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EFFECTS OF TWO PHYTOTHERAPIC FORMULATIONS CONTAINING *Glycine max* (L.) MERR ON MALE RAT FERTILITY

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ABSTRACT: **The present study evaluates the effects of two commercial phytotherapic formulations containing *Glycine max* (L.) Merr. (Soy) (Phytotherapic A and Phytotherapic B) on Wistar rat fertility. Doses were based on the manufacturer's recommendation and increased in a logarithmic scale. The animals were divided into six experimental groups and a control group, which received distillated water. Three groups were treated with Phytotherapic A and three groups were treated with Phytotherapic B. The doses were: 4.3mg.kg^{-1}.day^{-1}, 21.5 mg.kg^{-1}.day^{-1} and 43 mg.kg^{-1}.day^{-1}, respectively, for GPA1, GPA2, GPA3, and GPB1, GPB2, GPB3. The males were treated during ninety-one days, before and during the mating. Female Wistar rats were treated before and during the mating, pregnancy and lactation. The total number of spermatozoa, their daily production sperm morphology, histopathology and weight of sexual organs were evaluated. The results showed the interference of the phytotherapic formulations A and B in the total number of spermatozoa and in the sperm morphology in a dose-dependent manner.**

*(Key words: soy; **Glycine max**; phytotherapic; male rats; fertility; toxicity)*

EFECTOS DE DOS FORMULACIONES FITOTERAPÉUTICAS QUE CONTIENEN *Glycine max* (L.) MERR SOBRE LA FERTILIDAD DE RATAS MACHOS

RESUMEN: **El presente estudio evalúa los efectos de dos formulaciones comerciales que contienen fitoterapéuticos Glycine max (L.) Merr. (Soja) (fitoterapéutico A y fitoterapéutico B) sobre la fertilidad de ratas Wistar. Las dosis se basaron en las recomendaciones del fabricante y el aumento en una escala logarítmica. Los animales se dividieron en seis grupos experimentales y un grupo control, que recibió agua destilada. Tres grupos fueron tratados con un fitoterapéutico y tres grupos fueron tratados con fitoterapéutico B. Las dosis fueron: 4.3mg.kg^{-1}.day^{-1}, 21,5 mg.kg^{-1}.day^{-1} y 43 mg.kg^{-1}.day^{-1}, respectivamente, para GPA1, GPA2, GPA3 y GPB1, GPB2, GPB3. Los machos fueron tratados durante noventa y un días, antes y durante el apareamiento. Las Hembras Wistar fueron tratadas antes y durante el apareamiento, el embarazo y la lactancia. Número total de espermatozoides, la producción diaria de espermatozoides, la morfología del esperma, la histopatología y el peso de los órganos sexuales fueron evaluados. Los resultados mostraron la interferencia de las formulaciones fitoterapéuticos A y B en el número total de espermatozoides y en la morfología de los espermatozoides de una manera dosis-dependiente.**

*(Palabras clave: soja; **Glycine max**; fitoterapéuticos; ratas machos; fertilidad; toxicidad)*

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INTRODUCTION

Soybean is a leguminous species of the Fabaceae family, rich in phytochemical compounds, especially isoflavones. Products derived from the soybean (such as flour, milk, or tofu) contain significant concentrations of isoflavones.

Isoflavones are members of the polyphenol group and their chemical structure is closely related to human estrogen. They are capable of inducing marked hormonal effects and are designated phytoestrogens (1).

There is extensive literature covering the use of soybean for prevention and treatment of several diseases. The therapeutic indications include: milk substitution for allergic children, management of pre- and post-menopausal symptoms, prevention of osteoporosis, prevention and treatment of cancer, prevention of cardiovascular diseases, and as an adjuvant to the handling of diabetes. However, many contradictory studies report toxic effects of isoflavones on fertility.

Moreover, there is substantial bibliography regarding potential adverse outcomes from the ingestion of soybean and its isoflavones. Among others, interference in the immunological system, changes in thyroid function, reduction of vitamins and minerals absorption, impairment of the myelinization process, triggering of cancer and damage to DNA chain have been recorded (2, 3, 4, 5).

The best documented effects are related to the activity on reproduction. With this regard, soybean and its isoflavones are classified as endocrinal disrupters (6, 7, 8). *In vitro* studies have shown that genistein induces apoptosis of testicular cell lines, and inhibits their growth and proliferation. Also, it may interfere with percentage of sperm motility and modulate sperm capacitation, acrossome reactions, and fertilizing ability. Histopathologic findings in males included ductal alveolar hyperplasia and hypertrophy in the mammary glands; aberrant or delayed spermatozoa and decreased sperm in the epididymis (9, 10, 11, 12).

The consumption of pharmaceutical products based on soybean and claimed as natural substances has grown exponentially, representing a real danger for public health since many of the active principles of soybean have not been well evaluated. Therefore, studies about the toxicity of such phytotherapics, besides assessment of their safety and efficacy on rat fertility have become a matter of highest necessity.

The present study has evaluated the effects of two phytotherapic preparations on the fertility and reproductive performance of male Wistar rats (13). It is part of a larger appraisal about the reproductive toxicity of soybean-based phytotherapic substances that was recommended by the Federal Drug Administration (FDA) and the Organization for Economic Cooperation and Development (OECD).

METHODS

Phytotherapic formulations

The phytotherapic formulations used in the experiments were acquired in local pharmaceutical establishments, containing the same batch number and date of manufacture and were within the validity period in all cases. The corporate name for Phytotherapic A was Ache Laboratórios Farmacêuticos S/A, and for Phytotherapic B was Herbarium Laboratório Botânico Ltda. The declared composition in the package leaflet of Phytotherapic A was dry extract of *Glycine max* (L.) Merr. 40% in 150mg capsule and of Phytotherapic B was dry extract of *Glycine max* (L.) Merr 40% in the 75mg capsule. The experimental doses were obtained based on the manufacturer's recommendations as follows: G1 – the therapeutic dose (4.3mg.kg^{-1}), G2 – five times therapeutic dose (21.5 mg.kg^{-1}), G3 – ten times therapeutic dose (43mg.kg^{-1}). The phytotherapic formulations were prepared through the dilution of the contents of the capsules, using distilled water as a vehicle, stored in amber vial and kept under refrigeration.

Quantification of isoflavones

Confirmation of the isoflavones levels per capsule of the Phythoterapic was performed by high performance liquid chromatography (HPLC).

HP1100 liquid chromatograph (Agilent, CA, USA) consisting in quaternary pump, degasser, autosampler, diode array detector (DAD) were used under chromatographic conditions described by César et al. (14). Conjugates malonyl-glucosides and total isoflavones as aglycones were calculated based on their molecular weight. The content of isoflavones found in the sample was: daidzin: 8.93%, genistin: 3.89%, Daidzein: 25.75%, Genistein: 16.61% (GPA) and, daidzin: 8.04%, genistin: 3.05%, Daidzein: 25.6%, Genistein: 15.65% (GPB). The results ensured the isoflavones levels produced by the laboratories.

Animals

The 24 males and 72 females albino Wistar rats from the Center of the Reproduction and Experimentation of Laboratory Animals of the Universidade Federal do Rio Grande do Sul (UFRGS) were kept under a day/night cycle (lights on 9:00 am to 9:00 pm), room temperature

21ºC ± 1, and 50% ± 5 relative humidity. The animals received a standard pellet diet (Nuvital CR 1®, Paraná, Brazil) and tap water *ad libitum* throughout the experiment. The rats were adapted to these conditions in their own animal quarters for 2 weeks before starting the experiment. Breeding, housing, and experimental procedures followed guidelines published in the NIH Guide for Care and Use of Laboratory Animals and were in accordance with current Brazilian regulations including approval by the Research Ethics Committee of UFRGS.

Mating procedure

Males were housed individually in a cage with wood shavings as bedding. Three virgin females were placed into a male cage for 2 hours each (7:00 am to 9:00 am) and vaginal smears were collected (9:00 am) and examined for the presence of sperm. The mating procedure was repeated from Monday to Friday for 3 weeks.

Treatment schedule

The animals were divided into 7 groups composed of 8 males and 24 females each. A control group received only distillated water vehicle (CG). The other six groups received the therapeutic doses of Phytotherapic A and Phytotherapic B, five times the therapeutic dose, and ten times the therapeutic dose (4.3mg.kg^{-1}.day^{-1}, 21.5 mg.kg^{-1}.day^{-1} and 43 mg.kg^{-1}.day), respectively GPA1, GPA2, GPA3, GPB1, GPB2 and GPB3. All animals in the experimental groups were dosed once daily by gavage, the volume of administration was equivalent to 10ml.kg^{-1}. Male rats were dosed for 91 days (70 days before mating and 21 during mating). Females were dosed before mating (14 days) and during mating (21 days), pregnancy (21 days), and lactation periods (21 days).

Animal evaluation

All males and females were assessed daily for weight development, mortality, and toxicity signs. Pregnant females were monitored for weight gain, signs of abortion, dystocia, and prolonged duration of pregnancy.

Fertility evaluation

On the 21st day of pregnancy, half of the females was anaesthetized with tiletamin/ zolazepan 50% and euthanatized by decapitation. After the collection of uterus and ovaries, resorptions as well as living and dead fetuses were counted and the number of implantation sites was determined (data not shown).

Male examination procedure

All male rats were euthanized by decapitation after tiletamin/zolazepan 50% anesthesia at the end of the mating period and necropsied. Organs were inspected macroscopically, weighed and fixed in 10% neutral buffered formalin for routine processing and light-microscopic evaluation of sections stained with hematoxylin-eosin. One animal/group had its testis removed immediately after being euthanized. The testis was fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin-eosin for histological examination.

Spermatid and sperm numbers

Testes and epididymis were removed after the animals were euthanazed. The testis was rinsed and homogenized in 10 ml 0.9 % NaCl containing 0.5 % triton X-100 at medium speed in a Fisaton 720® tissuemizer for 1 min, after removal of the albuginea tunic. The number of homogenization-resistant spermatids was counted in a hematocytomer (Neubauer). The cauda epididymis was also rinsed, homogenized, and spermatozoa counted in the hematocytomer.

The number of sperm and daily sperm production was determined as follows: Number of sperm (S) = C_s x FC x V; and daily sperm production – C_d x FC x V: 6, 1; S = total number per animal. FC = chamber factor (1.250). V = dilution (10^6). C_s = number of sperms counted. C_d = number of homogenization-resistant spermatids counted.

Sperm morphology assessment

To assess the percentage of morphologically abnormal sperm (defects in head, body or tail piece), the ductus deferens was rinsed with 1M 0.9 % NaCl and a sperm suspension was subsequently obtained. An aliquot of sperm suspension was stained with 2 % eosin to assess the percentage of morphologically abnormal sperm. Two hundred sperm/animal were analyzed microscopically at a magnification of 400 times and were recorded as being either morphologically normal or abnormal. The abnormal sperm was classified according to defects in head or in cauda. The head alteration categories were outstanding, malformation and missing. The cauda alteration categories were outstanding, broken and cauda with intense folding.

Statistical analysis

Data were analyzed by one-way analysis of variance. Bonferroni test was used to identify differences between groups in the control group. Proportions were analyzed by the Chi-square test. Statistical evaluation was performed using Excel and SPSS for Windows programs, and $P< 0.05$ was considered significant.

RESULTS

Body weight gain and toxicity

The administration of phytotherapics for 91 days prior to mating and during the mating period did not induce death or toxicity. There were no statistically significant differences in body weight gain among the groups at the three doses of Phytotherapics A and B (data not shown).

Organ weights

Treatment with Phytotherapic A resulted in statistically significant differences in absolute and/or relative weights of livers, epididymides, and seminal vesicle. Testis, prostate, kidneys, livers, spleens and hearts of the animals examined did not show statistically significant differences. The treatment with Phytotherapic B showed statistically significant differences in absolute weights of epididymides. Testes, accessory glands (prostate and seminal vesicle), kidneys, livers, spleens and hearts of the animals examined did not show statistically significant differences.

Histology

Light microscopic evaluation did not reveal morphological alterations in the examined organs of male rats treated with Phytotherapic A and B at the three doses.

Sperm number and daily sperm production

The number of sperm in the caudal epididymides in male rats treated with Phytotherapic A and B resulted in statistically significant differences at a higher dose (43 $mg.kg^{-1}.day^{-1}$) (Fig 1). Daily sperm productions also resulted in statistically significant differences, but at a lower dose (4.3 $mg.kg^{-1}.day^{-1}$) (Fig 2).

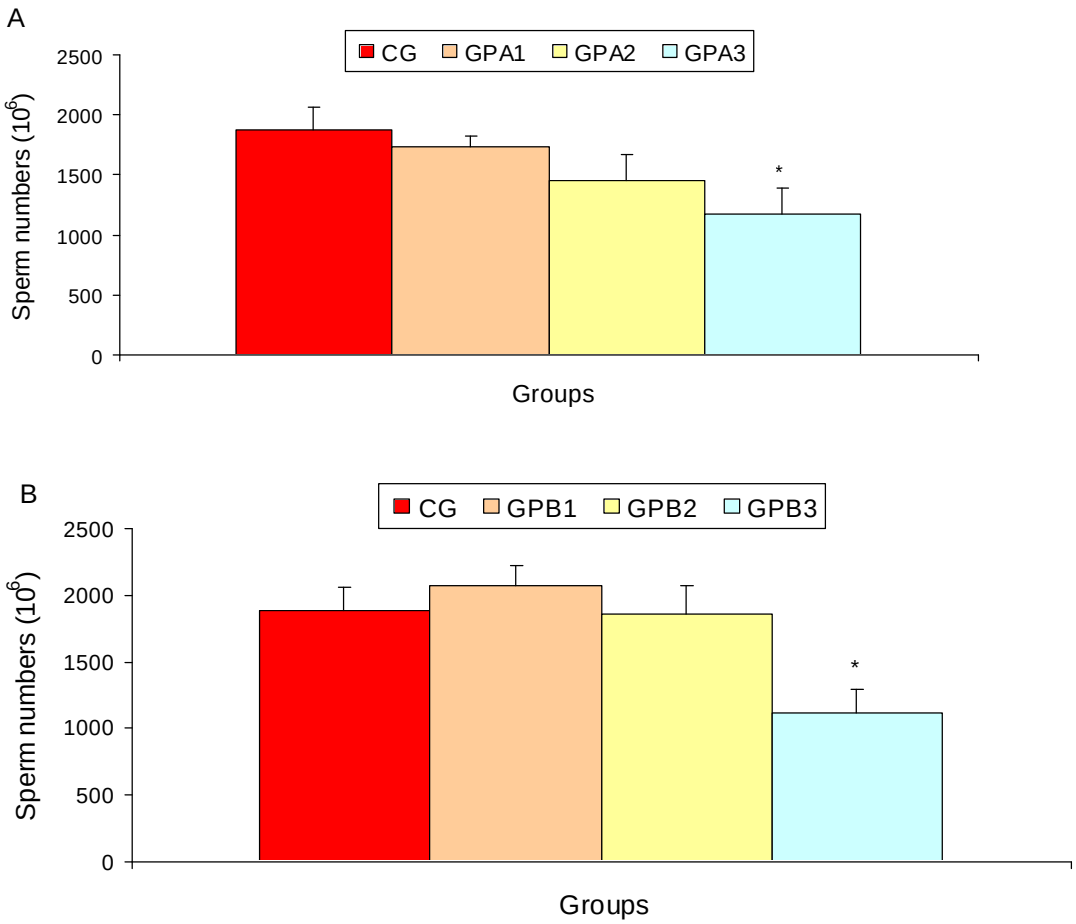

FIGURE 1. Number of sperm/group in male rats treated with Phytotherapic A (Graph A) and Phytotherapic B (Graph B) at three doses and the control group for 91 days (prior and during mating). Data were analyzed by ANOVA and Bonferroni test. Values are means/group. * Significantly different (P<0. 05) from the control group./ *Número de espermatozoides/grupo de ratas macho tratadas con Fitoterapéutico A (grafico A) y Fitoterapéutico B (grafico B) en tres dosis y el grupo control de 91 días (antes y durante el apareamiento). Los datos analizados por ANOVA y test de Bonferroni. Los valores son medias / grupo. * Significativamente diferente (p <0. 05) del grupo control.*

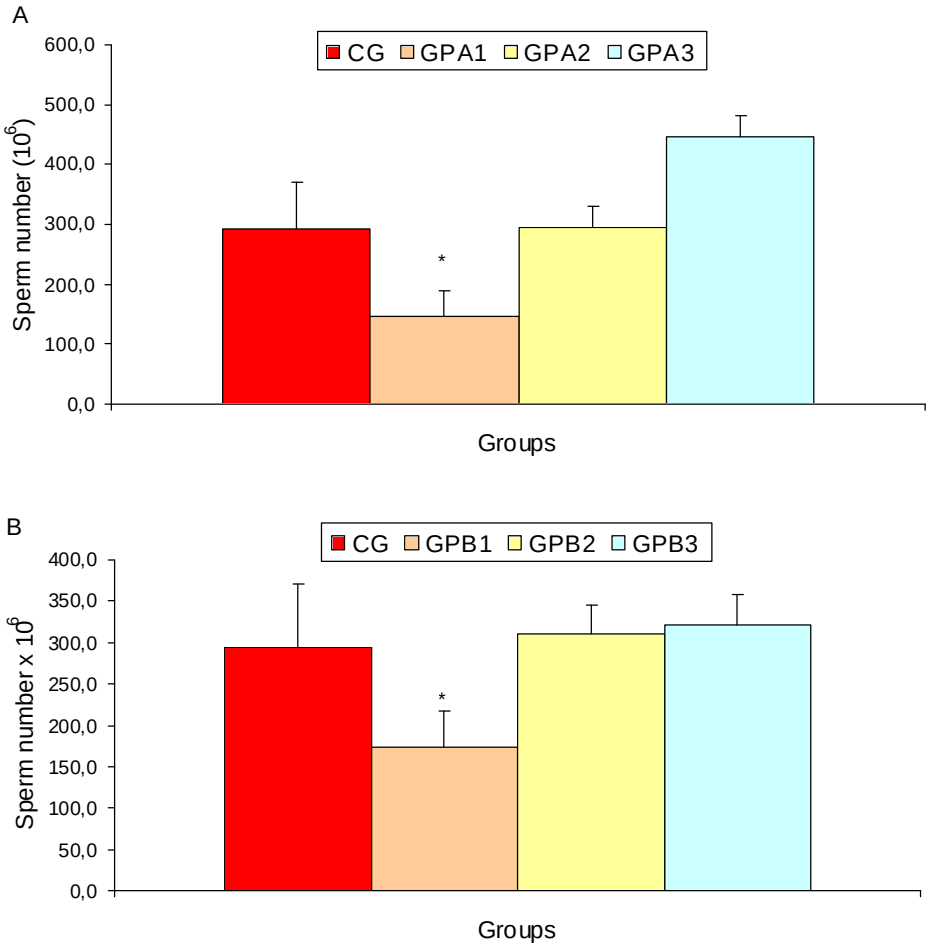

FIGURE 2. Daily sperm production in male rats treated for 91 days with Phytotherapic A(Graph A) and Phytotherapic B (Graph B) at three doses and the control group. Data were analyzed by ANOVA and Bonferroni test. Values are means/group. * Significantly different (P<0. 05) from the control group./ *La producción de espermatozoides al día en las ratas macho tratadas durante 91 días con Fitoterapéutico A (grafico A) y Fitoterapéutico B (grafico B) en tres dosis y el grupo control. Los datos analizados por ANOVA y test de Bonferroni. Los valores son medias/grupo. * Significativamente diferente (p <0. 05) del grupo control.*

Sperm Morphology

The number and percentage of morphologically abnormal sperm in males treated with Phytotherapic A and B resulted in statistically significant differences from the control group at the three doses (Fig 3).

DISCUSSION

The phytoestrogens of the soy are molecules of plant origin with numerous biological properties, but the best known is that they behave like weak estrogens. Evidence from animal studies suggests that the ingestion of very high amounts of phytoestrogens may affect fertility. Sheep exposed to great amounts of clover forage presents infertility, ewes fed with estrogenic forage may suffer impaired ovarian function, often accompanied by reduced conception rates and increased embryonic loss. In cattle, phytoestrogens cause irregular estrus, nymphomania, anestrus, and ovarian cysts (15).

In males, estrogenic compounds can be toxic to testicular tissue in rodents and humans (16). Fertility of the human male is particularly susceptible to agents that reduce the number or quality of sperm produced. Compared to many other species, human males produce fewer sperm in relation to the number of sperm required for fertility (17, 18). As a result, many men are subfertile or infertile (18). If the number of normal sperm per ejaculation is sufficiently low, fertilization is unlikely and an infertile condition exists (19). However, isoflavones are able to exert non-genomic actions potentially injurious to male fertility (20). Rodents can also be sensitive to the effects of isoflavones, as

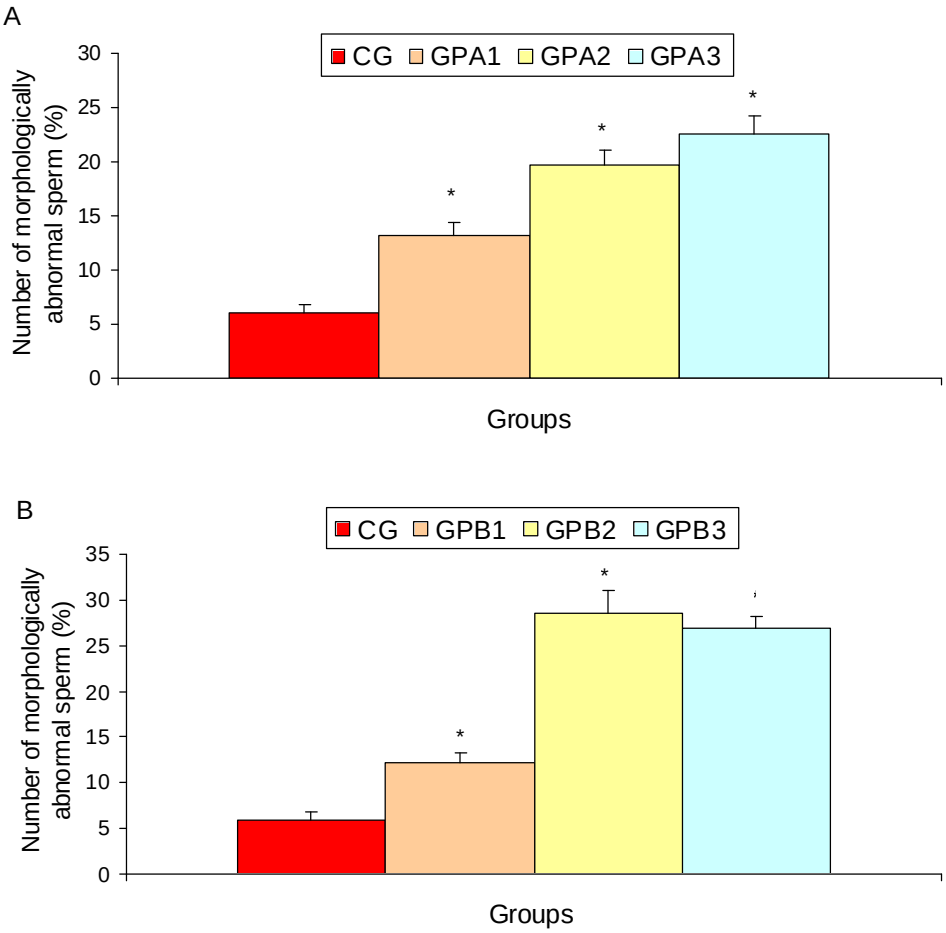

FIGURE 3. Number of morphologically abnormal sperm in male rats treated orally during 91 days with Phytotherapic A (Graph A) and Phytotherapic B (Graph B) at three doses and the control group. Data were analyzed by the Chi-square test. *Significantly different (P<0.01) from the control group./ *Número de espermatozoides morfológicamente anormales en las ratas macho tratadas por vía oral durante 91 días con fitoterapéutico A (grafico A) y Fitoterapéutico B (Grafico B) en tres dosis y el grupo control. Los datos analizados por la prueba de Chi-cuadrado. *Significativamente diferente (p<0,01) del grupo control.*

genistein can inhibit the growth and proliferation of testicular cells. In mice, it was shown that genistein can deregulate the spermatogenesis and reduce the number of sperm in the epididymis (9).

Various studies show that treatment of male rats with estrogenic chemicals reduces testicular and epididymal sperm concentration and Sertoli cell number, alters testicular gene expression, and causes rete tubule distension and reduced epithelial height in the efferent ducts (21, 22, 23, 24).

Global declines in semen quality were suggested to be associated with enhanced exposure to environmental chemicals that act as endocrine disrupters as a result of our increased use of pesticides, plastics, phytoestrogens and other anthropogenic materials (25). Considerable toxicology data based upon laboratory and wildlife animal studies suggest that exposure to certain endocrine disrupters is associated with reproductive toxicity, including abnormalities of the male reproductive tract (cryptorchidism, hypospadias), reduced semen quality, and impaired fertility in the adult (26, 27, 28, 29, 30).

In humans, a study assessed 99 male partners of subfertile couples as the intake of isoflavones in the diet. As a result it was observed that there was an inverse association between soy food intake and that sperm concentration significant remained after accounting for age, abstinence time, body mass index, caffeine and alcohol intake and smoking. In conclusions, these data suggest that a higher intake of soy foods and soy isoflavones is associated with a lower sperm concentration (31).

Data from this study suggest that male rats exposed to the daily oral administration of the two phytotherapic preparations (Phytotherapic A and Phytotherapic B) for 91 days prior to and during the mating period did not have any systemic toxicity. However, both were able to reduce the epididymis weight, without promoting histological alterations. Both phytotherapic formulations decreased the total number of sperm stored in the epididymis at the highest dose and increased the sperm morphology changes in a dose-dependent manner.

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 its lack of cytoplasm, sperm in the epididymis is susceptible to damage from reactive oxygen species (32).

An increase in abnormal sperm morphology has been considered as evidence that the agent has gained access to germ cells (33, 34). Sperm morphology profiles are relatively stable and characteristic in a normal individual (and a strain within a species) over time.

The litters of males exposed to Phytotherapic A and Phytotherapic B and the controls negative and positive were also evaluated for overall and sexual development, and behavior on the open field. No change related to the administration of isoflavones was found (35).

To identify possible effects of isoflavones on male fertility, reproductive parameters were evaluated in Wistar-Unilever rats receiving dietary exposure to PTI G-2535, a characterized mixture of soy-derived isoflavones containing 45% genistein, 23% daidzein, and 4% glycitein. Rats received chronic dietary exposure to the soy isoflavone mixture (200 or 2000 mg/kg diet) for a minimum of 12 months. Dietary exposure to isoflavones induced no gross toxicity or alterations in body weight gain. Histopathologic evaluations demonstrated that testicular morphology was similar in all study groups (36).

In conclusion, this study suggests that both Phytotherapic formulations have acted on the epididymis of the animals, causing decrease dose-dependent in the weight of the organ without causing a decrease in animal weight, reducing the number of sperm at the highest dose used and increasing the percentage of spermatozoa with morphological alterations however, the results reinforce the data observed in related literature, concerning the effects of soy isoflavones on male fertility.

CONFLICT OF INTEREST

None.

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