Reproductive Toxicology 81 (2018) 168–179

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Maternal supplementation with corn oil associated or not with di-*n*-butyl phthalate increases circulating estradiol levels of gerbil offspring and impairs sperm reserve

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ARTICLE INFO

Keywords:
Di-*n*-butyl phthalate
Corn oil
Lipid metabolism
Sperm reserve
Sperm motility

ABSTRACT

This study evaluated the consequences of gestational exposure to di-*n*-butyl phthalate (DBP) for testicular steroidogenesis and sperm parameters of the adult gerbil and the interference of corn oil (*co*), a vehicle widely used for administration of liposoluble agents, on DBP effects. Pregnant gerbils received no treatment or were treated from gestational day 8 to 23 via gavage with 0.1 mL/day of *co* only or containing DBP (100 mg/kg/day). Maternal *co* intake enhanced serum estradiol levels and testicular content of ERα, and reduced sperm reserve of adult offspring. Gestational DBP exposure caused dyslipidemia, increased serum and intratesticular estradiol levels and reduced sperm reserve and motility. Thus, maternal *co* supplementation alters circulating estradiol and impairs sperm quantity and quality of offspring. Gestational DBP exposure alters lipid metabolism and testicular steroidogenesis and worsens the negative effects of *co* on the sperm reserve and motility of gerbil. Therefore, *co* interferes with the reproductive response to DBP.

1. Introduction

Endocrine disrupting chemical (EDC) exposure during critical periods of development, such as the fetal period and lactation, may lead to transient effects or permanently affect the homeostatic mechanisms of the endocrine system, causing physiological alterations in adulthood [1–5]. Phthalate esters are a class of EDC of growing interest for the scientific community. Phthalates are chemicals used as plasticizers in several types of plastics, mainly PVC (polyvinyl chloride) [6], being found in cosmetics, children's toys, food products and hospital supplies [7]. EDC forms non-covalent bindings with the polymers with which they are mixed [8], so, at high temperatures, they can be released into food, beverage, and other products with which they come into contact [8,9].

Certain phthalates, particularly di-*n*-butyl phthalate (DBP), have been shown to interfere with the development of the male reproductive tract of the offspring when administered to pregnant rats [7,10–12]. Mylchreest et al. [7] demonstrated that rats exposed to DBP (0.5 to 500 mg/kg/day) during late gestation showed dose-dependent alterations in reproductive development, causing reduction in anogenital distance, increase in nipple number, reduction in epididimal weight, besides malformations in the testis and epididymis, and hypospadias at the highest doses. In addition, gestational exposure to DBP can reduce male fertility by decreasing testicular and epididimal sperm counts

Abbreviations: AR, androgen receptor; CAT, catalase; DBP, di-*n*-butyl phthalate; DSP, daily sperm production; EDC, endocrine disrupting chemical; ERα, estrogen receptor α; E_2, estradiol; GPx, glutathione peroxidase; HDL, high-density lipoprotein; LXRα, liver X receptor α; MDA, malondialdehyde; PPARγ, peroxisome proliferator-activated receptor γ; SOD, superoxide dismutase; T, testosterone; TBARS, thiobarbituric acid reactive substance; TNFα, tumor necrosis factor α

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https://doi.org/10.1016/j.reprotox.2018.08.011
Received 16 February 2018; Received in revised form 7 August 2018; Accepted 9 August 2018
Available online 11 August 2018
0890-6238/

[13,14].

Another consequence of phthalate toxicity is a reduction in testosterone synthesis during the fetal period and, sometimes, in adult life [10,15,16]. According to Giribabu et al. [16], rats exposed to 100 and 500 mg/kg/day of DBP during gestation present a reduction in serum testosterone levels due to an alteration in steroidogenic enzyme expression 3β-HSD and 17β-HSD. This reduction was also accompanied by impaired fertility, since the animals had decreased sperm count and motility [16]. Due to these results, the authors classify DBP and other phthalates as anti-androgenic compounds [10,17]. At the same time, other studies have demonstrated the estrogenic activity of phthalates, which are capable of binding to estrogen receptors (ER) [18,19]. Despite the anti-androgenic classification of phthalates, *in utero* exposure of rats to 100 mg/kg/day of DBP from gestational day 12 to postnatal day 21 altered neither testosterone levels in adult rats nor sperm counts in the testis and epididymis, sperm morphology and motility [12].

The vast majority of experimental studies focusing on the action of phthalates on reproductive organs have been performed on classical rodents such as the mouse [16,20–22] and the rat [7,10–17]. It is therefore relevant to expand our knowledge of the reproductive effects of phthalates on other rodents, such as the Mongolian gerbil. Mongolian gerbils were introduced into the Americas as a new experimental proposal in the 1950s [23] and since then they have been increasingly used in different areas of biomedical research. In our own laboratory, the gerbil has been chosen as a very useful rodent for evaluation of the prostate response, particularly because of the fact that the high frequency of the prostatic gland in the females, in comparison with other classical rodents, makes it possible to compare the prostatic response between the sexes. We therefore examined the response of the prostatic gland of the gerbil to different agents such as hormones, endocrine disruptors and dietary components [24–28]. However, the testicular response of the gerbil to phthalates is largely unknown. Experimental and epidemiological studies indicate that a variety of EDCs, including phthalates, inappropriately regulate lipid metabolism, promoting adipogenesis and obesity [29,30]. Evidence has shown that the lipid metabolism of Mongolian gerbil in response to dietary lipid components is more similar to that of the human than that of other rodents, such as the rat [31]. Thus, the gerbil may be considered to be a more suitable model for the evaluation of agents that may affect the lipid metabolism in terms of comparison with the human species.

Most of the studies found in the literature make use of corn oil as the diluent vehicle of the liposoluble compounds to be tested, such as DBP, when administered via gavage [4,11,16,32,33]. Recent data obtained in our laboratory have shown that small amount of corn oil, such as that used to administration of liposoluble agents, may alter sexual steroids and reproductive physiology of the Mongolian gerbil. Corn oil can play a major role in the human diet, since it provides essential fatty acids and vitamin E, besides being a source rich in polyunsaturated fats, such as omega-6 [34] and phytosterols [35]. Then, the intake of corn oil by pregnant gerbils can potentially alter the development of the reproductive organs. Additionally, considering the composition of corn oil, it is relevant to examine whether this vehicle can interfere in the phthalate effects. Thus, the aim of the present study was to investigate whether gestational exposure of pregnant gerbils to di-*n*-butyl phthalate impacts on the sperm parameters, lipid metabolism, testicular steroidogenesis and oxidative stress markers of adult male offspring. In addition, we evaluated the consequences of corn oil administration to pregnant gerbils on these parameters and the possible interference on di-*n*-butyl phthalate effects.

2. Material and methods

2.1. Animals and experimental design

Mongolian gerbils (*Meriones unguiculatus*) were maintained in the Animal Breeding Center of São Paulo State University (UNESP), Institute of Biosciences, Humanities and Exact Sciences (IBILCE, São José do Rio Preto, Brazil) in polyethylene cages with shaving substrate, under controlled light (12-hour light/dark cycle) and temperature (22 ± 2 °C) conditions. Gerbils had free access to food and water. The experimental procedures were approved by the Institutional Committee for Ethics in Animal Experimentation of the Institute (093/2014 CEUA / UNESP).

In order to obtain the gerbils from different experimental groups, thirty-six families were formed using one male gerbil at 14 weeks of age and one female at 12 weeks of age. The age of the females for family formation was chosen based on the studies of Marston and Chang [36] and the age of the male was based on a study of spermatic reserve that shows that full sexual maturation only occurs after 12 weeks of age [37]. Day 0 of pregnancy was confirmed by vaginal swabs. Afterwards, the families were randomly divided into three groups according to the treatment of the pregnant females. The treatment occurred by gavage (via oral), from gestational day 8 to 23. This period was chosen because gerbil implantation occurs on gestational day 8 and the gestation lasts from 24 to 26 days [38]. Following previous procedures adopted to minimize the distress associated to gavage in rats [39], all the female gerbils of the groups to be subjected to gavage were handled daily for two weeks prior in order to ease gavage administration. The number of pups per littermate varied between 6 and 8 and, at weaning, only one male per litter was chosen and placed in individual cages until the end of the experiment. Thus, three groups of adult male gerbils (16 week old) were used in this study (n = 12 animals per group): Control group (C) – control animals, born from intact mothers, without any treatment; Oil group (O) – animals exposed during gestation to 0.1 mL/day of commercial corn oil (Cargil, Brazil); Phthalate group (Ph) – animals exposed during gestation to 0.1 mL/day of corn oil containing 100 mg/kg of DBP (Sigma-Aldrich, 524980, MO, USA). The dose of 100 mg/kg/day of DBP is the lowest dose at which adverse effects begin to occur in the reproductive system of male rats (LOAEL) [7].

The male gerbils were weighed and euthanized by CO_2 inhalation, followed by decapitation for blood collection. Euthanasia was performed between 7:30 and 11:00 a.m. The weights of the testes and epididymis were measured, and the gonadosomatic index (GSI) was determined based on the formula [(testicular weight/body weight) × 100].

2.2. Metabolic characterization

The body weights of offspring at weaning (5weeks old) and in adulthood (16 weeks old) were determined and the adiposity index was estimated by the formula [(sum of epididymal, retroperitoneal and visceral fat/body weight) × 100], according to Taylor and Phillips [40]. The glucose concentrations were determined at the end of the experiment with the Accu-Chek Active monitor (Roche Diagnostics, Switzerland), from blood drops taken from the cervical region.

The lipid profile was evaluated by colorimetric-enzymatic tests to assess total cholesterol, high-density lipoprotein (HDL), non-HDL and triglycerides levels (In vitro Diagnóstica Ltda, MG, Brazil). The test was realized in serum samples, after blood centrifugation (1200 g, 20 min). Each sample was evaluated in triplicate in a Thermo Scientific Evolution 300 UV–vis spectrophotometer (Thermo Fisher Scientific, MA, USA). The triglycerides/HDL ratio was calculated as a predictor of insulin resistance, as proposed by Fan et al. [41].

2.3. Sperm counts

Spermatids resistant to homogenization (stages 13, 14 and 15 of spermiogenesis) [42] and spermatozoa present in the caput/corpus and cauda of the epididymis were estimated as described by Robb et al. [43], with modifications according to Fernandes et al. [44]. After homogenization of the testes and epididymis regions in 0.9% NaCl with 0.5% TritonX100, testicular and epididimal samples were diluted 10×

and 20×, respectively. Counts were performed in Neubauer chambers (Laboropitk Ltd, UK). To determine the daily sperm production (DSP), the number of spermatids per testis was divided by 5.81, which is the number of days in which mature spermatids resistant to homogenization are present in the seminiferous epithelium [42]. DSP per gram was calculated in order to determine the efficiency of the spermatogenic process [45,46]. Sperm reserve was obtained by the number of spermatozoa in the cauda of the epididymis multiplied by 2. The sperm transit time through the caput/corpus and cauda of the epididymis was obtained by dividing the number of spermatozoa present in each of these regions by the DSP [47].

2.4. Sperm motility

Estimation of sperm motility was performed immediately after euthanasia, using the cauda of the epididymis. Sperm was obtained through perforations made with the aid of a needle in 1.0 mL of modified HTF medium (Human Tubal Fluid, Irvine Scientific, CA, USA) at 34 °C A Makler counting chamber (Sefi-Medical Instruments, Israel) warmed to 34 °C was loaded with 10 µL of sperm solution. The evaluation of sperm motility was assessed by visual estimation (100 spermatozoa per animal, in duplicate) under a phase-contrast microscope (Olympus BX60, Olympus Corporation, Japan) at 200× magnification. Spermatozoa were classified as motile with progressive movement, motile without progressive movement and immotile [48].

2.5. Histological alterations and apoptosis index

The testes were removed and fixed in Bouin's fluid for 12 h. Fragments were washed several times in 70% alcohol, processed for inclusion in Paraplast (Merck, Germany) in a TP1020 processor (Leica Microsystems Brazil, China) and used for general histological analysis.

Histological sections stained with Hematoxylin-Eosin (HE) were used for the general histological analysis. The entire histological section from each testis fragment was examined, and the number of seminiferous tubules affected by some type of histopathological alteration was counted and expressed as a percentage of the total number of seminiferous tubules of the section. Two histological sections of two distinct fragments per animal were evaluated in five animals per group.

Apoptotic cells were detected using the DNA fragmentation assay associated with cell death, based on the TUNEL reaction, according to the kit instructions (ApopTag Plus in situ, Apoptosis Detection Kit, Millipore, 57101, CA, USA). Briefly, after digestion with proteinase K (#JA 1477, Calbiochem, Germany), the histological sections were submitted to procedures designed to inactivate endogenous peroxidase (3% H_2O_2 in PBS, 5 min), and then incubated with the enzyme deoxynucleotidyl terminal transferase (TdT) for 40 min at 37 °C. At the end of the reaction, the sections were incubated with peroxidase, revealed with diaminobenzidine (DAB) and counterstained with Harris' Hematoxylin. The estimation of the apoptosis index was determined by the ratio of the number of apoptotic cells in the entire histological section and the corresponding section area. This analysis was performed in five histological sections of five distinct animals per group.

The images were obtained with a Pike F-505C VC50 camera (Allied Vision Technologies, Germany) attached to an Olympus VS10 Virtual Microscope Slide Scanning System (Olympus Corporation, Japan).

2.6. Protein expression in testicular samples

The protein content of the peroxisome proliferator-activated receptor γ (PPARγ), liver X receptor α (LXRα), androgen receptor (AR) and estrogen receptor α (ERα) were quantified in testis samples by Western blotting. Total extracts were obtained from 100 mg of tissue fragments, homogenized in RIPA buffer (Sigma, MO, USA) with a cocktail of protease inhibitors (Sigma, MO, USA) and Triton X100. The homogenate was centrifuged at 18,000 g for 20 min at 4 °C and the supernatant was stored at -80 °C. The protein dosage was quantified by the Bradford method [49] and 30 µg of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare, Italy). Blots were blocked with 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.2% Tween-20) for 30 min at room temperature, followed by overnight incubation at 4 °C with primary antibodies, according to Table 1. Thereafter, the membranes were washed in TBST for three 10-min cycles, incubated for 1 h with a specific secondary horseradish peroxidase–conjugate antibody (Table 1) at room temperature, and washed in TBST for three 10 min cycles once more. Protein expression was normalized to the β-actin values (Table 1). Antibody detection was revealed using the ECL chemiluminescent detection kit (GE Healthcare, Italy). The membranes were revealed in G: BOX Chemi XRQ photodocumentator (Syngene Synoptics Ltd, UK) and the band densities were analyzed in a densitometry program - Image J 1.34 (Wayne Rasband, Research Services Banch, National Institute of Mental Health, MD, USA).

Table 1
Description of the antibodies and protocols used in the immunoblotting.

Primary antibody		Dilution	Secundary antibody	Dilution
Anti-PPARγ	Abcam (MA, USA) - 209350	1:400	Anti-rabbit	1:4,000
Anti-LXRα	Abcam (MA, USA) - 106464	1:200	Anti-rabbit	1:10,000
Anti-AR	Santa Cruz Biotechnology (CA, USA) - sc-816	1:300	Anti-rabbit	1:3,000
Anti-ERα	Santa Cruz Biotechnology (CA, USA) - sc-8005	1:200	Anti-mouse	1:7,000
Anti-β-actin	Santa Cruz Biotechnology (CA, USA) - sc-47778	1:1000	Anti-mouse	1:10,000

2.7. Testicular TNFα concentration

Testicular fragments were homogenized in lysis buffer (50 mM Tris-HCl and 150 mM NaCl) with a Protease Inhibitor Cocktail Set I (Cat. No. 539131, Calbiochem-Merck, Germany). The homogenate was centrifuged at 18,000 g for 10 min at 4 °C. Supernatant was used for total protein dosage [49] and determining of the proinflammatory cytokine tumor necrosis factor α (TNFα). TNFα was quantified by capture/sandwich ELISA using specific commercial kits from R&D System (DY510, MN, USA), and the results were normalized by concentration of total proteins.

2.8. Hormone levels

After euthanasia, blood samples were centrifuged (1200 g, 20 min) and the serum obtained was frozen at −80 °C for hormone analyses. Serum testosterone (T) and estradiol (E_2) levels were analyzed in duplicate by capture/sandwich ELISA using specific commercial kits, according to the instructions of the manufacturer (Testosterone EIA Kit, No. 582701, and Estradiol EIA Kit, No. 582251, Cayman Chemical Company, MI, USA).

Intratesticular hormone concentration was performed after the testes were processed for extraction of steroids, according to Pinto-Fochi et al. [50]. Testis fragments were homogenized in PBS and incubated in a tube with diethyl ether for 10 min initially at room temperature and subsequently on dry ice. The supernatants were removed and transferred to another tube and stored until complete evaporation of the diethyl ether. The material was suspended in PBS and stored at −80 °C until hormone determining. T and E_2 concentrations were also analyzed in duplicate using ELISA capture/sandwich and specific commercial kits (Testosterone Test System, code 3775-300, Monobind Inc., CA, USA, and Estradiol EIA Kit, No. 582251, Cayman Chemical Company, MI, USA).

The readings were performed in a SpectraMax Plus 384 microplate reader (Molecular Devices, CA, USA).

2.9. Activity of antioxidant enzymes

Testicular and epididimal fragments were weighed and stored at −80 °C. Then, the fragments were homogenized in 1: 4 vol of Tris-HCl buffer (0.2 mM, pH 7.5) with 1 mM EDTA, 1 mM DTT, 0.5 M sacarose and 0.15 M KCl, and centrifuged at 10,000 g for 20 min at 3 °C. The supernatant was centrifuged once more at 50,000 g for 60 min at 1 °C. The new supernatant fraction was collected, aliquoted and used to determine the enzyme activities of Catalase (CAT) [51], Glutathione peroxidase (GPx) [52] and Superoxide dismutase (SOD) by kit, according to the instructions of the manufacturer (Sigma-Aldrich, No. 19160, MO, USA). The total protein content was determined by the Bradford method [49]. Analyses of CAT and GPx were performed in a Thermo Scientific Evolution 300 UV–vis spectrophotometer (Thermo Fisher Scientific, MA, USA). SOD activity was determined with a Victor TM X3 microplate reader (Perkin ElmerR, MA, USA). The aforementioned analyses were also applied to the hemolysate obtained through blood samples collected, immediately after euthanasia, in polyethylene tubes containing heparin.

2.10. Oxidized biomolecule levels

As oxidation marker, the levels of colored adducts produced by the reaction of oxidized biomolecules with thiobarbituric acid (TBA) [53] were measured in plasma samples and fragments of testis and epididymis. Plasma samples were obtained after centrifugation of the hemolysate (850 g, 10 min). Testicular and epididimal fragments were weighed and homogenized in 1:3 vol of Tris-HCl buffer (0.1 M, pH 8). Following the addition of 300 µL TBA, the homogenate was incubated at 90 °C for 40 min and, subsequently, incubated on ice to stop the reaction. Afterwards, 600 µL n-butanol was added to the homogenate, which was centrifuged at 2,500 g for 5 min. The supernatant was collected and used for oxidized biomolecule determination. Products were detected by spectrophotometry (SpectraMax Plus 384 microplate reader, Molecular Devices, CA, USA), according to Uchiyama and Mihara [54].

2.11. Statistical analysis

Statistical analyses were performed by Statistica 8.0 software (Statsoft. Inc., OK, USA), while the graphics were done using GraphPad Prisma 5.01for Windows (GraphPad Software, CA, USA). First, the data were checked for normality using the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was used. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effects on metabolic parameters

The intake by pregnant mothers of corn oil alone or containing DBP did not affect the body weight and the adiposity index of the offspring when compared to the offspring of untreated mothers (Table 2). Neither the glucose levels (C: 81.2 ± 3.5; O: 78.6 ± 2.9; Ph: 84.0 ± 3.0 mg/dL; $p = 0.49$) nor the triglyceride/HDL ratio (C: 3.3 ± 0.5; O: 3.2 ± 0.5; Ph: 4.1 ± 0.6; $p = 0.59$) were affected. Regarding the lipid profile, the O group showed an increase non-HDL cholesterol serum levels in relation to the C group (~37%; $p = 0.53$) (Fig. 1). When the Ph and C groups were compared there was an increase in serum levels of total cholesterol (~43%), non-HDL cholesterol (~66%) and triglycerides (~90%) (Fig. 1).

Table 2
Biometric data of the animals from the Control (C), Oil (O) and Phthalate (Ph) groups.

n = 12 per group	Experimental groups		
	C	O	Ph
Body weight at 5th weeks old (g)[a]	27.8 ± 0.7	24.0 ± 1.4	24.6 ± 1.1
Body weight at 16th weeks old (g)[b]	70.6 ± 1.0	67.6 ± 1.1	68.8 ± 1.5
Adiposity index (%)[a]	2.89 ± 0.2	2.48 ± 0.2	2.81 ± 0.2
Testicular weight (mg)[a]	537.5 ± 12.1	513.9 ± 8.0	524.8 ± 9.7
Gonadosomatic index (%)[a]	0.76 ± 0.01	0.76 ± 0.01	0.76 ± 0.01
Epididimal weight (mg)[b]	199.2 ± 4.1	186.0 ± 4.0	194.2 ± 4.5

Values expressed as median ± SEM.
[a] ANOVA followed by Tukey's test.
[b] Kruskal-Wallis test followed by Dunn's test.

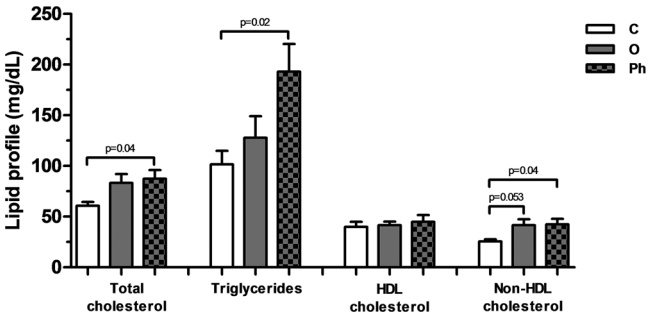

Fig. 1. Lipid profile of the gerbils from the Control (C), Oil (O) and Phthalate (Ph) groups. n = 10 animals per group. Values are expressed as mean ± SEM. Values $p < 0.05$ were considered significant (ANOVA test followed by Tukey's test).

3.2. Effects on sperm parameters

The gerbils of the O and Ph groups did not show any change in testicular and epididimal weights nor in GSI in relation to the gerbils from the C group (Table 2). There were no alterations in the testicular sperm counts and DSP of the O and Ph groups in relation to those in the C group (Table 3). The sperm reserve was reduced by ~21% in the O group and ~68% in the Ph group in relation to the control animals, since the number of spermatozoa in the caput/corpus of the epididymis was reduced in the Ph group and the number in the cauda of the epididymis was decreased in the O and Ph groups (Table 3). The sperm transit time through the epididymis was not altered in the Ph group, however, it decreased by about 16% in the O group when compared to the C group (Table 3).

DBP exposure during gestation had a negative effect on sperm motility, with a reduction in the rate of spermatozoa with progressive movement and an increase in the rate of spermatozoa without progressive movement and immotile spermatozoa in relation to the animals of the C and O groups (Fig. 2). In the O group there was also an increase in the rate of spermatozoa without progressive movement when compared to the C group (Fig. 2).

3.3. Effects on testis histology, apoptosis and expression of nuclear receptors

Histopathological analysis did not indicate tissue lesions in the testis of the animals of both groups, except for an increase in the number of seminiferous tubules presenting premature detachment of the germinative epithelium in the O group in relation to the C group (Fig. 3). Four out of five animals evaluated per group showed premature cell detachment in both O and Ph groups (C: 0 ± 0; O: 1.42 ± 0.46; Ph: 0.72 ± 0.38% of the total tissue section area).

The relative density of apoptotic cells in the testis did not change in

Table 3
Testicular and epididymal sperm counts, and sperm transit time through the epididymis of gerbils from the Control (C), Oil (O) and Phthalate (Ph) groups.

n = 12 per group	Experimental groups		
	C	O	Ph
Sperm number in the testis ($\times 10^6$)	77.4 ± 4.1	73.3 ± 3.7	66.1 ± 2.4
Sperm number per gram of testis ($\times 10^6$)	146.0 ± 6.8	145.8 ± 7.0	130.9 ± 4.5
DSP ($\times 10^6$)	13.3 ± 0.7	12.6 ± 0.6	11.4 ± 0.3
DSP per gram ($\times 10^6$)[a]	25.1 ± 1.2	25.1 ± 1.2	22.5 ± 0.8
Sperm number in the caput/corpus of the epididymis ($\times 10^6$)	13.8 ± 0.8	12.0 ± 0.5	10.1 ± 0.5[a]
Sperm number per gram of caput/corpus of the epididymis (x10^6)	194.1 ± 8.8	181.4 ± 8.9	149.6 ± 6.2[a,b]
Sperm number in the cauda of the epididymis ($\times 10^6$)	166.3 ± 7.8	130.6 ± 5.9[a]	130.4 ± 6.7[a]
Sperm number per gram of cauda of the epididymis ($\times 10^6$)	1266 ± 42.2	1077 ± 36.3[a]	1061 ± 32.7[a]
Sperm reserve in the epididymis ($\times 10^6$)[b]	332.6 ± 15.6	261.3 ± 11.8[a]	260.7 ± 13.3[a]
Sperm transit time in the epididymis (days)			
Caput/corpus	1.05 ± 0.07	0.97 ± 0.05	0.90 ± 0.06
Cauda	12.62 ± 0.46	10.48 ± 0.43[a]	11.46 ± 0.46
Total	13.67 ± 1.72	11.70 ± 1.36[a]	12.36 ± 1.67

Values expressed as mean ± SEM.
The superscripts a, b indicate statistical difference among the groups: a $p < 0.05$ versus C; b $p < 0.05$ *versus* O (Kruskal-Wallis test followed by Dunn's test).
[a] Espermatogenic efficiency.
[b] Value per cauda of the epididymis multiplied by 2.

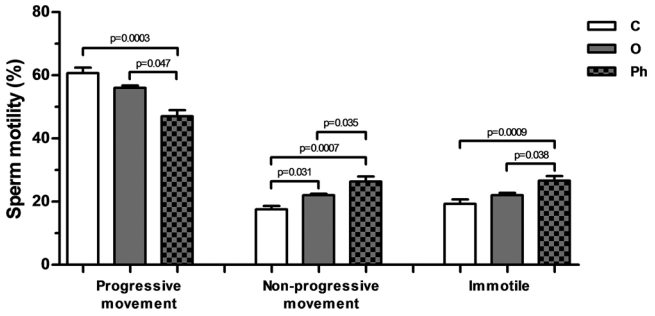

Fig. 2. Sperm motility of the animals from the Control (C), Oil (O) and Phthalate (Ph) groups. Sperm were analyzed according to the movement type: progressive movement, non-progressive movement and immotile. n = 10 animals per group. Values are expressed as mean ± SEM. Values $p < 0.05$ were considered significant (Kruskal-Wallis test followed by Dunn's test).

the O group, but increased by about 48% in the animals exposed to DBP in comparison to the C animals (Fig. 4).

Testicular expression of the nuclear receptors PPARγ, LXRα and AR did not vary in the O and Ph groups (Fig. 5A–C). On the other hand, ERα content in the testis was approximately 78% higher in the O group when compared to the C group (Fig. 5D). ERα expression did not vary in the Ph group when compared with the control group (Fig. 5D).

No differences were observed in testicular concentrations of TNFα among the groups (C: 4.54 ± 0.18; O: 5.23 ± 0.29; Ph: 4.89 ± 0.46 × 10^{-8} pg/mL; p = 0.34).

3.4. Effects on sexual steroids

No alterations were observed in serum and intratesticular T levels among the groups (Fig. 6A and B). When compared to the C group, serum levels of E_2 increased by about 52% and 54% in the O and Ph groups, respectively (Fig. 6C). Intratesticular E_2 levels were approximately 86% higher in the Ph group in relation to the C and O groups (Fig. 6D).

3.5. Effects on oxidative stress markers

In relation to the activity of the antioxidant enzymes, CAT and SOD activity was higher in the blood of the O and Ph animals when compared to the animals of the C group (Fig. 7A, G), while GPx activity was unchanged among the groups (Fig. 7D). In the testis, the antioxidant activity of CAT was lower in the O group than in the C group (Fig. 7B), and no changes were observed in GPx and SOD activity among the groups (Fig. 7E and H). In the epididymis, there was no change in the activity of CAT, GPx and SOD enzymes in both O and Ph groups (Fig. 7C, F and I).

Thiobarbituric acid reactive substance (TBARS) assay demonstrated that there was an increase by about 80% in plasmatic oxidized biomolecule levels of the Ph animals in comparison to the C animals (Fig. 7J). No changes were observed in the levels of testicular oxidized biomolecules among the groups (Fig. 7K). However, there was a reduction of approximately 46% in epididymal oxidized biomolecule levels in the Ph group compared to the O group (Fig. 7L).

4. Discussion

The present study with gerbils demonstrated that the maternal intake of a small amount of corn oil (100 µl/day), a vehicle widely used for the dilution of liposoluble agents, led to persistent alterations in the sex steroid metabolism, causing an increase in both circulating estradiol and the content of ERα in the testis of the male offspring at adulthood and a reduction in the sperm reserve. It was also demonstrated that gestational exposure to 100 mg/kg/day of DBP in the same volume of corn oil further reduced the sperm reserve and impaired sperm motility of the adult offspring. The alterations caused by DBP were associated with perturbations in the intratesticular synthesis of estradiol and systemic alterations in the lipid metabolism.

4.1. Effects on adiposity and the lipid metabolism

DBP and other EDCs are known to act as obesogenic agents, promoting obesity by altering the lipid metabolism and adipogenesis process [29]. In the present study, it was verified that the animals exposed to DBP were dyslipidemic, presenting higher levels of serum triglycerides, total cholesterol and non-HDL cholesterol. Thus, gestational exposure to DBP causes dyslipidemia in gerbils in adulthood. Mice exposed *in utero* to the diethylstilbestrol (DES) deregulator also showed higher serum triglyceride levels, as well as leptin and interleukin 6 (IL-6) at two months of age, even before they become overweight and obese [3]. These animals presented higher adiposity and obesity from the third month of life, suggesting that high levels of triglycerides, leptin and IL-6 would be important early markers of the metabolic syndrome [3]. In comparison with mice exposed during gestation to DES [3], the data obtained here for the gerbil, after DBP exposure, also reveal alterations in total cholesterol and non-HDL cholesterol. Experimental

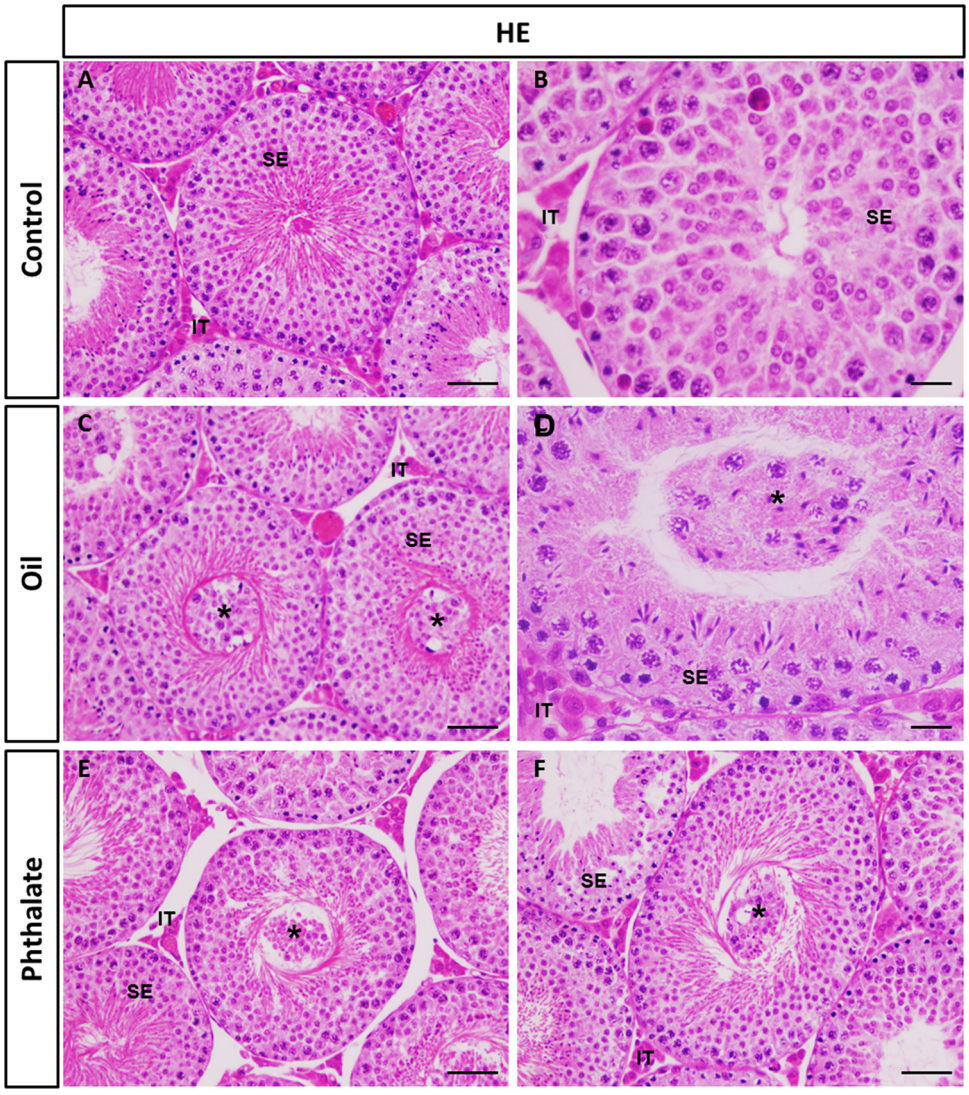

Fig. 3. Testicular histological sections from the Control, Oil, and Phthalate groups stained with Hematoxylin-Eosin (HE). A and B represent testicular sections with normal appearance. C, D, E and F present sections containing seminiferous tubules with premature detachment of germinative cells (*). Two histological sections of two distinct fragments per animal were evaluated in five animals per group. IT: interstitial tissue; SE: seminiferous epithelium. Bars: 50 μm (A, C, E and F) and 20 μm (B and D).

feeding of gerbils with a high-fat diet for two weeks showed that gerbils exhibit a higher susceptibility to hypercholesterolemia than other rodents, which is related to the stable expression of sterol regulatory element binding protein (SREBP)-2 in the liver [55]. These findings may explain the hypercholesterolemia detected in the present study for gerbils after DBP exposure and not for other rodents [3].

It should be noted that isolated exposure to corn oil also marginally altered non-HDL cholesterol levels, which were ~ 63% higher in the O group than in the C group ($p = 0.053$). Clinical and experimental studies have implicated this plant oil, as well as corn fiber oil, in the regulation of serum cholesterol levels of adult individuals [35,56,57]. A study by Poveda et al. [58] demonstrated that the consumption of 0.2 mL/day of corn oil by female Wistar rats for two weeks enhanced the serum HDL cholesterol levels. The authors suggest that the high concentration of tocopherols, a vitamin E constituent present in corn oil, may improve the lipid profile and increased HDL cholesterol levels [58]. It is known that fatty acids in the maternal circulation can be transported through the placenta and captured by the liver of the fetus, being subsequently esterified and released as triglycerides in the circulation [59]. In postnatal life, the lipid metabolism and, consequently, the profile of circulating lipids is largely determined by hepatic function [60]. Other studies indicate that the offspring of mice fed a high-fat diet during gestation have impaired hepatic function in adulthood [61]. Our results corroborate the data of Gregorio et al. [61], indicating that, despite not affecting adiposity in early adulthood, the consumption of a small amount of corn oil during pregnancy may interfere with the lipid metabolism of the offspring in a different manner from that occur in our own consumption, increasing cholesterol levels [58]. It was not the aim of this study to evaluate the mechanisms by which corn oil and DBP impairs the lipid metabolism, which would require other analyses of the liver and adipose tissue, which were not predicted in this study. However, it is known from the literature that phthalates can act in the induction of metabolic syndrome by interfering with the signaling of LXRα, PPARγ and ERα [22,62–64]. Our data suggest that ingestion of the corn oil diluent vehicle interferes with the lipid metabolism, especially of cholesterol, and DBP potentiates these disorders, increasing serum triglycerides levels, demonstrating an additive or synergistic effect of the two compounds. It has been hypothesized that oils containing high levels of omega-3 polyunsaturated fatty acids, such as canola and fish oil, could counteract some of the adverse effects induced by phthalates.

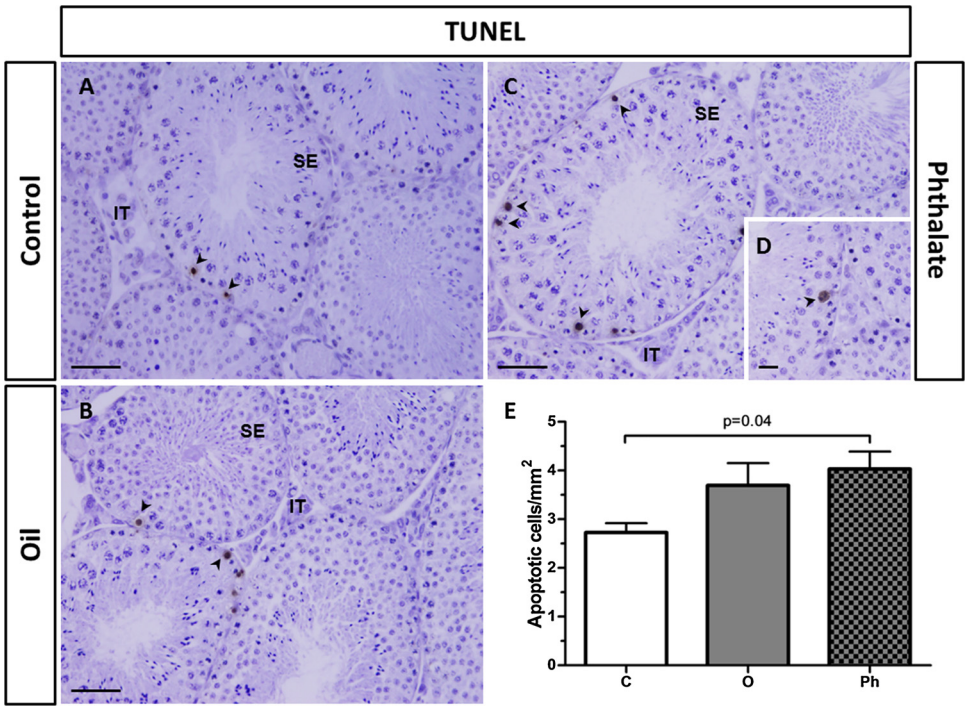

Fig. 4. Histological sections of gerbil testes submitted to TUNEL reaction to detect apoptotic cells in the Control (C; A), Oil (O; B), and Phthalate (Ph; C and D) groups. Arrowheads indicate cells in apoptosis. SE: seminiferous epithelium; IT: interstitial tissue. Bars: 50 μm (A, B, and C) and 20 μm (D). E. Relative density of apoptotic cells in the C, O and Ph groups. Five histological sections of five distinct animals per group were evaluated. Values are expressed as mean ± SEM. Values $p < 0.05$ were considered significant (Kruskal-Wallis test followed by Dunn's test).

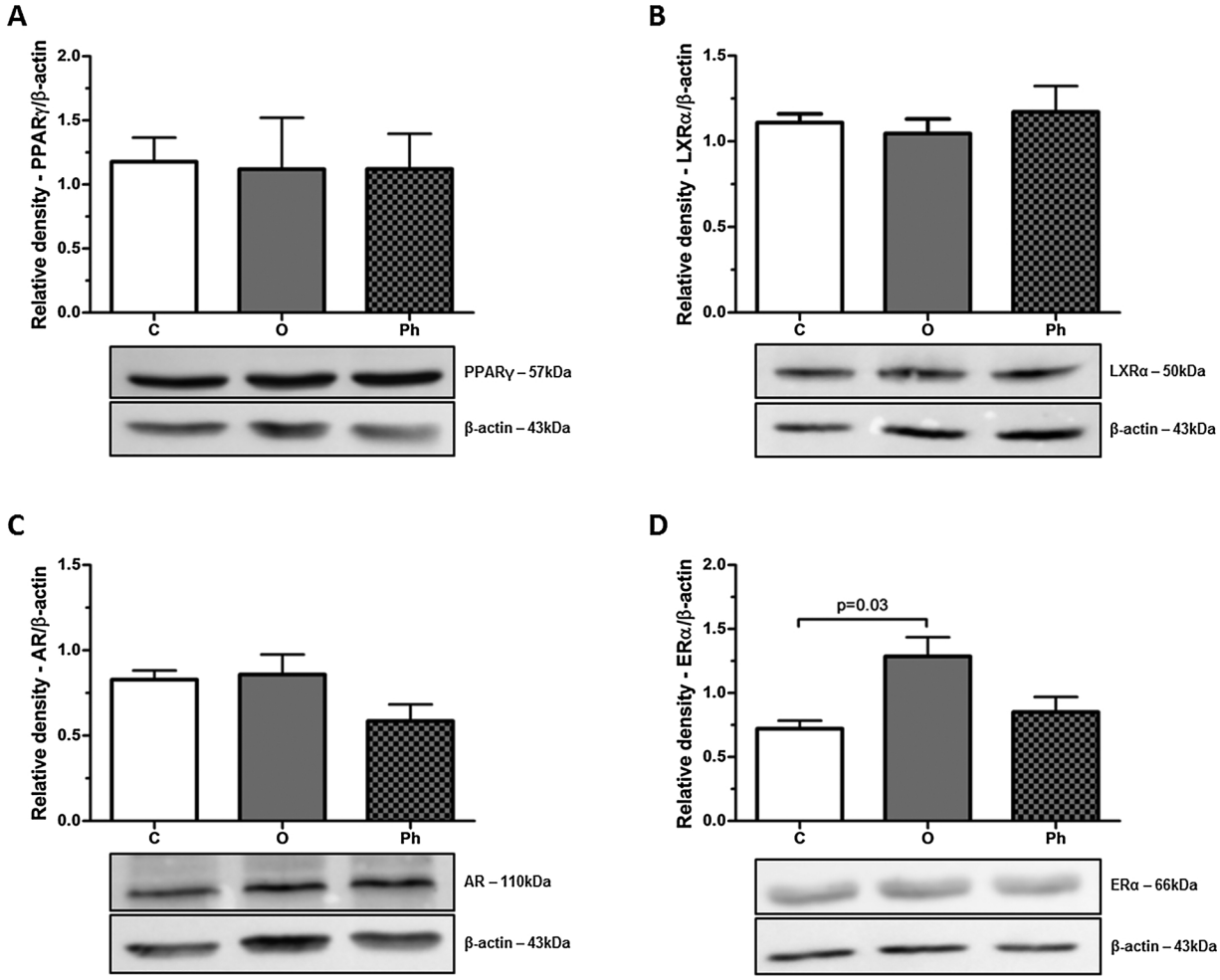

Fig. 5. Determination of the expression of the peroxisome proliferator-activated receptor γ (PPARγ; A), liver X receptor α (LXRα; B), androgen receptor (AR; **C**) and estrogen receptor α (ERα; D) in the testis of gerbils from the Control (C), Oil (O) and Phthalate (Ph) groups, normalized by β-actin, which was used as a positive control. n = 4 animals per group. Values are expressed as mean ± SEM. Values $p < 0.05$ were considered significant (Kruskal-Wallis test followed by Dunn's test).

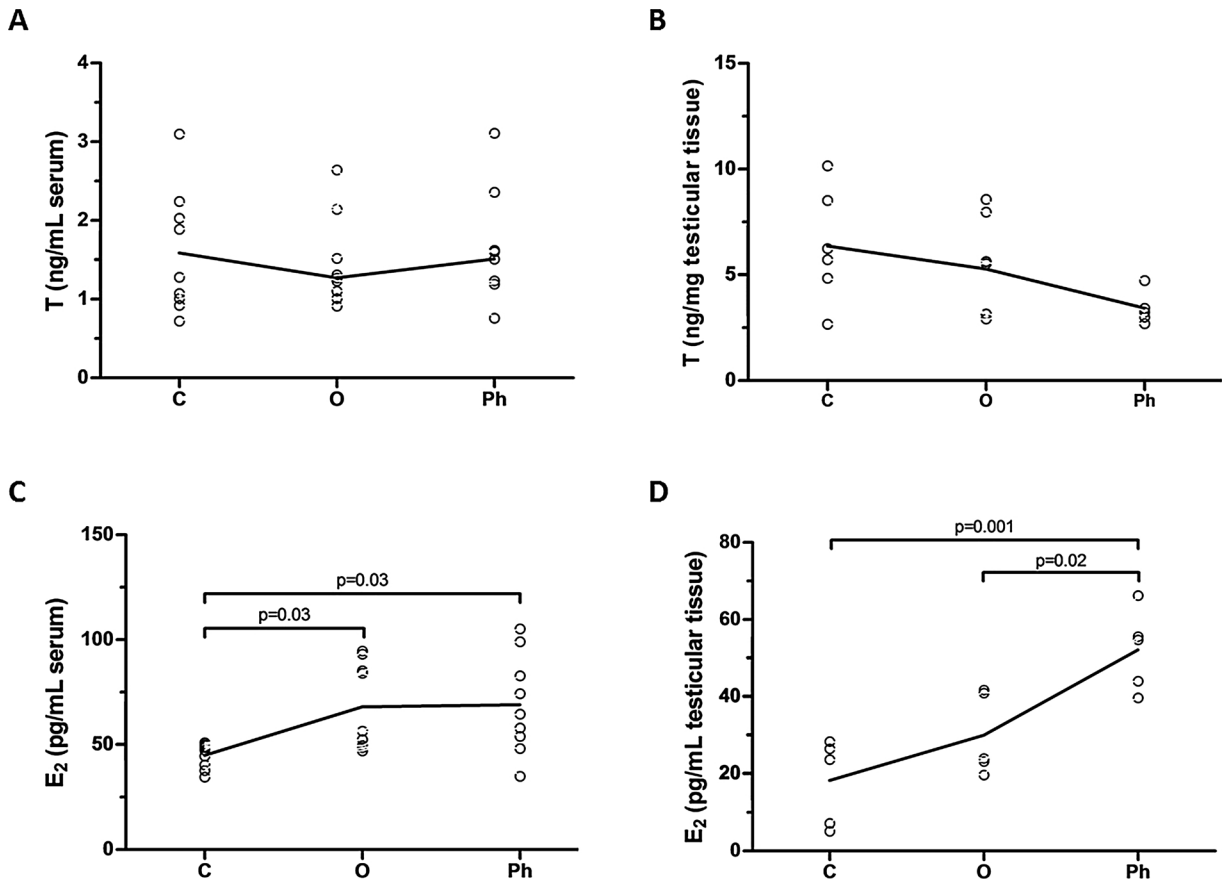

Fig. 6. Hormone profile of the animals from the Control (C), Oil (O) and Phthalate (Ph) groups. A and B. Serum and intratesticular testosterone (T) levels, respectively. C and D. Serum and intratesticlar estradiol (E_2) levels, respectively. Serum and intratesticular dosages were performed in 10 and 7 animals per group, respectively. Individual values (circles) and mean value (line) are represented. Values $p < 0.05$ were considered significant (A. Kruskal-Wallis test followed by Dunn's test; B, C and D. ANOVA test followed by Tukey's test).

4.2. Effects on sexual hormones

Disorders caused by EDCs in critical periods of development, such as the fetal period, may be transitory or affect the homeostatic mechanisms of the endocrine system permanently [1–5]. We found that gestational exposure of gerbils to 100 mg/kg/day of DBP increases the serum and intratesticular estradiol levels. However, these changes occur without impacting the serum and intratesticular levels of testosterone. It is known that intrauterine exposure of other rodents to phthalates reduces testosterone synthesis during the fetal period [15,65,66], leading to reproductive damage that persists up to adulthood [15,67]. However, Giribaru et al. [16] observed that rats exposed during gestation to the same DBP dose used here exhibited a decrease in serum testosterone levels in adulthood. Therefore, in comparison with other rodents, the gerbil appears to be less susceptible to DBP effects regarding the regulation of circulating testosterone, being the main impact in circulating and intratesticular estradiol levels. The aromatase enzyme is responsible for the conversion of testosterone and androstenedione to estradiol and estrone, respectively [68]. Thus, it is possible that gestational exposure to DBP may have induced the overexpression and increased aromatase enzyme activity by testicular cells, culminating in higher estradiol levels.

Comparing the hormonal changes of the gerbils exposed to DBP with offspring from mothers that ingested only corn oil, higher circulating estradiol levels were observed without alterations in intratesticular levels of this hormone. Pearlman [69] introduced the concept that the plasma level of a steroid was related to its production rate divided by its metabolism rate. This relationship is designated as the metabolic clearance rate [70], and it has proved to be a powerful tool in the study of steroid physiology. Higher estradiol serum levels in the O group may be due to alterations in the metabolic clearance rate, caused by reduction in hepatic metabolism of steroids, or damage in the mechanism of blood transport and cell entrance. It should be noted that, although the pregnant females were treated with a small amount of corn oil (100 µl/day), it corresponds to about 0.83 mg of phytosterols, which can result in an effective physiological response in hormone-regulated organs, such as the liver and the reproductive organs. In conclusion, these data indicate that maternal corn oil alters the systemic metabolism of estradiol in adulthood.

4.3. Testicular effects and sperm parameter alterations

Studies have shown that exposure to phthalates, such as DBP and DEHP and their monoesters, increases the expression of PPAR in the testis, culminating in damage to the male reproductive system [71]. Ryu et al. [72] treated prepuberal rats at 750 mg/kg/day of DBP for 30 days and observed a significant increase in PPARγ levels in the testis. Testicular PPARγ content was unchanged in the gerbils showing that the present *in utero* exposure to DBP did not affect the expression of this receptor. We also found no alterations in the LXRα, AR or ERα contents after DBP exposure. Increases in germ-cell apoptosis are often observed after exposing experimental animals to various testicular toxicants [73,74]. In this study, gestational exposure to DBP increased by about 48% the relative density of apoptotic cells in the testis. LXRα and PPARγ had been implicated in increased rates of cell death. LXRα-deficient mice present an increased apoptosis of the germ cells [75]. Ryu et al. [72] demonstrated that PPARγ might mediate DEHP-induced apoptosis in testicular cells. However, the increase in apoptosis in the

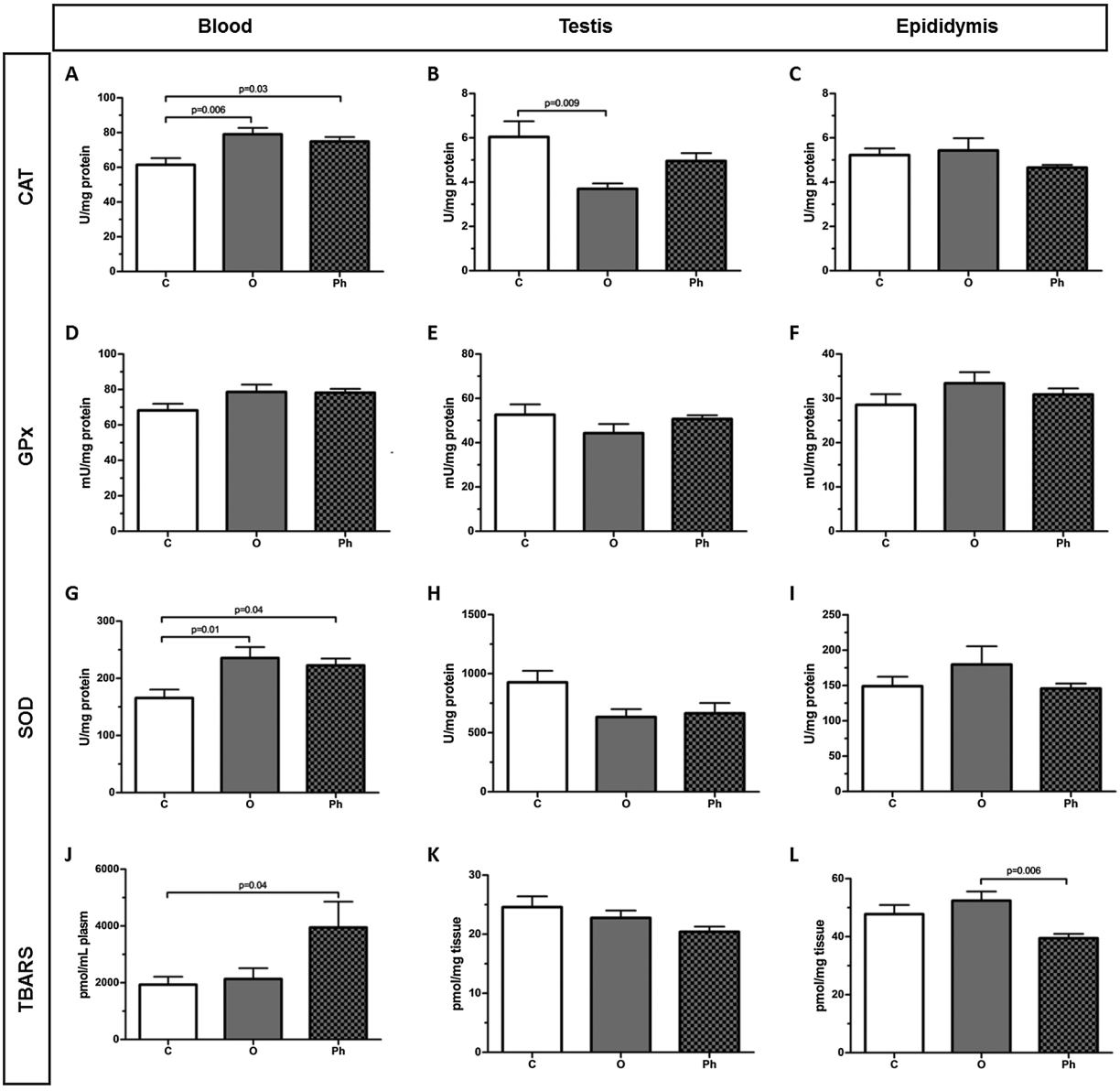

Fig. 7. Oxidative stress markers in blood samples and testicular and epididimal fragments of gerbils from the Control (C), Oil (O) and Phthalate (Ph) groups. Antioxidant activity of the Catalase (CAT; A, B and C), Glutathione peroxidase (GPx; D, E and F) and Superoxide dismutase (SOD; G, H and I) enzymes was determined. Oxidized biomolecule levels were determined by thiobarbituric acid reactive substance (TBARS) assay (J, K and L). n = 7 animals per group. Values are expressed as mean ± SEM. Values $p < 0.05$ were considered significant (A, B, D, E, F, G, H, I, J and L. ANOVA test followed by Tukey's test; B and K. Kruskal-Wallis test followed by Dunn's test).

testis of the gerbils exposed to DBP was not related to alteration in PPARγ and LXRα expression. Higher intratesticular estradiol concentration may be responsible for apoptosis-induction in the testis [76].

Among other adverse effects of DBP exposure are decreases in the weight of the epididymis, testis and accessory sex glands [77–79]. Gerbils exposed to 100 mg/kg/day of DBP during gestation did not present alterations in testis and epididymis weights and in GSI, as well as other abnormalities in the reproductive system. DSP was unchanged either, but decreased sperm concentration in the epididymis and markedly impaired sperm motility. Considering that motility is one of the most important sperm parameters used for evaluation of semen quality [80,81], we can conclude that high doses of DBP during pregnancy compromised sperm motility and the reproductive function of the gerbil. Giribabu et al. [16] verified that prenatal exposure to DBP alters the reproductive function of Wistar rats by reducing sperm concentration and motility.

The acquisition of sperm motility is dependent on the presence of androgens [82] and of an adequate process of sperm maturation in the epididymis [83,84]. Although estradiol is also essential for male fertility [85,86], high estrogen levels are detrimental to epididymal function. A study by Goyal et al. [32] demonstrated that the administration of low doses (≥ 8 μg/day) of DES, an estrogenic compound, to adult rats for 12 days reduced the epididymal weight and the sperm concentration in this organ, and impaired sperm motility without altering daily sperm production, providing relevant data for studying the role of estrogens in the function of the epididymis. In addition, regulation of the amount of cholesterol in the sperm plasma membrane during maturation is determinant for sperm motility [87]. Our data revealed no variations in circulating and intratesticular testosterone levels in the animals exposed to DBP, however serum and intratesticular levels of estradiol were higher in these animals, as well as total and non-HDL cholesterol and triglyceride levels, which may be improperly modulating the process of sperm maturation.

Regarding the reproductive alterations caused by the corn oil, we

verified that gestational exposure to this plant oil increased the rate of seminiferous tubules presenting premature detachment of germ cell. Reame et al. [88] suggest, in their study of obesity induction in Wistar rats fed a high-fat diet, that the increase in the number of tubules with immature germ cell detachment was due to the reduction in serum testosterone levels. As already reported, these levels were unchanged in the animals exposed to corn oil, but we observed higher serum estradiol levels. In this group, a higher testicular expression of ERα was also observed, even without presenting higher intratesticular estradiol levels. It is known that spermatogenesis is dependent on steroid hormones, and alterations in testosterone and estradiol levels, especially high estrogen levels, result in impairments to male fertility [86–89]. The animals exposed to corn oil alone also presented a reduction in the sperm reserve and an increase in the rate of sperm without progressive movement. Moreover, the sperm transit time through the epididymis was reduced in this group. These results demonstrate that gestational exposure to corn oil also compromised the sperm quantity and quality and caused alterations in testicular histology and ERα content. Since these animals were found to have high levels of non-HDL cholesterol, the mechanisms involved in these reproductive disorders are probably related to the high levels of circulating cholesterol and estradiol.

One aspect that should be mentioned concerning the possible interferences of the maternal gavage in the metabolic and reproductive results detected to corn oil- treated group. Gavage is the most common technique for drug administration in pharmacokinetic and toxicological studies, with increasing number of researchers chosen this method [90]. However, up to date few studies have investigated, in a controlled manner, the level of stress induced by oral gavage *per se* and by restraint used to accomplish this procedure [39,91]. According to previous study with rats, in the present study the female gerbils of all groups to be subjected to gavage were manipulated daily for two weeks before gestation aiming to minimize the distressed associated to this procedure. Recently, McDonnell-Dowling et al. [90] also used the habituation to the technique to examine if oral gavage, as a route of drug administration, during pregnancy and lactation affected neurodevelopmental and behavioral outcomes in the offspring of rat dams. These authors concluded that daily oral gavage during gestation and lactation has no significant effect on rat dams. They also showed that gavage treatment of dams had no effect on developmental parameters in their offspring, including birth weight, pinna unfolding, fur appearance, anogenital distance, body weight, and neonatal death, concluding that oral gavage is a safe and feasible route of administration for use during pregnancy and lactation. So, we consider that the alterations detected in corn oil-treated group were caused by this plant oil and not as a result of gavage.

4.4. Effects on oxidative stress markers

Some effects of environmental contaminants are caused by the generation of reactive oxygen species (ROS) in both intra- and extracellular spaces of cells, leading to cell death and tissue injury [92,93]. Oxidative stress is known to be a major factor regulating the vitality and functionality of mammalian spermatozoa *in vitro* [94]. Sperm plasma membrane, being rich in polyunsaturated fatty acids, is highly susceptible to ROS attack and, consequently, to peroxidation of lipid bilayers [95]. Zhou et al. [95] revealed a dose-dependent epididymal toxicity of DBP, demonstrating that exposure to graded doses of DBP disrupts the epididymal structure and function by impacting the antioxidant enzyme activity (GPx and SOD) and increasing lipid peroxidation, thereby causing oxidative stress in the epididymis of the rats exposed to this phthalate (500 mg/kg/day) for two weeks in adulthood. However, antioxidant enzyme activity and lipid peroxidation were unchanged at 100 mg/kg/day of DBP [33]. The marker most frequently used to indicate the level of lipid peroxidation or oxidized biomolecules is malondialdehyde (MDA) [96,97], which can be determined by many different techniques [98]. MDA can interfere in protein biosynthesis by forming adducts with DNA, RNA and protein [99]. Our data indicated that there was no change in CAT, GPx and SOD activities, and the levels of oxidized biomolecules were, unexpectedly, lower in the epididymis of the animals exposed to DBP during gestation. These results suggested that *in utero* exposure to 100 mg/kg/day of DBP was not a high enough dose to impact the mechanisms involved in oxidative stress regulation in this organ. Thus, it is probable that the sperm motility damage observed in this study was not caused by these mechanisms.

Data regarding the oxidative stress markers in the testis did not reveal any alterations in the antioxidant enzyme and TBARS levels of the animals in the Ph group. On the other hand, blood CAT and SOD activities were higher both in animals exposed to DBP and in animals exposed to corn oil, while plasmatic TBARS levels increased only in those gerbils exposed to DBP. These data show that gestational exposure to 100 mg/kg/day of DBP augmented oxidative stress in the blood, even with the increase of the antioxidant response observed. The results confirm the importance of blood evaluation, which acts in the protection of lesions in the organs and also reflects the systemic condition of the organism.

A particularly relevant finding of this study concerns the use of corn oil as a vehicle. As verified throughout the study, most of the effects of gestational exposure to DBP are observed when compared to the control group. Corn oil is one of the major diluent vehicles of liposoluble substances to be administered in animal models orally [4,11,16,32,33,77]. Our results indicate that daily intake of corn oil by pregnant females can cause metabolic alterations in the offspring. In addition, it can result in damage to steroidogenesis, altering the profile of sexual steroids, and compromise semen quantity and quality. As mentioned above, changes in sperm parameters were not due to the reduction in DSP, but probably as a result of problems in epididymal function. These data indicate that the use of this agent as a vehicle for the administration of oral substances is not adequate in studies on the reproductive system or metabolic parameters. This is a cause for concern, which needs a lot of attention and better investigation, since the real effects of the drug tested may be being camouflaged by corn oil.

5. Conclusion

In conclusion, maternal intake of corn oil, a widely used diluent vehicle, led to alterations in the lipid metabolism and circulating estradiol levels, increasing testicular ERα expression and reducing sperm quantity and quality of the gerbil in adulthood. Our results also indicate that gestational exposure to DBP causes perturbations in the lipid metabolism and in the intratesticular synthesis of estradiol of the gerbil, with resulting damage to the sperm parameters. These findings provide important data about DBP exposure in other mammalian species, and point to an important issue regarding the use of corn oil as a diluent vehicle.

Funding

This work was supported by São Paulo State Research Foundation - FAPESP [Grant to Rejane Maira Góes: 2013/16368-7, and doctoral fellowship to Ana Carolina Negrin: 2014/04146-2] and by National Counsel of Technological and Scientific Development - CNPq [Grant to Rejane Maira Góes: 308367/2014-6].

Conflicts of interest

The authors declare no conflicts of interest

Acknowledgements

The authors are gratefully to Mr Luiz Roberto Faleiros Júnior for the technical assistance, and to Prof Peter James Harris for the English-language revision of this paper.

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