephart, 2001; Pan et al., 2008; WHO, 2012).

Moreover, innumerous studies suggest that glyphosate-based herbicides, a group of herbicides widely used in soy crops, are potencially harmful to the endocrine system, especially the formulations containing the surfactant polyoxyethyleneamine, even in concentrations lower than the acceptable limits (Dallegrave et al., 2002; Dallegrave et al., 2007; El-Shenavy, 2009; Gasnier et al., 2009; Mesnage et al., 2012). Studies show that these compounds and their metabolites are major contaminants in surface waters and they generally persist in agricultural products, posing a relevant risk to human and animal health (Bohm et al., 2008; Clair et al., 2012; Dalsenter et al., 1999; Dallegrave et al., 2007). Glyphosate exposure is also related to metabolic disorders, like neurodegenerative damage (Astiz et al., 2012; Cattani et al., 2014) and oxidative stress (Çağlar and Kolankaya, 2008; El-Shenavy, 2009; Mesnage et al., 2012).

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Considering that children have an exclusive milky diet, they are at a higher risk because of their lower body mass and the period of development that the exposure occurs, and, the presence of phytoestrogens might enhance the endocrine-disrupting properties of glyphosate (Thongprakaisang et al., 2013). In light of these aspects, this study aimed to evaluate the reproductive toxicity of a diet rich in soy milk, supplemented or not with glyphosate, during prepubertal period in male Wistar rats.

2. Materials and methods

2.1. Products

Roundup Original®, produced by Monsanto, was used in the experiment. Soy milk (5,2 g protein per serving) was used as feed control.

2.2. Experimental model

The experiment was conducted in 23 days old male Wistar rats, divided into four groups (five animals per group). The treatment was administered daily, for 35 days, through gavage, considering a volume of 10 mL/kg. The control group received saline solution, the soy milk control group received soy milk, the glyphosate 50 group received soy milk plus 50 mg/kg glyphosate and the glyphosate 100 group received soy milk plus 100 mg/kg glyphosate. The doses were chosen according to preliminary studies (Dallegrave et al., 2007).

The dosing solutions were prepared weekly. They were maintained in amber glass bottles in the refrigerator (4 °C) to avoid photodegradation and heat.

The animals were housed in individual cages, allowing individualized evaluation of body weight gain and toxicity signs (respiratory function, piloerection, diarrhea, cyanosis and mucosal pallor). They were housed in a temperature- and humidity-controlled environment. Food and water were provided *ad libitum* and animals were subjected to a 12 h light/dark cycle. This project was approved by the Ethics Committee on Animal Use of University of Passo Fundo (protocol number 008/2014). After 35 days of treatment, in the end of childhood (puberty begins at day 50, approximately) (Caceres et al., 2015), animals suffered euthanasia under anaesthesia with ketamine/xylazine (100 mg/kg and 10 mg/kg).

2.3. Evaluation of sexual organs and spermatozoa production

Testes, epididymis, prostate and seminal vesicle were excised and their weight evaluated according to whole bodymass of each animal (prostate without involucre and seminal vesicle without its content).

Testes were removed from the tunica albuginea, put in 10 mL of saline solution 0,9% with Triton-X 0,05%, and homogenized during one minute. 100 μL of the mix of each testicle were diluted in 900 μL of saline solution 0,9%. After the dilution, the spermatids resistant to homogenization (stages 17–19) were counted in Neubauer chamber.

Cauda epididymis were sliced into small parts, put into 10 mL of saline solution 0,9% with Triton-X 0,05% and homogenized. Spermatozoa were counted in Neubauer chamber.

To assess the percentage of morphologically abnormal sperm (detected in the head or tail piece) the deferens ducts were rinsed with 0.5 mL 0.9% NaCl (for 65-day-old animals) and a sperm suspension was obtained. An aliquot of sperm suspension was carefully stained with 2% eosin to prepare a smear on the slide. Fifty sperm per animal were analyzed microscopically at 400 × magnification and the morphology of sperm was recorded according to the presence or absence of defects found in the head or tail of the spermatozoon (adapted by Dallegrave et al., 2007).

For histologic evaluation, 5 testis per group were fixed in Bouin's solution, included in paraffin, sectioned at 3 μm and stained with hematoxylin/eosin. One hundred essentially round seminiferous tubules per testis (stages VII and VIII) were measured at 200 × magnification to assess the mean tubule diameter, accepting a deviation of 5% in the x vs. y ratio, with an imaging system Image J. The number of round and elongating spermatids were counted in round tubule cross sections at stage VII of the seminiferous epithelium cycle, via light microscopy. And also, normal and degenerated Sertoli cells per tubule and Leydig cells around of these were counted. On counting Sertoli cells we considered the nucleoli and on Leydig cells we counted the nuclei, and the magnification was 400×. The proportion of degenerated cells in relation to normal cells was calculated.

2.4. Hormonal analysis

In the day of the euthanasia, blood was collected into serum separating tubes, and immediately sent to laboratory to analyze testosterone and free T_4 serum levels, according to recommendations of the pubertal development assay of OECD (Organisation for Economic Co-operation and Development) (OECD, 2010). This assay aims to identify chemicals able to interact with androgen receptors and thyroid, and able to interfere with hormones production. This assay also detects compounds that may modify pubertal development through changes in hipothalamic-pituitary-gonadal axis. The prepubertal period is extremely susceptible to substances that might interfere with endocrine system (OECD, 2010).

2.5. Statistical analysis

Data distribution was considered normal after Shapiro-Wilk test, thus statistical comparison was performed with One-Way ANOVA followed by Bonferroni *post hoc* tests. Significance was accepted at $p < 0.05$ *vs* Control.

3. Results

3.1. Hormonal analysis

A significant decrease in testosterone serum levels was observed in the serum of animals that received only soy milk without glyphosate supplementation and in animals treated with glyphosate compared to control (Fig. 1) (see Fig. 6, Table 1). No significant difference was observed on free T_4 levels (*data not shown*).

3.2. Clinical evaluations

No significant difference was observed on feed, body weight gain and water intake of the animals during the period of study. No toxicity signs were observed either (*data not shown*).

3.3. Evaluation of sexual organs and sperm count

The evaluation of relative weight of sexual organs revealed that treatment supplemented with glyphosate (both doses) led to a significant increase in cauda epididymis weight compared to control, as shown in Fig. 2. In prostate, seminal vesicle, testes and epididymis relative weight, no significant difference was observed (see Fig. 6, Table 1).

A significant reduction in spermatids resistant to homogenization in both glyphosate groups was observed, compared to control (Fig. 3). No significant differences were observed in sperm count

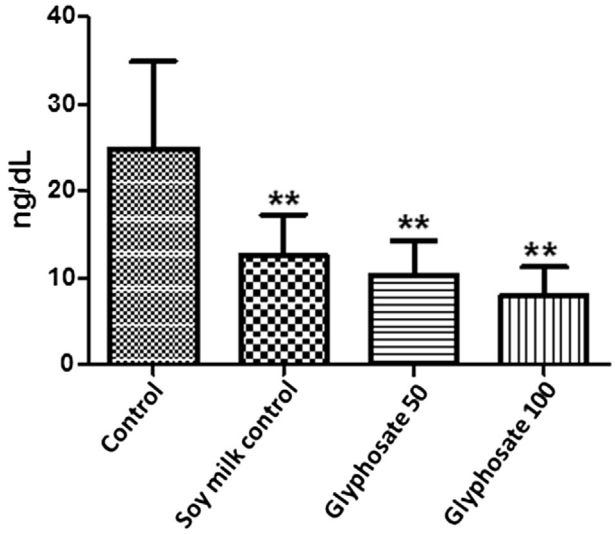

Fig. 1. Testosterone levels. Results are presented as means ± standard deviation (n = 5 animals per group). Statistical analysis was performed with One-Way ANOVA followed by Bonferroni *post hoc* test (**$p < 0.01$ *vs* Control).

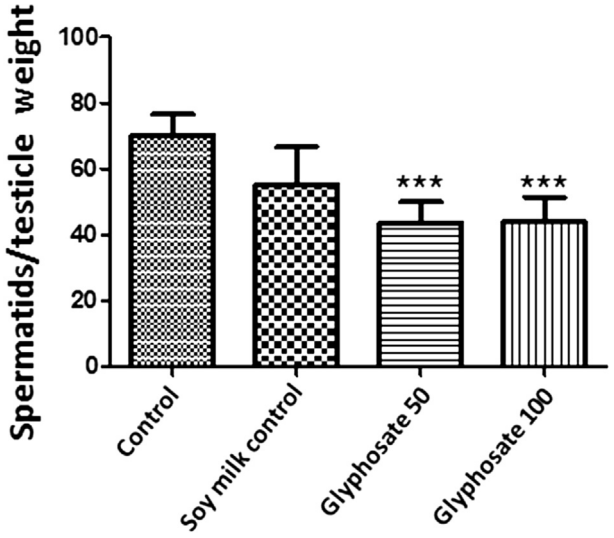

Fig. 3. Spermatids/testicle weight. Results are presented as means ± standard deviation (n = 5 animals per group). Statistical analysis was performed with One-Way ANOVA followed by Bonferroni *post hoc* test (***$p < 0.001$ *vs* Control).

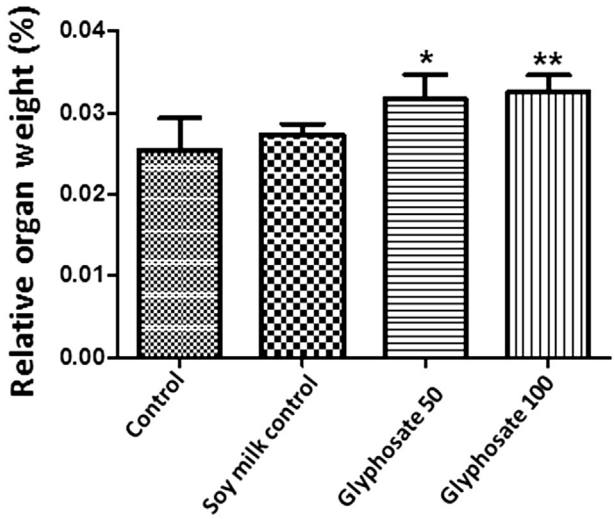

Fig. 2. Relative weight of cauda epididymis. Results are presented as means ± standard deviation (n = 5 animals per group). Statistical analysis was performed using One-Way ANOVA followed by Bonferroni *post hoc* test (*$p < 0.05$ *vs* Control; **$p < 0.01$ *vs* Control).

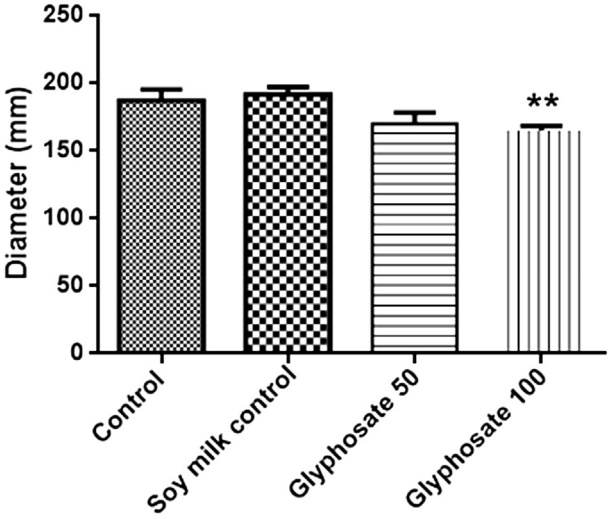

Fig. 4. Diameter of seminiferous tubules. Results are presented as means ± standard deviation (n = 5 animals per group). Statistical analysis was performed with One-Way ANOVA followed by Bonferroni *post hoc* test (**$p < 0.01$ *vs* Control).

(data not shown).

On the evaluation of the diameter of seminiferous tubules, a significant difference was observed between the group Glyphosate 100 compared to group Soy milk control (Fig. 4).

On the evaluation of sperm morphology, a significant difference was observed on the Glyphosate 100 group with both the Control and Soy milk control groups (***$p < 0.001$ *vs* Controls). The main sperm alterations were coiled and irregular tail (Fig. 5).

4. Discussion

The present study evidenced a decrease in testosterone in animals receiving a rich soy milk diet during prepubertal development. This change occurs in all groups receiving soy milk, supplemented or not with glyphosate. Endocrine disruption was more evident in groups supplemented with glyphosate, as highlighted by decrease in the spermatids resistant to homogenization, the increase in the relative weight of the epididymis tail and increase in abnormal sperm morphology compared to control group. Histopathologic evaluation evidenced a decrease in the diameter of seminiferous tubules in animals receiving 100 mg/kg of glyphosate compared to soy milk group, decrease in round spermatids in glyphosate 100 mg/kg group compared to control, decrease in Sertoli cell and, increase in percentage of degenerated Sertoli and Leydig cells in all groups compared to control.

Spermatogenesis is controlled by gonadotrophins produced by the pituitary gland, through hypothalamic-pituitary-gonadal axis action. The luteinizing hormone (LH) acts on Leydig cells in the testes, inducing the secretion of testosterone, hormone that stimulates spermatogenesis process and is responsible for the characteristics of the male body (Guyton and Hall, 1988; O'Hara and Smith, 2015). The follicle-stimulating hormone (FSH) acts on Sertoli cells in testes, which have the function of nourishing and

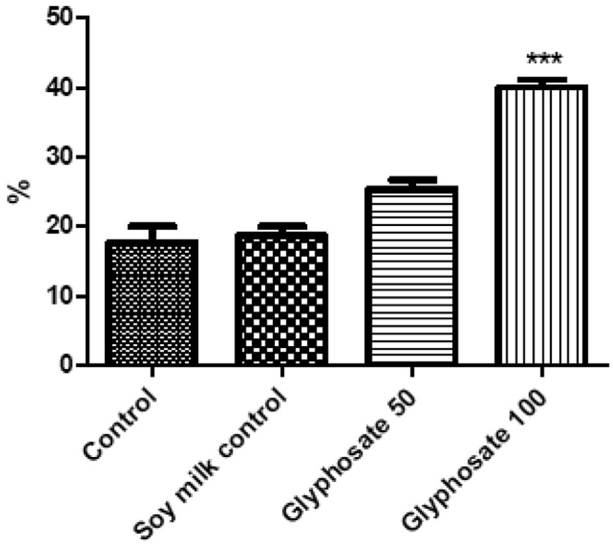

Fig. 5. Sperm morphology. Results are presented as means ± standard deviation (n = 3 animals per group). Statistical analysis was performed with One-Way ANOVA followed by Bonferroni *post hoc* test (***$p < 0.001$ *vs* Control and ***$p < 0,001$ *vs* Soy milk control).

protecting the developing sperm cells (O'Hara and Smith, 2015). In the present study, due to sample limitations, we focused on testosterone changes and did not evaluate FSH and LH levels. However, the FSH and LH levels probably decreased, as observed by Romano et al. (2012), who observed glyphosate-induced changes in LH and FSH mRNA expression in rats treated in gestational period to 50 mg/kg of glyphosate.

In our study, decreases in testosterone serum levels suggests a disruption in hypothalamic-pituitary-gonadal axis or a direct effect on Leydig cells, as shown in a previous study using rat Leydig cells (Clair et al., 2012). In a research conducted by Cavalli et al. (2013) it was suggested that a glyphosate-based herbicide leads to Sertoli cell death through oxidative stress in prepubertal testes after a 30-min of cell exposure. Since Sertoli cells are important during spermatogenesis process, these findings help to explain testosterone decreased levels in our study, since the Sertoli cell number was decreased and the percentage of degenerated Sertoli cell was increased. Besides, we observed an increase in the percentage of degenerated Leydig cells, another reason for the great decrease in testosterone observed in our study. It also explains the fact that we did not observe decrease in seminiferous tubules weight but we did in testosterone levels. The degenerated Leydig cells aren't able to maintain testosterone production in normal levels.

Interestingly, testosterone decreased levels and percentage of degenerated Sertoli and Leydig cells were also observed in the soy milk group without glyphosate supplementation. Soy-based products contain phytoestrogens (eg. genistein and daidzein), substances known as having estrogenic properties due to the structure similar to female hormones, the oestrogens, especially the 17 β-estradiol (Lund and Lephart, 2001). Various studies evidence the estrogenic properties of phytoestrogens (Lephart et al., 2004; Lund and Lephart, 2001; Setchell and Clerici, 2010). An increase in proliferative activity of developing Leydig cells followed by a suppression of their steroidogenic capacity in adulthood and decrease in testosterone serum levels in the offspring of dams feeded with soybean diet in the neonatal period was already described (Napier et al., 2013). Moreover, rats treated in juvenile period with daidzein (20 mg/kg and 100 mg/kg) showed a reduction in testosterone serum levels (Pan et al., 2008).

Regarding the relevance of these results for sexual male development, more studies are necessary to elucidate whether these effects are a consequence of ingestion of soy milk *per se* or glyphosate residues in soy milk are responsible for the effects considering that soybeans frequently contained elevated levels of glyphosate residues, sometimes above the allowed limits (Bohm et al., 2008).

Despite the clear alterations in testosterone serum levels observed in this and in prior studies, it is worth mentioning that these changes are not always observed. The offspring of dams treated with glyphosate in the doses of 50, 150 and 450 mg/kg during pregnancy and lactation had not shown any significant alterations in testosterone serum levels (Dallegrave et al., 2007). Furthermore, another study demonstrated an increase in testosterone serum levels in male offspring exposed to glyphosate (50 mg/kg) during the critical period of sexual hypothalamic differentiation, the perinatal period (Romano et al., 2012). These differences from our study are probably due to the different dose regimen and the different periods of administration. In these studies, the exposure period was during pregnancy and in a short period after birth. Our study considered a subchronic prepubertal exposure, as recommended by OECD guidelines. Furthermore, the administration of soy milk in our study might be another reason for the testosterone serum levels decrease.

In spite of the absence of significant differences in sperm count, the spermatids resistant to homogenization count showed a significant decrease in both groups treated with glyphosate compared to control and the number of round spermatids decreased in the exposure to 100 mg/kg of glyphosate compared to control. This result corroborates the occurrence of endocrine-disrupting effects linked to glyphosate. In a prior study, rats exposed to a commercial formulation of glyphosate during pregnancy and lactation in the doses of 150 and 450 mg/kg evidenced reproductive toxicity, characterized by decrease and vacuolization in spermatids of male rats. It was also observed an increase in the percentage of abnormal spermatozoa (Dallegrave et al., 2007). Likewise, rats exposed to a glyphosate-based herbicide during the period of 23–53 days old promoted a delay in the beginning of puberty, suggesting that chronic exposure to this formulation leads to endocrine disruption in the hypothalamic-pituitary-gonadal axis (Romano et al., 2008). The decrease in the spermatids resistant to homogenization may be due to the decreased levels of serum testosterone or LH secretion, since these hormones are essential for all spermatogenesis process, allowing the development from the stages of spermatocytes, to spermatids and then spermatozoa (Guyton and Hall, 1988; O'Hara and Smith, 2015). Another hypothesis is that decrease in spermatid numbers reflect Sertoli cell damage or direct effects on the Leydig cells, both observed in our study.

The increase in cauda epididymis weight suggests the occurrence of oedema in sexual organs, what evidences acute reproductive toxicity. The increase in cauda epididymis weight was also demonstrated by Romano et al. (2008), who administered glyphosate doses of 50 mg/kg once daily, from day 18th in pregnancy until five days after birth. Spermatogenesis in rats takes approximately 56 days, and begins in puberty. In this study, animals were euthanized at the beginning of puberty (65 days old), what explains that we only observed significant difference in spermatids count and not in sperm count. However, the reduction of spermatids may impact on sperm quality (Creasy, 1997; Dallegrave et al., 2007; Guyton and Hall, 1988). In our study, we observed an increase in abnormal sperm morphology in the group exposed to 100 mg/kg of glyphosate compared to both the Control group and the Soy milk control group, which corroborates the endocrine disruptor effects of glyphosate exposure in prepubertal period.

Considering the fact that the decrease in seminiferous tubules

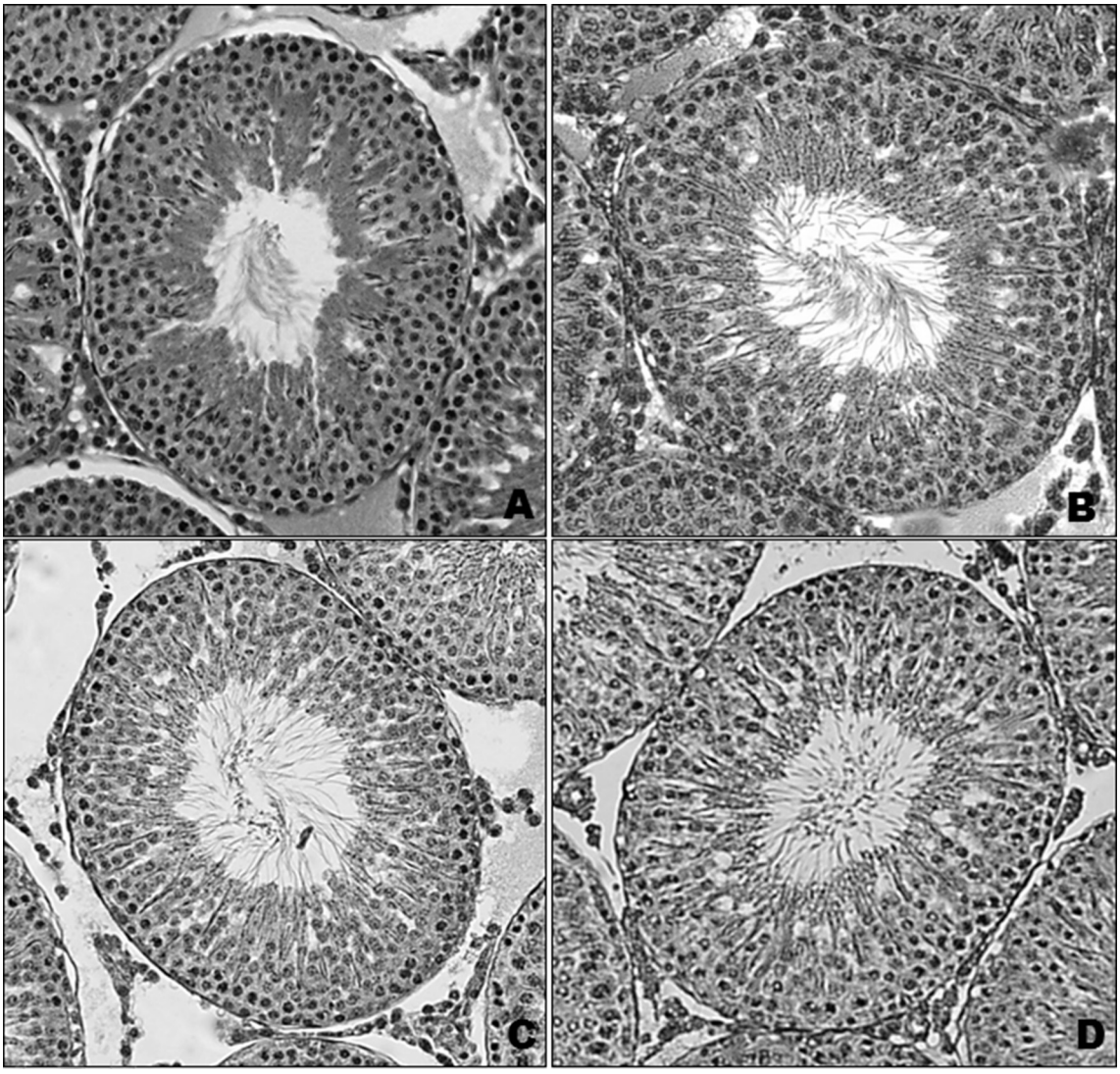

Fig. 6. Testicular histology 400× magnification. A: Control group, B: Soy milk group, C: Soy milk +50 mg/kg glyphosate, D: Soy milk +100 mg/kg glyphosate.

Table 1
Histopathological evaluation of testes. Data are expressed as mean ± standard deviation (n = 5). Statistical analysis was performed using One-Way ANOVA followed by Bonferroni *post hoc* test (*$p < 0,05$ *vs*. Control,**$p < 0,01$ *vs*. Control).

Parameters	Groups					
	Rats (n)	Control	Soy milk	Glyphosate 50	Glyphosate 100	p (ANOVA/Bonferroni)
Round spermatids number	5	218.6 ± 36,63a	185.8 ± 30,90a	180.4 ± 19,65a	127.4 ± 53,83b	0.011*
Elongated spermatids number	5	143,0 ± 42,05	106.6 ± 21,10	120.0 ± 22,43	105.0 ± 31,42	0.214
Sertoli cells number	5	91.4 ± 51,51a	29.6 ± 17,42b	25.2 ± 8,07b	28.4 ± 14,53b	0.005**
Leydig cells number	5	97.6 ± 22,38	99.8 ± 35,70	88.4 ± 33,50	62.0 ± 23,21	0.197
Sertoli cells degeneration (%)	5	10.3 ± 4,33a	83.7 ± 25,92b	101.3 ± 48,94b	68.0 ± 34,92b	0.003**
Leydig cells degeneration (%)	5	10.3 ± 3,40a	44.4 ± 3,77b	53.4 ± 30,84b	66.3 ± 25,27b	0.003**

diameter was significant in the higher dose administered (100 mg/kg) this effect is probably a consequence of glyphosate exposure, without interference of phytoestrogens. This result is related to the decrease in spermatids resistant to homogenization count in animals treated with both doses of glyphosate, since spermatozoa are produced in seminiferous tubules. In contrast, the studies performed by Dallegrave et al. (2007) and Romano et al. (2008) observed no significant differences in this parameter in animals treated with commercial formulations of glyphosate in different periods of treatment.

Despite the sample limitations in this study, the results presented here show enough evidence that glyphosate impairs male reproductive system by affecting innumerous components in a prepubertal exposure.

5. Conclusions

To the best of our knowledge, this study demonstrate for the

first time a relevant endocrine disruption of a soy milk rich diet during prepubertal period. Sexual development is more affected in groups receiving soy milk supplemented with glyphosate.

Conflict of interest

The authors declare that they have no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.12.030.

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