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EFFECTS OF CHRONIC TREATMENT WITH SOY DERIVED ISOFLAVONES ON REPRODUCTIVE HEALTH OF MALE RABBITS

EFEITOS DO TRATAMENTO CRÔNICO COM ISOFLAVONAS DA SOJA NA SAÚDE REPRODUTIVA DE COELHOS MACHOS

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ABSTRACT: The aim of this study was to investigate the effects of chronic exposure to soy isoflavones concentrate on the morphology of reproductive organs, semen quality, puberty age, serum levels of testosterone and sexual behavior of male rabbits. With this purpose, pregnant female rabbits were randomly assigned to receive orally 2.5mg (ISF 2.5) or 10mg (ISF 10) of soy isoflavones/kg of body wt/day. The animals of control group were manipulated as the other groups and received placebo (corn starch). All the rabbits were maintained on a soy-and alfafa-free diet throughout the gestation and lactation. Their male offspring received the same treatments from weaning to 33 weeks of age. Chronic exposure to isoflavones did not induce statistically significant alteration in the age at puberty, semen volume, daily sperm output, sperm concentration, motility, vigor and abnormalities. Also, isoflavones exposure had no effects on serum testosterone levels or sexual behavior in any group. Histopathologic evaluation did not reveal alterations in the testis, epididymis, prostate and pro-prostate glands of the rabbits. Taken together, these results show that gestational, lactational and post-lactational exposure to soy isoflavones, in doses comparable to those found in soy-containing animal and human diets, has no adverse effects on the reproductive parameters of male rabbits.

KEYWORDS: Phytoestrogens. Isoflavones. Reproduction. Rabbits.

INTRODUCTION

Isoflavones (ISF) are a class of phytoestrogens (PE) found principally in soybeans and soy-protein foods. The estrogenic activity of these compounds was first described in 1946, in a syndrome known as clover disease in sheep (KURZER; XU, 1997). After grazing on pastures of subterranean red clover, Australian ewes suffered from a severe reproductive disorder that resulted in permanent infertility. Since then, several investigations have been performed to elucidate the biological action of PE in human and animal tissues.

It has long been known that PE exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals (THIGPEN et al., 1999), but little is known about the effects of developmental and life-time exposure to PE in males, which have lower number of estrogen receptors (KURZER and XU, 1997). Male genital abnormalities have been noticed in men and in a variety of animal species following prenatal, neonatal or post pubertal exposure to estrogens, as evidenced in populational and experimental studies (STILLMAN, 1982; ARAI et al., 1983). Considering that the potency of the PE such as

genistein or daidzein, can even exceed that of estradiol, especially in infants who are fed soy-based formula as a sole source of nutrition (GREIM, 2004), the safety of these agents must be better investigated.

Recent studies show that chronic or transient PE exposure appears to be innocuous for testicular or epididimal sperm counts of rats (AWONIYI et al., 1997; ROBERTS et al., 2000; NAGAO et al., 2001; SHIBAYAMA et al., 2001; FIELDEN et al., 2003; FAQI et al., 2004; JUNG et al., 2004; LEE, KANG; JUNG, 2004). Although, *in vitro* studies have shown that genistein induces apoptosis of testicular cell lines, and inhibits their growth and proliferation (KUMI-DIAKA, RODRIGUEZ; GOUDAZE, 1998). Also it may interfere with percentage of sperm motility and modulate sperm capacitation, acrosome reactions and fertilizing ability (KUMI-DIAKA and TOWNSEND, 2001; ADEOYA et al., 2003), with genistein being considerably more potent than 17 β -estradiol (ADEOYA et al., 2003). Furthermore, these agents may alter the volume of the sexually dimorphic nucleus of brain (LEPHART, ADLERCREUTZ; LUND, 2001; LEPHART, WEST; WEBER, 2002) and promote deleterious alterations in the sexual behavior of rats (WHITTEN, PATISAUL; YOUNG, 2002;

WISNIEWSKI et al., 2003), in which adult males were less likely to mount, intromit and ejaculate during mating tests.

To improve the knowledge about the influence of PE on male reproductive health, the present study investigated the effects of the chronic exposure to soy isoflavones on: (1) morphology of the reproductive organs; (2) semen quality; (3) age at puberty; (4) serum testosterone levels; and (5) sexual behavior of male rabbits.

MATERIALS AND METHODS

Animals

With due compliance of the international guiding principles for animal research, the experimental protocol was reviewed and approved by the University of Brasília Institute of Biological Sciences Ethical Committee to Animal Use. One male New Zealand and 10 female rabbits between 8 and 10 months of age were started in this study. They were housed individually in steel cages equipped with automatic watering systems. Animals were kept on natural photoperiod and environmental temperature.

Treatments

Females were randomly assigned to a control (n=4) and two treatment groups (n=3/group). The animals from the treatment groups received 2.5mg (ISF 2.5) or 10mg (ISF 10) of soy isoflavones/kg of body wt/day (Soy Isoflavones, 40% - DEG Importação de Produtos Químicos Ltda - São Paulo - SP). The animals of the control group received corn starch as placebo. The proper dose of isoflavones as well as placebo was inserted directly into the oral cavity after rabbit immobilization. All animals were kept on a soy- and alfafa-free diet, in which soy and alfafa proteins were replaced with cottonseed meal and meat meal protein. The three groups received water and food *ad libitum*. Soy isoflavones concentrate used in the present study is similar to commercially available soy-derived products, currently used for hormonal replacement, and for the prevention or treatment of various hormone-dependent diseases. The most common dose used in these cases is 1 mg/kg body wt/day.

To minimize the genetic influence, females were mated by the same buck. At the fifth week of age, male offspring were numbered and surplus pups were randomly excluded from the study. Remaining six pups of each isoflavones-treatment groups and ten pups of the untreated control group received the same treatments that were given to

their respective mothers from weaning (5 weeks) to 33 weeks of age. As a consequence, these animals were exposed to isoflavones indirectly via maternal consumption during gestation and lactation and directly by oral route from weaning to adulthood. During the exposure period, the animals were monitored daily for health status. Body weights and food consumption were measured weekly throughout the experimental period.

Ejaculates collection and evaluation

The artificial vagina (AV) for semen collection was built based on a model described by Andrade et al. (2002). Its mucosae was filled with warm water (60° C), and it was used when the inner temperature was between 45° and 50° C. A collector tube was attached onto one of the edges, and the free edge was positioned to penis intromission. Before semen collection, bucks were allowed one false mount and, at the subsequent mounting, the AV was adequately positioned on the dorsum of the stimulus female allowing penis intromission.

From 14 to 25 weeks of age, semen samples were collected once a week to evaluate the sexual maturation. After this period, the animals were collected every other day for 5 weeks, permitting a total of 17 collections. The first seven samples were used to stabilize sperm output and were not included in the analysis, so the daily sperm output was quantified using the last 10 ejaculates (THOMPSON; BERNDTSON, 1993). This procedure minimizes the possibility of false detection of treatment-induced changes.

After removing and weighing the gel mass, ejaculate volume was recorded in the graduated tube attached to the artificial vagina. Just after the ejaculation, a semen drop was mixed with the same volume of heated saline on a warm (37 °C) slide. The sperm motility (0-100%) and vigor (0-5) were subjectively evaluated in light microscope (400X). For determination of sperm concentration, ejaculates were diluted 1:100 in a 4% formol/0.9% saline solution and counted twice in Neubauer haemocytometer (GmbH+Co., Brandstwiete 4, 2000 Hamburg 11, Germany) using a light microscope (Nikon Eclipse C600, Japan) (400X). The sperm morphology was examined on semen smears stained with Congo red and Gentian violet solutions.

Age at Puberty and Sexual Behavior

The age at puberty was considered when sperm counts stopped to raise. The ages (in weeks),

which males showed stabilized ejaculate values were used for data analysis.

To quantitatively measure the males' sexual behavior, the time of reaction (latency to begin mounting); the interval between two consecutive ejaculations in the artificial vagina; and the mounting reflex were determined.

The time of reaction (time elapsed from the moment of subjecting a doe to the buck and mounting) and the interval between two consecutive ejaculations into the artificial vagina were measured in seconds using a stopwatch. The mounting reflex (indicative of sexual interest) was considered when rabbits were capable to mount and complete the copulation and was quantified in days of age. Values significantly higher or lower than those from the control group indicate decreased or increased libido, respectively.

Tissue collection and evaluation

At 33 weeks of age, males were killed via jugular exsanguination, after barbiturate anesthesia. Blood samples were centrifuged at 1600 rpm for 8 minutes, and serum was stored at -20°C until analysis. Serum testosterone was assayed by automated Kimiluminescence (ACS 180 plus Bayer equipment) using manufacturer protocol.

Testes, epididymides, prostate and prostate glands were dissected and weighed. For histopathological evaluation, portions of the organs were fixed in Bouin's solution, washed with

running water, and transferred into 70% ethanol before being processed for 24 h in an automated processor (Leica TP 1020 – Histology & E. M. Products – SP, Brazil). Subsequently they were embedded in paraffin wax, sectioned at $5\ \mu\text{M}$ thickness, and stained with hematoxylin and eosin. The sections were carefully examined for the presence of abnormalities.

Statistical Methods

As all data were found to be normally distributed, different parameters were analyzed using Analysis of Variance (ANOVA). When a significant treatment effect was found, differences among groups' means were assessed by Tukey's test. Values were expressed as mean \pm SD (Standard Deviation). Statistical analyses were carried out using SAS System for Windows, SAS Institute Inc., Cary, NC, USA.

RESULTS

Ejaculate parameters

The mean values of the ejaculate volume, total motile sperm per ejaculate, vigor of motile sperm, percentage of sperm abnormalities, sperm concentration and daily sperm output are summarized in table 1. The differences in these parameters between the groups were not statistically significant ($P>0.05$).

Table 1. Effects of soy isoflavones treatment (2.5 and 10mg/Kg BW/day) on semen characteristics of rabbits

	Groups		
	Control	ISF 2.5	ISF 10
Volume (ml)	0.54 \pm 0.0	0.52 \pm 0.0	0.51 \pm 0.0
Motility (%)	77.5 \pm 4.0	80.1 \pm 3.6	79.1 \pm 5.2
Vigor (0-5)	3.1 \pm 0.3	3.2 \pm 0.2	3.2 \pm 0.1
Abnormal sperm (%)	25.3 \pm 1.2	21.5 \pm 4.6	23.0 \pm 4.1
Sperm concentration ($\times 10^6/\text{ml}$)	197.1 \pm 35.5	204.6 \pm 17.5	223.8 \pm 17.0
Daily sperm output ($\times 10^6$)	104.7 \pm 16.3	108.4 \pm 6.8	114.6 \pm 7.0

Data are shown as group means \pm SD of 10 ejaculates per animal. No intergroup differences were significant at the 5% level of confidence.

Rabbits treated with 10mg of soy isoflavones/kg body wt/day presented ejaculates with motile sperm earlier than the those treated with 2.5 mg/kg body wt/day and the untreated control group ($P<0.05$), however this difference disappeared in the adult males.

Reproductive organs weight and morphology

Absolute (data not shown) and relative weights of the testes, epididymis, prostate and prostate were similar in all studied groups (Table

2). Gross morphology is unlikely to be affected by long-term administration of isoflavones. Testes shown apparently normal pattern of spermatogenesis in the seminiferous tubules. Sex accessory glands presented a typical simple columnar epithelium with no evidence of pathological changes. This secretory tissue gives rise extensive branching folds projecting into the acinar lumen, which are peculiar of the species. Also, no evidence of pathological changes was observed in the epididymal duct of the caput,

corpus, and cauda epididymides among the treatment groups. Results from the histological

approach of the reproductive organs support the *in vivo* observations.

Table 2. Food intake, body and reproductive organs weight of rabbits treated with soy isoflavones (2.5 and 10mg/kg BW/day)

	Groups		
	Control	ISF 2.5	ISF 10
Body weight BW (g)	3754.4 ± 179.50	3684.1 ± 112.80	3595.4 ± 121.85
Food intake ¹ (g)	112.1 ± 8.00	114.0 ± 5.25	110.5 ± 13.80
Testes/BW (mg/g)	1.53 ± 0.06	1.54 ± 0.03	1.53 ± 0.02
Epididymides/BW (mg/g)	0.61 ± 0.04	0.64 ± 0.01	0.63 ± 0.00
Prostate/BW (mg/g)	0.205 ± 0.09	0.198 ± 0.00	0.196 ± 0.00
Proprstate/BW (mg/g)	0.198 ± 0.01	0.196 ± 0.00	0.193 ± 0.00

Values are given as mean ± SD of 10 ejaculates per animal. No intergroup differences were significant at the 5% level of confidence; ¹ Average values obtained during post-natal weeks 29 to 33.

Age at puberty, sexual behavior and serum testosterone concentration

The age at puberty (mean and standard deviations) in the different groups was: control, 161 ± 11.4 days; ISF 2.5, 170 ± 9 days; and ISF 10, 163 ± 11.8 days. These data did not reach statistically significant differences.

Chronic treatment of rabbits with soy isoflavones did not cause significant statistical

differences in the measurable parameters employed to the sexual behavior assessment (Table 3). A subjective evaluation based on simple observation of the mating routine for semen collections (over 25 mates per animal), corroborates the objective analyses. In addition, serum concentrations of testosterone did not differ among the groups.

Table 3. Effects of soy isoflavones treatment (2.5 and 10mg/kg BW/day) on the time of reaction, interval between two consecutive ejaculations into the artificial vagina (Δt), and serum testosterone levels

	Groups		
	Control	ISF 2.5	ISF 10
Time of reaction (sec.)	3.7 ± 0.7	3.9 ± 0.2	3.6 ± 0.2
Δt (sec.)	97.0 ± 7.5	95 ± 10.9	105.8 ± 13.4
Testosterone levels (ng/dl)	800 ± 141	750 ± 115	726.3 ± 112.6

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No intergroup differences were significant at the 5% level of confidence.

DISCUSSION

In vivo data show that PE have a wide range of biologic effects at doses and plasma concentrations considered normal in human diets. The doses reported to be biologically active in humans (0.4 to 10 mg/Kg body wt/day) are lower than doses generally reported to be active in rodents (10 to 100 mg/Kg body wt/day), although some studies have reported rodent responses at lower doses (WHITTEN; PATISAUL, 2001). Some estimative of isoflavones intake from soy-based diets consumption ranges from 6 to 11 mg/kg/body wt/day in human infants (SETCHELL et al., 1998), 1 to 1.5 mg/kg body wt/day in human adults (STRAUSS et al., 1998), 0.6 to 4.5 mg/kg/body wt/day in cats (COURT; FREEMAN, 2002) and 1.6 to 3.5 mg/kg body wt/day in monkeys (SHARPE et al., 2002). The doses of soy isoflavones we find to be safe for male

reproductive health are similar to the amounts consumed by human and animal eating soy-based diets.

McClain et al. (2005) reported atrophy of testes and absence of spermatozoa in the epididymus of Beagle dogs treated with genistein, but at a very high dose of this purified isoflavone. A comparable dose of genistein (\approx 500 mg/kg/day) was also required to induce persistent structural changes in rats (STRAUSS et al., 1998), similar to those observed in mice treated with potent synthetic estrogens. However doses of PE within dietary exposure levels had failed to induce major effects in sperm counts of rats (ROBERTS et al., 2000; SHIBAYAMA et al., 2001; FIELDEN et al., 2003; FAQI et al., 2004; JUNG et al., 2004; LEE, KANG; JUNG, 2004), and men (MITCHELL et al., 2001). In this context, it is opportune to consider that almost all commercially available rodent diets contain substantial levels of PE (THIGPEN et al.,

1999; BROWN; SETCHELL, 2001; LEPHART, WEST; WEBER, 2002), and no reports of impaired reproductive performance has been registered in these animals, as well as in rabbits fed a soy meal-based diet (CARDOSO; BÁO, 2006).

The effect of the PE on the rabbits' reproductive system seems to depend on the compound, exposure dose and period of administration. Total isoflavones exposure at doses ranging from 2 to 5mg/Kg of BW every other day for a subchronic period had several beneficial effects on semen characteristics and sexual behavior of male rabbits (YOUSEF, EL-DEMERDASH; AL-SALHEN, 2003; YOUSEF, ESMAIL; BAGHDADI, 2004), but rabbits treated with daidzein at 0.1 mg/Kg/day showed erectile dysfunction (SRILATHA; ADAIKAN, 2004). In contrast, results of the present study did not show either, beneficial nor deleterious effects on reproductive function and behavior of male rabbits. Also, a soy meal-based diet (17% of diet) did not change semen quality, reproductive organs morphology and sexual behavior of rabbits, as we have shown earlier (CARDOSO; BÁO, 2006).

Semipurified isoflavone concentrate exposure did not induce alterations in reproductive organs weight and morphology in this investigation, which is in accordance with studies in rats (CASANOVA et al., 1999; ROBERTS et al., 2000; SHIBAYAMA et al., 2001; FIELDEN et al., 2003; OHNO et al., 2003; FAQI et al., 2004; LEE, KANG; JUNG, 2004). However, there appears to be rats strain differences in the effects of PE on prostate structure. Decrease in prostate weight and scamous metaplasia of collector ducts was

observed in Outbred Han NMRI rats treated with 2.5 mg/Kg of genistein for 7 days (STRAUSS et al., 1998); the same dose for a greater period (5 weeks) did not cause lesions in ICR rats prostate (LEE, KANG; JUNG, 2004). In addition, doses of genistein 4 to 16 times higher had no effects on Wistar rats prostate weight (OHNO et al., 2003). Such differences like those discussed earlier in rabbits are not well explained due to the paucity of comparative data about the absorption, metabolism and bioavailability of PE. Moreover, these divergences motivate intense discussions about the possible undesirable side effects of these compounds on the human health.

In conclusion, our findings show that isoflavone exposure at normal human and animal dietary levels, which are recognized to have beneficial role in the prevention of several types of chronic diseases, do not impact male function and behavior of male rabbits.

This study provides additional data about the consequence of PE exposure on the reproductive male health, however the results may not be integrally extrapolated to females and other species, since several differences has been reported among species, and in the compound, dose and route of administration of these substances.

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RESUMO: O objetivo deste estudo foi investigar os efeitos da exposição crônica a concentrado de isoflavonas da soja sobre a morfologia dos órgãos reprodutivos, qualidade do sêmem, idade a puberdade, níveis séricos de testosterona e comportamento sexual de coelhos machos. Com este propósito, fêmeas gestantes foram aleatoriamente designadas para receber, por via oral, 2,5mg (ISF 2,5), ou 10mg (ISF 10) de isoflavonas da soja por quilo de peso corporal por dia. Os animais do grupo controle foram tratados como os dos demais grupos e receberam placebo (amido de milho). Todos os coelhos foram alimentados com dieta livre de soja e alfafa ao longo da gestação e lactação. A prole macho recebeu os mesmos tratamentos, da desmama até 33 semanas de vida. A exposição crônica a isoflavona não causou alterações estatisticamente significativas na idade a puberdade, volume de sêmem, emissão diária de espermatozoides, concentração espermática, motilidade, vigor e morfologia espermática. Em adição, a exposição à isoflavonas não resultou em efeitos negativos sobre os níveis de testosterona, ou no comportamento sexual dos coelhos. A avaliação histopatológica também não revelou qualquer alteração nos testículos, epidídimos, e nas glândulas próstata e pré-próstata dos animais. Os resultados deste estudo apontam que a exposição gestacional, lactacional e pós-lactacional a isoflavonas da soja, em doses comparáveis àquelas em que homens e animais estão expostos ao consumirem dieta a base de soja, não causa efeitos adversos nos parâmetros reprodutivos de coelhos machos.

PALAVRAS-CHAVE: Fitoestrógenos. Isoflavonas. Reprodução. Coelhos. Estrógenos ambientais. Toxicologia.

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