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A single neonatal administration of soybean oil and/or tamoxifen permanently affects testis histomorphology in adult rats

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Abstract

The aim of this study was to evaluate the effects of administration of tamoxifen (Tx) and its vehicle, soybean oil (SO), during the critical period of hypothalamic sexual differentiation in newborn male rats in the context of gonadal histomorphology during adulthood. The animals were randomly divided into 3 groups (n = 5 each). One hour after birth, one group was treated subcutaneously with 200 µg of Tx using commercial SO (20 µL) as a vehicle, another group was treated with only 20 µL of SO, and the control group received no treatment. All rats were weighed and sacrificed by cervical dislocation on day 90 post-treatment. Testicles were removed, weighed and processed for histological evaluation. The single administration of Tx and/or SO during the critical period of sexual differentiation of the hypothalamus permanently altered testicular histomorphology, spermatogenesis, and body weight in adulthood. Alterations included vacuolization and reduction in the number of spermatogonia and Sertoli cells. The administration of Tx reduced testicular weight, the diameter and area of the seminiferous tubules, and the height of the germinal epithelium and increased the intertubular space. Soybean oil by itself reduced the number of spermatocytes and spermatids to a greater extent than Tx. There was no effect on the number of Leydig cells. The possibility that soybean oil can act as an endocrine disruptor deserves greater attention and opens the possibility for the development of new pest control methods.

Keywords: Endocrine disruptors; Tamoxifen; Soybean oil; Sexual differentiation of the hypothalamus; Testicular morphology.

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Additional information and declarations can be found on page 9

Introduction

During development, in the "critical period" of brain sexual differentiation, the brains of male rats are exposed to higher levels of testosterone of testicular origin, which increases at the end of gestation, primarily two hours after birth (Anne *et al.*, 2011). In neurons, testosterone is converted to estrogens by the P450 aromatase enzyme . Estrogens may act in two different stages: an "organization" phase during the prenatal and early postnatal periods, and an "activation" phase in adulthood

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Vol. 3 | No. 2 | April-June | 2016

(Phoenix *et al.*, 1959). The organizational effects of estradiol induce sexual differentiation of the hypothalamus (Lauber *et al.*, 1997) and actively regulate the neural circuits (Barraclough, 1966; Simerly *et al.*, 2002), which are responsible for the tonic secretion of gonadotropins (Finkelstein *et al.*, 1991; Bagatell *et al.*, 1994) and reproductive behaviors characteristic of males (Lauber *et al.*, 1997 and Simerly, 2002). Estrogens are also involved in spermatogenesis and sperm maturation in the epididymis (Balasinor *et al.*, 2001).

Exposure to exogenous endocrine-disrupting chemicals (EDCs, of natural or synthetic origin) that mimic or block the effects of endogenous sex steroid hormones (Wilson and Davies, 2007) can send mixed messages to the body, causing developmental abnormalities with irreversible effects. The role of estrogens in sexual reproduction has been confirmed by the use of antiestrogens such as tamoxifen (Tx; Z-1-[4-(2-dimethylamino-ethoxy)-phenyl]-1, 2-diphenyl-1-butane) (Barraclough, 1967; Dörner and Staudt, 1968; Döhler *et al.*, 1984), a synthetic drug commonly used in breast cancer treatment. Its mechanism of action remains unclear, but it is known that it exerts several biological effects. It may act as a complete estrogen agonist or antagonist depending on the dose, target organ, sex, and species (Gill-Sharma *et al.*, 1993; Furr and Jordan, 1984; Macnab *et al.*, 1984). In addition to Tx, there are other chemicals that can alter the hormonal milieu, affecting growth, sexual development and behavior.

In male mice, Tx is detrimental for testicular and accessory gland development after neonatal administration (24 hours post-birth; 20 or 100 µg/d for 5 days; Iguchi and Hirokawa, 1986) and renders male mice infertile (Taguchi, 1987). Tx impedes the interaction of estradiol with its receptor in the hypothalamus, in addition to inhibiting the aromatase enzyme, leading to cerebral feminization (Döhler *et al.*, 1985). In adults, a reversible effect of Tx on the hypothalamus-hypophysis axis has been reported (Gill-Sharma *et al.*, 1993); Balasinor *et al.* (2002) observed a dose-dependent reduction in fertility due to embryo loss in females mated to male rats treated with Tx.

In our group, Herrera *et al.* (2013) found that a single subcutaneous (sc) dose of Tx (200 µg) in rats one hour after birth modified the expression of certain hypothalamic genes. To advance on those studies, an experiment was designed to examine the expression of 11 specific genes in the hypothalamus of male rats within the first six hours after treatment at one hour after birth with Tx in SO, SO or control. Some of the animals were weighed at weekly intervals and sacrificed as adults at 90 d of age, when both testicles were removed, weighed and subjected to histological analysis. Observations of the changes in testicular morphology in those animals are presented in this communication.

Materials and methods

Animals

The animals were obtained from and housed in the Production and Animal Experimentation Laboratory Unit (UPEAL) of the Universidad Autónoma Metropolitana, campus Xochimilco (UAMX), in a controlled environment (21°C) with 12-hour light-dark inverted cycles (lights off at 4:00 AM) as well as food (Rodent Diet 5001, LabDiet) and water *ad libitum*.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 | No. 2 | April-June | 2016

Nine Wistar rats (6 females and 3 males) were used as breeders. The males were 90 day-old rats that weighed more than 400 g. Their reproductive capacity was evaluated based on their copulatory behavior, the presence of a seminal plug in females after copulation, and pregnancy confirmation. The females were 60-day-old rats that weighed more than 200 g. Their estrous cycle was monitored daily through vaginal cytology for at least 3 cycles to confirm their endocrine-reproductive normality. Estrous rats were placed in a cage with a male to obtain a first pregnancy and to corroborate their reproductive capacity. After the first litter, a second round of selection was conducted based on the observed maternal behavior. Finally, only rats that had estrous cycles, gestation, and normal litters, as well as adequate maternal behavior, were selected.

Two weeks after the first delivery, new estrous cycles were monitored again. At estrous, one female and one male were placed in a cage again for 3 hours. The beginning of pregnancy was verified by the presence of sperm through vaginal cytology and/or the formation of a seminal plug. Pregnant rats were housed in cages under the conditions previously described. Because our main goal was to analyze the effects of early administration (1 hour after birth) of Tx and its vehicle (SO) on testicular microscopic morphology in adult rats, strict control of the moment of fertilization and constant monitoring of mothers were necessary, primarily at the end of gestation, to identify the exact time of birth.

The time of birth of each animal was considered hour zero. Newborn males were separated and divided randomly into 3 groups of 5 animals each. An hour after birth, treatment was administered: one group was treated subcutaneously with 200 µg of Tx (Sigma-Aldrich- Sigma-Aldrich Corporate Offices, 3050 Spruce St. St. Louis, MO 63103, U.S.A.) diluted in commercial SO (20 µL), another group was treated subcutaneously only with SO (20 µL), and the control group received no treatment. Tamoxifen was used to induce brain feminization. Soybean oil alone was given to avoid confounding the effects of treatment due to the vehicle. Treated rats were returned to their mothers until weaning (21 days) and then fed in the same manner as the other animals.

Collection and processing of samples

All rats were weighed weekly until 90 days post-treatment and sacrificed by cervical dislocation. Immediately, testicles were removed in a block, dissected under a stereoscopic microscope, weighed on an analytical balance, perfused and fixed in Bouin solution for 8 hours. Subsequently, they were dehydrated in a decreasing ethanol series, cleared in xylene, and embedded in paraffin to obtain cross-section slides (5 microns) on a rotary microtome (Leica RM2125 RT). Finally, hematoxylin-eosin staining was performed for morphological evaluation, and the testicle cross-sections were observed under a microscope.

Morphological evaluation

Only round tubules were digitized for analysis in 20X and 40X magnification fields. Ten tubules were analyzed for each testicle (20 tubules for each rat). Measurements of diameter, area, height of the germinal epithelium, and interstitial space were made from the scanned sections with the LSM5 software (Carl Zeiss, Ger-

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 I No. 2 I April-June I 2016

Table 1. Mean ± SD for testicular weight and body weight at 90 days.

Characteristic	Control	Soybean oil	Tamoxifen
Testicular weight (g)	5.2 ± 0.3^a	5.5 ± 0.2^a	4.4 ± 0.3^b
Body weight (g)	443.3 ± 15.1^a	400.5 ± 14.1^b	362.0 ± 8.6^c

Testicular weights and body weights of 90-day-old rats. Distinct superscripts within each line indicate statistically significant differences ($P < 0.05$).

many) using the marking tools. The photographed cross-sections were used to identify the tubular stages and to count cells using the tubular morphology method (Berndtson, 1977; Hess and França, 2007), which is based on the shape of the spermatid nuclei, the occurrence of meiotic divisions, and the position of spermatids in the seminiferous epithelium (Farias *et al.*, 2014). All slides were analyzed by 3 veterinary morphologists, and the individual results were averaged for this report.

Statistical analysis

A Shapiro Wilks test was performed to determine the normality of the data for the different parameters studied. Depending on the normality of distribution, data were analyzed with a Kruskal-Wallis test (morphology and cell counts) or one-way ANOVA (body weight and testicular weight). The results are expressed as the mean values ± SE (standard error) and mean values ± SD (standard deviation), respectively. Values analyzed were derived from 5 animals per group for body weight and testicular weight and from 10 tubules randomly taken from each testicle. PAST software (Paleontological Statistics Package for Education and Data Analysis, Huang *et al.*, 2013) was used for all statistical calculations.

Results and discussion

The results for testicular weight and body weight at 90 days post-treatment are shown in Table 1. The average testicular weight was significantly lower in animals treated with Tx than in the control group or the SO-treated group (F: 47.19; degrees of freedom between groups: 2; degrees of freedom within groups: 12; $P < 0.001$). The average body weight at 90 days was lowest for the animals treated with Tx, followed by the group treated with SO, whereas the animals in the control group were the heaviest (F: 16.67; degrees of freedom between groups: 2; degrees of freedom within groups: 12; $P < 0.001$). Only the animals treated with Tx showed a significant reduction in tube diameter (H: 12.88; Hc: 13.00; $P < 0.005$), tubular area (H: 9.85; Hc 9.85; $P < 0.05$), and height of the germinal epithelium (H: 27.04; Hc: 27.05; $P < 0.001$) [Table 2]. In comparison to the control group, the intertubular space was largest in the animals treated with Tx, followed by those treated with SO.

The average results for cell count per tubule are shown in Table 3. The numbers of spermatogonia (H: 6,953; Hc: 6,959; $P < 0.05$) and Sertoli cells (H: 9.89; Hc: 10.31; $P < 0.05$) were significantly lower in the Tx and SO experimental groups in comparison to the control group. No significant differences in the number of Leydig cells were observed (H: 1.33; Hc: 1.33; $P < 0.5$). Compared to the control group, the males treated with SO showed the greatest reduction in the number of spermatocytes (H: 12.21; Hc: 12.22 $P < 0.05$) and elongated spermatids (H: 11.62; Hc: 11.64; $P < 0.05$), followed by the Tx-treated males.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 | No. 2 | April-June | 2016

Table 2. Testicle morphometry of 90-day-old male rats treated with tamoxifen and/or soybean oil 1 h post-birth (mean ± SE).

Characteristic	Control	Soybean oil	Tamoxifen
Tubule diameter (μm)	279.4 ± 8.7[a]	304.11 ± 11.0[a]	219.2 ± 6.4[b]
Tubule area (μm^2)	193,103.1 ± 10249.0[a]	163,359.0± 11495.1[a]	131,672.0 ± 3033.6[b]
Germinal epithelium height (μm)	45.0 ± 1.1[a]	42.0 ± 2.9[a]	30.4 ± 1.4[b]
Intertubular space (μm)	4.2 ± 0.4[a]	16.7 ± 2.2[b]	39.5 ± 4.1[c]

Testicular alterations induced by Tx and/or SO treatments. Distinct superscripts within each line indicate statistically significant differences ($P < 0.05$).

Table 3. Testicular cell counts of 90-day-old male rats treated with tamoxifen and/or soybean oil 1 h post-birth (mean ± SE).

Cell type (number)	Control	Soybean oil	Tamoxifen
Spermatogonias/tubule	66.7± 8.4[a]	46.1 ± 5.4[b]	40.3 ± 2.7[b]
Spermatocytes/tubule	149.3 ± 17.4[a]	64.9 ± 5.9[c]	97.2 ± 7.9[b]
Spermatids/tubule	171.7 ± 40.8[a]	41.6 ± 9.2[c]	113.3 ± 9.2[b]
Sertoli cells/tubule	7.4 ± 0.5[a]	4.7 ± 0.3[b]	5.0 ± 0.7[b]
Leydig cells/intertubular space	10.5 ± 0.5[a]	9.8 ± 0.6[a]	9.8 ± 0.6[a]

Testicular alterations induced by Tx and/or SO treatments. Distinct superscripts within each line indicate statistically significant differences ($P < 0.05$).

As shown in Fig. 1, testicles in the control group (A-B) showed evidence of seminiferous tubules with normal spermatogenesis. The SO-treated group (C-D) showed vacuolization in the seminiferous tubules, destruction of the germinal epithelium, and a reduction in the amount of germ and Sertoli cells; additionally, increases in the intertubular spaces and ungrouped Leydig cells were observed. These alterations were also noted in animals treated with Tx, which in addition showed a significant reduction in tubular diameter and tubular area as well as a larger intertubular space than either the control or SO groups.

The results revealed that during the critical period of sexual differentiation of the hypothalamus, a single administration of Tx and SO reduced body weight, testicular weight, and altered the gonadal histomorphology and spermatogenesis in adult rats. In our experiment, although no measurements of circulating hormone concentrations were performed, the effect on weight gain over 90 days in treated animals is an indication of insufficient testosterone stimulation, despite the lack of effects on the number of Leydig cells due to treatment. It must be noted that the edematous environment and the dispersion of cells in the intertubular space in treated animals, as well as the possible lack of proper gonadotropic stimulation, could reduce testosterone production. The disruption of normal gonadotropin secretion (and therefore testosterone) due to inadequate hypothalamic control caused by the treatments could explain the low numbers of Sertoli cells, which constitute the structural basis for the blood-testis barrier (Yu-hua *et al.*, 2015) and provide support for spermatogenesis. The diminished presence of FSH and testosterone in the seminiferous tubules could have affected Sertoli cell function and estrogen production, which is congruous with the observed reduction in germ cell numbers, most likely caused by increased apoptosis in an environment with vacuolization and tubular edema.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 | No. 2 | April-June | 2016

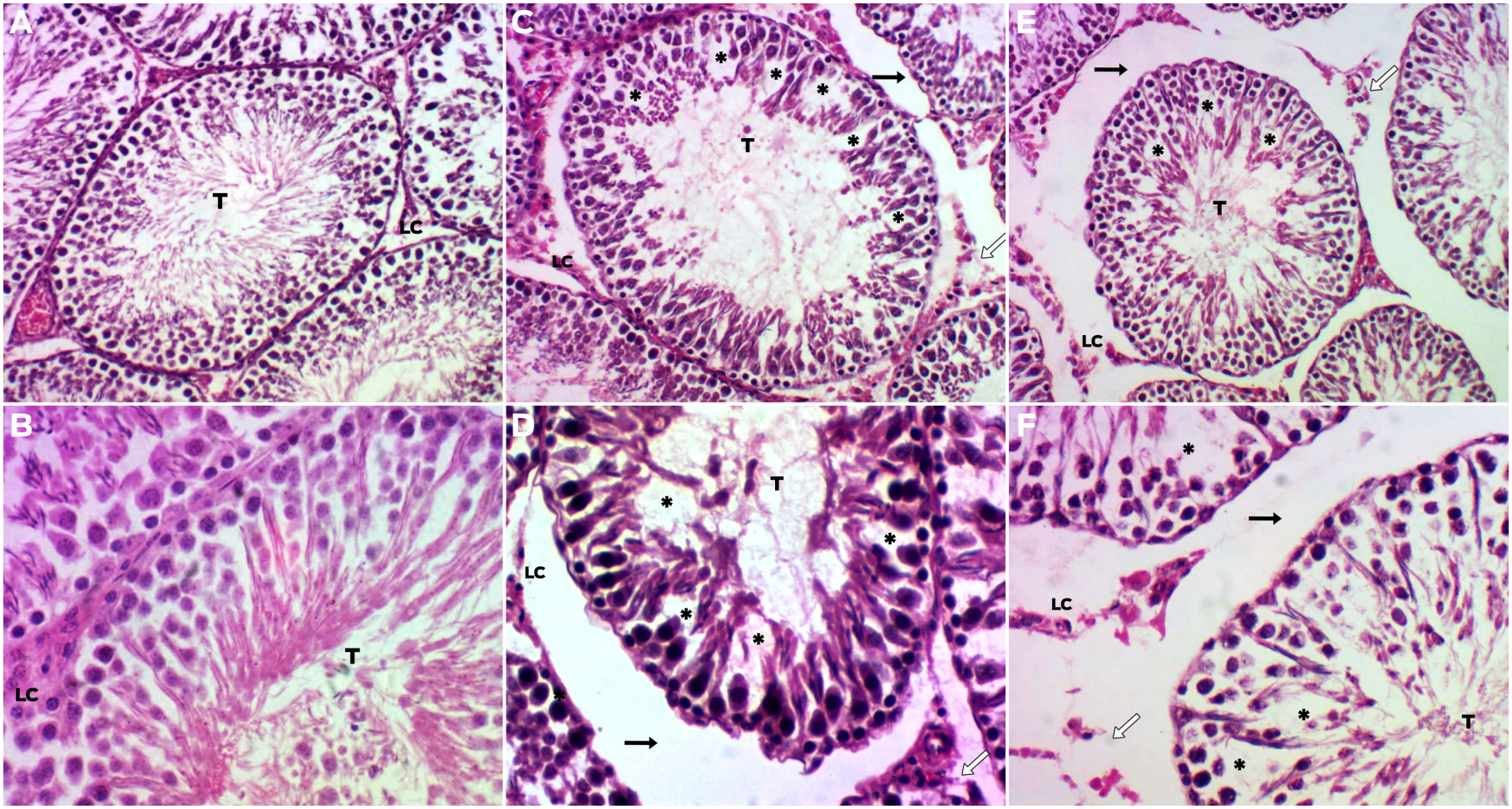

Figure 1. Representative hematoxylin-eosin-stained histological sections of testicles collected from the control group (A y B), rats treated with SO (C y D) and rats treated with Tx (E y F). A, C and E, 200X. B, D and F, 400X. T: Tubule. LC: Leydig cells. Histological changes induced by Tx and/or SO treatments can be observed. Black arrows point to the largest intertubular space. Dashed arrows in E and F indicate the ungrouping of the LC. Asterisks indicate the vacuoles in the seminiferous tubules.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 | No. 2 | April-June | 2016

The mammalian testis is characterized by the synthesis of steroid hormones and sperm production (Carreau *et al.*, 2006). Normal testicular development and spermatogenesis maintenance are primarily controlled by gonadotropins and testosterone (Carreau *et al.*, 1999). Their effects are modulated by local factors, among which estrogens are essential for the maintenance of the reproductive tract in males (Carreau *et al.*, 2003). Estrogens are synthesized in Leydig cells (Abney, 1999), Sertoli cells, and round and elongated spermatids (Hess *et al.*, 2001; Saunders *et al.*, 2005; Lambard *et al.*, 2005). In these cells, the existence of specific estrogen alpha (ERα) and beta (ERβ) receptors suggests paracrine and autocrine effects of estradiol (Carreau *et al.*, 2002; Scobie *et al.*, 2002). Estrogens act as survival factors in germ cells (Carreau *et al.*, 2003). It has been demonstrated that the P450 aromatase enzyme is required for the maturation of sperm and fluid absorption mechanisms in the efferent ducts (Lambard *et al.*, 2005). In the absence of estrogen receptors, fluid absorption in these ducts, in the *rete testis* and in the seminiferous tubules is reduced (Hess *et al.*, 2000) and accumulates in the testicles, causing damage to the germinal epithelium and therefore causing alterations in sperm development and fertility (Hess *et al.*, 2001), as was observed in our work. This suggests that the treatments influenced the mechanisms involved to provide the estrogenic action necessary for normal testicular function.

Under physiological conditions, estrogens play an important role in the normal development of the structure and function of the testicles and the male reproductive tract. In the testicle, estrogens depend on the availability of androgens for their synthesis; however, under experimental conditions, the neonatal administration of exogenous estrogenic substances orally, in feed or water, reduces sperm counts, plasma testosterone and the number of Sertoli cells (Goyal *et al.*, 2003; Sharpe *et al.*, 2003 and Atanossova *et al.*, 2005). In post-pubertal rats, Assinder *et al.* (2007) reported that weaned male rats fed for 24 days on a diet high in phytoestrogens demonstrated increased apoptosis of germ cells, a reduction in spermatic counts and a lumenal increase in the seminiferous tubules, without apparent involvement of the hypothalamus-hypophysis-testicle axis, but rather involving the paracrine and autocrine effects of estrogens in the testicle.

Soy and its derivatives are known sources of isoflavones, a particular class of phytoestrogens that may interact in the signaling pathways of endogenous estrogen (Cederroth *et al.*, 2012). Previous studies have shown that during critical periods of development, exposure to phytoestrogen induces adverse morphological and physiological effects on male sexual differentiation in mice (Roberts *et al.*, 2000; Wisniewski *et al.*, 2003). Levy *et al.* (1995) reported that treatment with genistein (derived from soy) reduces body weight (in males and females) and the anus-genital distance at birth (in males) and delays the onset of puberty. These results were confirmed by Wisniewski *et al.* (2005), who also observed phenotypic and behavioral disorders in male mice of mothers fed with 5 or 300 mg of genistein/kg of body weight during pregnancy and lactation. At 21 days after birth, the offspring exhibited permanent demasculinization: a reduction in sperm cells, testosterone and aggressive behavior and an increase in defensive behavior. In our study, the observation of vacuolation, decreased germinal epithelium height, and increased intertubular space constituted important findings. Other researchers, such as Perez-Rivero *et al.* (2009 and 2014), have previously reported these changes in dogs and vampire bats fed with rich phytoestrogen (coumestrol) diets. These observa-

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Vol. 3 | No. 2 | April-June | 2016

tions are compatible with those in our experiment but are associated with a single sc injection of SO.

Tamoxifen administration during the perinatal stage blocks the interaction of estradiol with its receptor in the hypothalamus and inhibits the aromatase enzyme (Döhler *et al.*, 1985), which is necessary for the masculinization of the neural circuits that modulate the behavior and the gonadotropin secretion typical of males in adulthood. Tx induces oxidative stress and apoptosis in albino mouse spermatozoa, morphological abnormalities (DNA adduct formation; Padmalatha and Vijayalaxmi, 2001) and seminiferous tubule distortion with germ cell detachment. In adult male rats, Tx (200, 400 or 800 mg/kg of body weight) caused multinucleated giant cells (D'Souza, 2003) and reduced fertility (Balasinor *et al.*, 2001; Gill-Sharma, 1993).

It is known that Tx may act as an estrogen antagonist or agonist on the reproductive axis of the rat (Bellido *et al.*, 2003), and SO can be a source of phytoestrogens. In this study, both substances were administered in the first hour after the birth of male rat pups, which is close to the critical time for hypothalamus sex differentiation; thus, the morphological changes observed in the testicles could be attributable to the effects of the treatments on hypothalamic nuclei controlling tonic gonadotropin secretion, with an additional effect on testicular steroidogenesis. The role of testosterone in testicular function and spermatogenesis is well documented. However, it is worth mentioning that in models of soy phytoestrogen administration in adult rodents, the disruption of testicular function can be independent of the circulating concentrations of gonadotrophins and testosterone.

Conclusions

This communication documents the irreversible changes on the testicular function and histomorphology of adult rats caused by a single, acute treatment of tamoxifen and/or soybean oil at one hour after birth. These changes may be associated with disruption of the normal course of sex differentiation of the hypothalamus during the known critical period for this process.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.364
Vol. 3 | No. 2 | April-June | 2016

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The Internal Committee for the Care and Use of Laboratory Animals of UAMX approved the procedures performed on the animals.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Alicia González-González, experimental development and analysis of results.
Everardo González-Padilla, in the discussion and analysis of results.
Francisco Fierro, in the analysis of results.
Mónica Salas-Rojas, in the morphometric analysis.
Ma. De Lourdes Juarez-Mosqueda, in sample processing.
Juan José Perez-Rivero, in morphometric and statistical analysis.
Clara Ortega-Camarillo and Marcela Vergara Onofre, in experimental design and supply of materials.

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Vol. 3 | No. 2 | April-June | 2016

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Vol. 3 | No. 2 | April-June | 2016

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