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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