

Oral exposure of rats to dienestrol during gestation and lactation: Effects on the reproductive system of male offspring

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ABSTRACT

This study was aimed at determining whether dienestrol (DIES) affects reproduction in male offspring of rats following oral maternal exposure during gestation and lactation. Pregnant rats were treated from GD 6 to PND 21. Animals received 0 (control-vehicle), 0.75, 1.5, 3.12, 6.25, 12.5, 50, 75 µg/kg bw/d of DIES. A control group -without vehicle- was also included. High DIES concentrations caused abortions at 75 and 50 µg/kg bw/d, while at 12.5 µg/kg bw/d had still miscarriages. Ten male rats per group were kept alive until PND 90 to ensure sexual maturity. Body and organ weights, anogenital distance (AGD) at PNDs 21 and 90, biochemical and sperm parameters like motility, viability, morphology, spermatozoa and resistant spermatid counts, and histopathology for sexual organs and liver were determined. An increase in organ weight (liver and sexual organs) and a decrease in AGD due to vehicle were found. A reduction of sperm motility and viability, and an increase of abnormal sperm morphology were caused by DIES, which provoked a dose-dependent prostatitis. Maternal exposure to DIES induced toxicity on the reproductive system of the male offspring, which could affect the capacity of fertilization.

1. Introduction

Chemicals are ubiquitous in our daily lives. Some substances, known as endocrine disruptors (EDCs), can interfere in the endocrine system producing adverse effects such as reproductive, neurological, immune and on development, among others. According to the WHO, EDCs and potential EDCs are mostly man made, found in different materials such as pesticides, metals, additives or food contaminants, personal care products, etc. (Monneret, 2017; Vilela et al., 2018).

According to the USEPA, an EDC is “an agent that interferes with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior” (Kavlock et al., 1996). EDCs can be classified into two categories: a) those occurring naturally (natural chemicals in human and animal food, like the phytoestrogens genistein and coumestrol and b) those synthesized (Diamanti-Kandarakis et al., 2009; Kabir et al., 2015).

EDCs have been involved in various alterations in reproductive function of males and females, increasing for example the incidence of breast cancer, abnormalities in growth and neurodevelopment, as well

as producing important changes in the immune system. Human exposure to this type of compounds occurs through ingestion of food and water, through inhalation of gases or environmental particles, and through the skin (Gore, 2016). They can also be transferred to the offspring through the placenta and breast milk. Therefore, pregnant women and children are vulnerable populations that can be affected by exposure to EDCs, whose harmful effects may appear years after exposure (Fleck et al., 2018; Kabir et al., 2015; Monneret, 2017; Vilela et al., 2018; WHO, 2012).

Diethylstilbestrol (DES) is a stilbene derivative with estrogenic activity comparable to that of estradiol-17β, the natural estrogen. It was synthesized in 1938 with the aim of finding an economic and effective oral estrogen. In 1971, a possible health risk was suspected, because there was a possible association of DES with a rare form of vaginal and cervical cancer (adenocarcinoma of clear ovarian cells seen in elderly women) in daughters of women treated with DES during pregnancy (Al Jishi and Sergi, 2017; Greenwald et al., 1971; Herbst et al., 1971; Reed and Fenton, 2013). Consequently, DES was banned as a prescription drug for women with high-risk pregnancies in 1971 in the USA and in 1978 in Europe (Giusti et al., 1995). In some cases, DES is still used as a

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therapy against castration-resistant prostate cancer, an aggressive form of prostate cancer (Turo et al., 2014).

In addition to its clinical use, DES was also widely used in the agricultural sector until 1979, when trans-placental contamination was found, being then removed from agricultural uses. In recent years, high concentrations of natural estrogenic steroid hormones (DES included) have been detected in water (Chen et al., 2010; Xu et al., 2011), sediments (Pimentel et al., 2016), foods (Heitzman, 1983; Zhang et al., 2008) and urine (Martín, 2000; Zhang et al., 2017).

Due to the trans-placental effects of DES, attention is now also paid to its biotransformation in the perinatal organisms, as well as to the biological activity of the metabolism. A product of DES dehydrogenation is Z, Z-dienestrol (Z, Z-DIES) and in lesser quantity E, E-dienestrol (E, E-DIES) (Metzler and Fischer, 1981). Dienestrol, was used to treat vaginal atrophy in postmenopausal women, applied as a vaginal cream. According to the European Chemicals Agency (ECHA, 2018), DIES is currently classified as a carcinogen, as well as possible toxic for reproduction of category 2 (it is suspected that affects fertility or damages the fetus).

Like DES, DIES was also used as an anabolic steroid for agricultural uses, in cattle, ovine and avian livestock (Jansen et al., 1985; Malone et al., 2009; Socas-Rodríguez et al., 2017). Oral administration caused an increase in the rate of growth, increased food efficiency and stimulated milk production. Recent studies have shown that despite the ban on use of these steroids in livestock, there are still traces of dienestrol (of the range of $\mu\text{g/kg}$ or ng/kg) in dairy products such as milks (Gao et al., 2015; Hu et al., 2014; Socas-Rodríguez et al., 2013, 2018; Stypuła-Trębas et al., 2015) cheeses, kefir (Socas-Rodríguez et al., 2017) and yogurts (D'Orazio et al., 2016). It can mean a high risk, especially for individuals undergoing development such as children and babies. Although babies are not in direct contact with this kind of foods, the mothers may result exposed to DES or DIES mainly through diet. Therefore, mothers could mean a potential source of contamination for newborns through breast milk and to fetuses via the placenta (Stefanidou et al., 2009). DIES has also been found in seawater (He et al., 2016) and in bovine muscle (Malone et al., 2009), which represents health risks, especially in risky population groups where small doses can have adverse effects on development (Stefanidou et al., 2009). Nowadays, data regarding the effect of DIES intake on the reproductive system of mammals are not available in the scientific literature. Based on the above, the present study was aimed at evaluating in rats if maternal exposure during gestation and lactation to DIES, could produce significant changes in the male reproductive system of the offspring.

2. Materials and methods

2.1. Chemicals

Dienestrol (DIES) (purity $\geq 95\%$, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in absolute ethanol and diluted with commercial sunflower oil to obtain final concentrations of 0 (control-vehicle), 0.75, 1.5, 3.12, 6.25, 12.5, 50 and 75 $\mu\text{g/kg}$ bw/day. No dose references of DIES in experimental rodents were available. Therefore, the doses of DES, found in the literature, were used to calculate DIES doses (Alworth et al., 2002; Newbold et al., 2004). Another control group, without vehicle (control), was also included.

2.2. Animals and experimental design

Mature female and male rats (220–238 g) were obtained from Charles River (Saint-Germain-Nuelles, France). Animals were housed in an animal room at $22 \pm 2^\circ\text{C}$, a relative humidity of $50 \pm 10\%$, and a 12-hr light/dark automatic light cycle (light: 08.00–20.00 h). All animals were allowed free access to food (Panlab rodent chow, Barcelona, Spain) and tap water *ad libitum*. After one week of acclimation, the

oestral cycle of females was evaluated during 15 consecutive days. After this period, female rats were mated with males (2:1) overnight. Vaginal smears were collected the next morning to detect the presence of sperm. The day of sperm detection was considered as gestation day 0 (GD 0). Positive females were individually housed in plastic cages. The use of animals and the experimental protocol, which was based on OECD Guideline 443 (2011), were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Catalonia, Spain).

At GD 6 pregnant females received the different doses of DIES administered by gavage. DIES was dissolved in 0.2% ethanol and after administered in a 1 ml/400 g bw of commercial sunflower oil (vehicle). Two control groups (control-vehicle and control) were included in this study. DIES was administered between GD 6 to postnatal day (PND) 21. Twice a week, the body weight of the animals, as well as food and water consumption was recorded. Four male pups per litter were followed during the lactation period. At PND 21, body weight was recorded and anogenital distance (AGD) was measured using a Vernier caliper.

From PND 21, male rat pups (2 per litter) were untreated until PND 90 in order to complete at least one spermatogenesis cycle and to reach sexual maturity. During all this period, animals received food and water *ad libitum*. Weekly body weights were measured. At the age of 90 days, rats were anesthetized with intraperitoneal metomidine/ketamine (0.5 mg/kg bw plus 75 mg/kg bw). AGD was measured using a Vernier caliper. Blood samples were collected of the inferior cava vein to perform biochemical tests. Immediately, liver and sexual organs (testicles, epididymis, seminal vesicles and prostates) were excised and weighted. The cauda of the right epididymis was employed to obtain the diffusion of sperm, which was used to determine motility, viability and morphology. Moreover, parts of the left testis and the left epididymis were immediately stored at -20°C for a subsequent count of the number of spermatids and spermatozoa, respectively. For histopathological exam, samples of liver and sexual organs were cleaned and putted into formaldehyde (4%) for 24 h.

The biochemical parameters, sperm and spermatid count, sperm motility, sperm viability and morphology, were determined according to previous studies of our research group (Garcia et al., 2017).

2.3. Biochemical parameters

Blood samples were collected into a 10 mL collection tubes. They were left for at least 30 min at room temperature. Serum was subsequently obtained by centrifugation at 1300 g for 10 min at 4°C , being immediately separated and stored at -80°C , or at 4°C , depending on the test assay. Concentrations of glucose, cholesterol, triacylglycerides, high density lipoprotein, total proteins, albumin, urea, creatinine, as well as the levels of the enzymes aspartate transaminase, alanine transaminase and alkaline phosphatase (QCA kits, Tarragona, Spain; Spinreact kits, Girona, Spain) were measured with a Cobas Mira automatic analyzer (Roche Pharmaceuticals, Basel, Switzerland) according to the instructions provided by the manufacturer (Garcia et al., 2017).

2.4. Sperm and spermatid count

Spermatozoa were obtained from the left epididymis. Samples of the left epididymis were collected, being immediately weighed and homogenized with 0.5 mL of Triton solution (0.01% Triton X-100 + 0.9% sodium chloride, Sigma-Aldrich, St. Louis, MO, USA). The homogenate was then diluted with 4.5 mL of Triton solution, being transferred into each chamber of Neubauer hemocytometer. The sperm was counted under a standard optical microscope (Olympus CX41) at 400-fold magnification.

In turn, resistant spermatids were obtained from the left testicle. The tunica albuginea was removed from a piece of left testicle. It was weighed and homogenized with 1 mL of Triton solution, being transferred into each chamber of Neubauer hemocytometer. The resistant

Table 1

Body weight (g) of male offspring of DIES-treated dams during gestation and lactation. Data are given at PND 21 and 90.

	Control	Control-vehicle	0.75 µg/kg bw/d	1.5 µg/kg bw/d	3.12 µg/kg bw/d	6.25 µg/kg bw/d
PND 21 (g)	35.86 ± 6.12	35.48 ± 6.23	35.25 ± 3.45	37.37 ± 5.35	37.35 ± 5.01	34.19 ± 8.40
PND 90 (g)	424.50 ± 36.59	400.40 ± 46.79	383.20 ± 22.20	397.90 ± 11.35	421.50 ± 43.00	384.50 ± 19.40

Values are expressed as means ± SD.

spermatids from steps 9 to 19 were counted under a standard optical microscope (Olympus CX41) at a 400-fold magnification (Garcia et al., 2017).

2.5. Sperm motility

Spermatozoa were collected from the right cauda epididymis, which was removed and cleaned. Cauda epididymis was immediately placed into a pre-warmed Petri dish with 5 mL of HBSS medium (Sigma-Aldrich), which was supplemented with 0.5% BSA (Sigma-Aldrich) at 37 °C, and minced with scalpels. Subsequently, there was an incubation for 10 min at 37 °C. After incubation and diffusion time, 20 µL of the suspension were placed into a warmed microscope slide and covered by a 24 mm × 60 mm warmed cover slip. Sperm motility was recorded with camera (Moticam 480), which was added to a standard optical microscope (Motic BA-300). The sperm motility was observed at a 400-fold magnification, being 200 sperms per rat counted throughout at least 10 fields.

Sperm motility parameters were analyzed as percentage of progressive motile sperm (PMS), non-progressive motile sperm (NPMS), and non-motile sperm (NMS). The assessment of sperm motility was done according to the WHO (2010) protocol (Garcia et al., 2017).

2.6. Sperm viability and morphology

Ten minutes after sperm diffusion, 10 µL of the suspension were placed into a pre-warmed microscope slide. Ten µL of eosin/nigrosine staining solution (1.67% Eosin Y + 10% Nigrosine) (Sigma-Aldrich) were added and gently mixed. A smear of the suspension was prepared and allowed to dry at room temperature.

Samples were analyzed under a standard optical microscope (Olympus CX31) at 1000 or 600-fold magnification. Two-hundred sperm per rat were counted to determine viability and morphology of sperm, respectively. Viable sperms were unstained (white), while non-viable sperms were stained (pink). Slight stained sperms (slight pink) were considered as live sperms, being counted as viable. Viability data were expressed as the percentage of viable and non-viable spermatozoa. The morphology was expressed as percentage of normal forms (Garcia et al., 2017).

Table 2

Organ weights (g) and relative organ weights (g/kg bw) of male offspring of DIES-treated dams during gestation and lactation.

		Control	Control-vehicle	0.75 µg/kg bw/d	1.5 µg/kg bw/d	3.12 µg/kg bw/d	6.25 µg/kg bw/d
Liver	g	11.69 ± 1.65	12.97 ± 0.94 ^a	13.39 ± 0.91 ^{ac}	15.91 ± 1.83 ^{bc}	15.69 ± 2.28 ^{bc}	16.81 ± 3.12 ^b
	g/kg bw	26.71 ± 1.81	36.21 ± 2.12 ^{*,ab}	34.42 ± 1.63 ^a	39.98 ± 4.32 ^{***,ab}	37.19 ± 3.46 ^{***,ab}	43.64 ± 7.30 ^{***,b}
Testicles	g	1.57 ± 0.07	1.81 ± 0.14 ^{***}	1.77 ± 0.13 ^{**}	1.81 ± 0.17 ^{**}	1.74 ± 0.12 [*]	1.64 ± 0.09
	g/kg bw	3.67 ± 0.22	4.54 ± 0.45 ^{***}	4.62 ± 0.40 ^{***}	4.55 ± 0.37 ^{***}	4.25 ± 0.42 [*]	4.28 ± 0.30 [*]
Epididymis	g	0.60 ± 0.06	0.65 ± 0.06	0.62 ± 0.04	0.66 ± 0.06	0.68 ± 0.04	0.63 ± 0.06
	g/kg bw	1.43 ± 0.12	1.62 ± 0.15 [*]	1.70 ± 0.13 ^{**}	1.66 ± 0.11 [*]	1.65 ± 0.12 [*]	1.65 ± 0.16 [*]
Prostate	g	0.66 ± 0.10	0.80 ± 0.14 ^{*,ab}	0.80 ± 0.14 ^{*,ab}	0.91 ± 0.12 ^{***,a}	0.91 ± 0.16 ^{***,a}	0.72 ± 0.07 ^b
	g/kg bw	1.54 ± 0.16	2.00 ± 0.22 ^{***,ab}	2.09 ± 0.31 ^{***,ab}	2.92 ± 0.25 ^{***,a}	2.08 ± 0.30 ^{***,ab}	1.88 ± 0.24 ^{*,b}
Seminal vesicle	g	1.24 ± 0.22	1.60 ± 0.10 ^{**}	1.56 ± 0.22 [*]	1.55 ± 0.19 [*]	1.53 ± 0.11 [*]	1.44 ± 0.10
	g/kg bw	2.96 ± 0.64	3.97 ± 0.73 ^{***}	3.95 ± 0.41 ^{***}	3.88 ± 0.45 ^{**}	3.65 ± 0.45 [*]	3.74 ± 0.28 [*]

Values are expressed as means ± SD.

* Significant different from the control (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$).abc Significant differences between oil-treated groups at $P < 0.05$.

2.7. Histopathology

Liver and sexual organs (right testis, right epididymis, seminal vesicle and prostate) of 6 rats per group were removed, weighed, and fixed in 4% formaldehyde at pH 7.4 for 24 h. Tissues were sliced and embedded in paraffin. Sections of 4-mm thickness were stained with hematoxylin-eosin for tissue morphological evaluation. Images were captured with a camera, which was added to an optical microscope. Different abnormalities in liver and sexual organ sections were evaluated. The grading system used was the following: 0 for no abnormalities, and 1, 2 and 3 for mild, moderate and severe abnormalities, respectively.

2.8. Statistics

Statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS v.25). To determine differences due to vehicle, a two-tailed *t*-test was conducted between control and control-vehicle groups. If no differences between these groups were found, ANOVAs were done with treated groups and the control-vehicle, if two control groups (with and without vehicle) were different both were included in the ANOVAs analysis. If variances were homogenous, ANOVA, followed by the Bonferroni's test was used. The Kruskal-Wallis test, followed by the Dunns post-hoc test, were used when variances were not homogeneous.

3. Results

3.1. Viability of DIES doses in pregnant females: litter survival

The higher doses of DIES (75 and 50 µg/kg bw/d) produced 100% of vaginal hemorrhages between GD 12–17, resulting in miscarriages. A similar finding was also observed at 12.5 µg/kg bw/d, causing 75% of abortions in pregnant rats. The lowest DIES doses (6.25, 3.12, 1.5 and 0.75 µg/kg bw/d) allowed the delivery of the offspring. Viability of pups was higher than 90% in all groups. Sex differentiation was possible from PND 1. Four male pups were selected to be examined until PND 21, while two male pups were allowed to mature until PND 90.

3.2. Body and organ weights

Table 1 summarizes body weights at PND 21 and 90. No significant differences could be noted. Organ (liver, testicles, epididymis, prostate and seminal vesicle) weights at PND 90 were recorded (Table 2). Regarding liver weight, the 6.25, 3.12 and 1.5 µg/kg bw/d doses produced a significant increase compared to the control-vehicle group. Furthermore, the 6.25 µg/kg bw/d group showed significant differences with respect to the 0.75 µg/kg bw/d group (Table 2). Oral treatment with higher doses of DIES led to a significant increase of the liver weight, which was attributable to DIES exposure. All groups showed an increase of relative liver weight compared to the control group, being the exception the 0.75 µg/kg bw/d group. In turn, the 6.25 µg/kg bw/d group showed a significant increase with respect to animals in the 0.75 µg/kg bw/d group. Commercial oil also affected testis weight and relative testis weight. In all groups, excepting the 6.25 µg/kg bw/d group, a significant increase was found in testis weight compared to the control group. All oil-treated groups showed a significant increase in relative testis weights.

Epididymis weight did not present significant differences. However, a significant increase of relative epididymis weight in all groups was noted with respect to control group. Prostate weight was also affected by the commercial oil. Almost all groups showed a significant increase on their prostate weight, compared to that of animals in the control group. Only at 6.25 µg/kg bw/d there were not differences. Moreover, significant differences between the 1.5 and 3.12 µg/kg bw/d groups with respect to the 6.25 µg/kg bw/d group were also observed (Table 2). A similar effect was noticed in relative prostate weight, all oil-treated groups were different to the control group and also significant differences were found between 6.25 and 1.5 µg/kg bw/d groups. Seminal vesicle was also affected by the commercial oil. Organ weight and relative organ weight significantly increased with regard to the control group. Only at 6.25 µg/kg bw/d there was not a significant increase in seminal vesicle weight (Table 2).

3.3. Anogenital distance (AGD) evaluation

AGD is a parameter widely used to show feminization effects in males. This parameter was measured at PNDs 21 and 90. Results are shown in Table 3. At PND 21, a significant decrease of AGD was observed in almost all treated groups with respect to the control group, but 1.5 µg/kg bw/d group. Groups 1.5 and 3.12 µg/kg bw/d also showed differences between them. Relative AGD showed a significant decrease in relation to the control group, while a difference between the control-vehicle and 0.75 µg/kg bw/d groups, compared to the 3.12 µg/kg bw/d group, was noted. The AGD of the 3.12 µg/kg bw/d group was the lowest. All these significant differences were not observed at PND 90.

At PND 90, a tendency to increase AGD was noted at the highest DIES doses, being all significantly different when compared to those in the control-vehicle group. This increase was not statistically significant at 1.5 µg/kg bw/d. Differences between the 1.5 and 6.25 µg/kg bw/d groups were also found. Regarding relative AGD, the 0.75 and 6.25 µg/kg bw/d groups showed significant differences with the control-vehicle group (Table 3). Finally, animals in the 6.25 µg/kg bw/d group showed a significant increase when compared to those in the 1.5 and 3.12 µg/kg bw/d groups (Table 3).

Table 3

Anogenital (mm) and relative anogenital distance (mm/kg bw) in male offspring of DIES-treated dams during gestation and lactation. Data are given at PND 21 and 90.

		Control	Control-vehicle	0.75 µg/kg bw/d	1.5 µg/kg bw/d	3.12 µg/kg bw/d	6.25 µg/kg bw/d
PND 21	mm	14.28 ± 1.41	12.04 ± 1.29 ^{*,ab}	12.08 ± 0.64 ^{*,ab}	12.57 ± 1.56 ^a	11.20 ± 1.02 ^{***,b}	11.28 ± 1.68 ^{***,ab}
	mm/kg bw	398.10 ± 40.17	350.10 ± 39.90 ^{***,a}	350.80 ± 31.46 ^{***,a}	338.40 ± 25.72 ^{***,ab}	312.20 ± 22.61 ^{***,b}	336.70 ± 32.42 ^{***,ab}
PND 90	mm	39 ± 2.12	39.03 ± 2.92 ^a	43.13 ± 2.17 ^{bc}	40.72 ± 3.04 ^{ab}	43.49 ± 2.70 ^{bc}	46.14 ± 2.14 ^c
	mm/kg bw	92.21 ± 8.14	95.95 ± 4.63 ^a	111.50 ± 5.13 ^{bc}	102.40 ± 8.80 ^{ac}	101.50 ± 8.90 ^{ac}	120.70 ± 5.33 ^b

Values are expressed as means ± SD.

* Significantly different from the control (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$).

^{abc} Significant differences between oil-treated groups at $P < 0.05$.

kg bw/d groups showed significant differences with the control-vehicle group (Table 3). Finally, animals in the 6.25 µg/kg bw/d group showed a significant increase when compared to those in the 1.5 and 3.12 µg/kg bw/d groups (Table 3).

3.4. Biochemical analysis

Total plasma protein (TP), albumin, and albumin/globulin (A/G) ratio, were used as indicators for hepatic and lymphocyte functions. Glucose was used as an energetic metabolism indicator, while triacylglycerids (TAG), cholesterol (Chol) and high density lipoproteins (HDL) were used as indicators of lipid metabolism. Blood urea nitrogen (BUN) and creatinine were used for the renal function, while aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were used as indicators of the hepatic function.

Table 4 summarizes the levels of these parameters for animals in control, control with vehicle, and the DIES-treated groups. A significant decrease in TP levels was found in all treated groups with commercial oil when compared to the control group. Regarding albumin levels, control-vehicle are decreased respect to the control group. All DIES-treated groups showed an increase of albumin levels with respect to the control-vehicle group. Probably, the DIES effect was masked by the commercial oil. The ratio A/G did not show significant differences.

Glucose concentrations showed a significant increase between the 3.12 µg/kg bw/d and control-vehicle group. In turn, TAG did not show any difference between groups, while Chol and HDL presented differences between groups, showing a general increase in DIES-treated groups. However, the differences did not reach the level of statistical significance for the 3.12 and 6.25 µg/kg bw/d groups for Chol, and for the 3.12 µg/kg bw/d group for HDL.

On the other hand, BUN levels showed differences between 1.5 and 3.12 µg/kg bw/d, which was probably due to the intrinsic variability of data. No differences in creatinine concentrations between groups were found. With regard to the enzymatic activities, AST showed a decrease at control-vehicle with respect to the control group. ALT activity were increased in the control-vehicle and 6.25 µg/kg bw/d groups with respect to the control group. Only statistical differences into DIES-treated group were found between 3.12 and 6.25 µg/kg bw/d groups. In similar way, the control-vehicle group ALP's activity was increased respecting the control group, also 0.75, 3.12 and 6.25 µg/kg bw/d groups were increased with respect to the control group. All DIES-treated groups tended to increase ALP activity, but only statistical differences between control-vehicle and 6.25 µg/kg bw/d could be noted. Also, significant differences were found between 1.5 µg/kg bw/d group with respect to 3.12 and the 6.25 µg/kg bw/d groups.

3.5. Sperm and spermatid count

Spermatozoa counts showed a significant increase at control-vehicle and 6.25 µg/kg bw/d when compared to that in the control group (Table 5). However, the rest of groups treated with vehicle (oil) only showed a tendency to increase the number of spermatozoa with respect to the control group.

Table 4

Serum biochemistry data for male offspring of DIES-treated dams during gestation and lactation.

	Control	Control-vehicle	0.75 µg/kg bw/d	1.5 µg/kg bw/d	3.12 µg/kg bw/d	6.25 µg/kg bw/d
TP (g/dL)	6.31 ± 0.40	5.30 ± 0.28***	5.22 ± 0.23***	5.47 ± 0.32***	5.35 ± 0.39***	5.53 ± 0.28***
Albumin (g/dL)	4.40 ± 0.24	3.84 ± 0.21***,a	4.30 ± 0.12 ^{bc}	4.32 ± 0.14 ^{bc}	4.57 ± 0.22 ^b	4.17 ± 0.27 ^c
A/G	2.37 ± 0.48	2.67 ± 0.33	3.60 ± 1.00	3.30 ± 1.23	4.94 ± 2.17	2.90 ± 0.64
Glucose (mg/dL)	177.80 ± 33.69	192.60 ± 22.10 ^a	238.30 ± 36.58 ^{ab}	201.60 ± 22.11 ^{ab}	244.90 ± 48.40 ^b	212.60 ± 34.65 ^{ab}
TAG (mg/dL)	67.56 ± 24.94	81.32 ± 45.22	100.00 ± 22.75	117.00 ± 22.60	108.00 ± 47.61	120.30 ± 57.03
CHOL (mg/dL)	51.75 ± 7.61	53.04 ± 8.61 ^a	65.12 ± 4.86 ^b	63.11 ± 5.20 ^{bc}	53.69 ± 6.18 ^{ac}	58.09 ± 9.76 ^{ab}
HDL (mg/dL)	35.29 ± 6.58	35.96 ± 5.38 ^a	43.69 ± 3.69 ^{bc}	42.84 ± 4.38 ^{bc}	38.45 ± 4.02 ^{ac}	47.73 ± 3.95 ^b
BUN (mg/dL)	30.03 ± 4.06	28.23 ± 2.80 ^{ab}	31.39 ± 5.60 ^{ab}	25.05 ± 2.15 ^a	33.34 ± 6.12 ^b	27.85 ± 4.25 ^{ab}
Creatinine (mg/dL)	0.50 ± 0.07	0.53 ± 0.09	0.57 ± 0.05	0.51 ± 0.03	0.54 ± 0.07	0.54 ± 0.06
AST (U/L)	114.90 ± 17.77	79.35 ± 14.76 ^{**}	105.80 ± 21.06	92.78 ± 13.85	95.39 ± 24.64	99.66 ± 23.62
ALT (U/L)	31.71 ± 5.39	38.40 ± 3.95 ^{*,ab}	40.48 ± 5.45 ^{ab}	37.44 ± 7.02 ^{ab}	35.46 ± 7.40 ^a	44.63 ± 8.27 ^{*,b}
ALP (U/L)	122.10 ± 12.13	219.60 ± 14.15 ^{*,ac}	248.40 ± 69.45 ^{***,abc}	161.80 ± 47.96 ^c	280.80 ± 88.98 ^{***,ab}	309.90 ± 50.93 ^{***,b}

TP. total proteins; A/G. albumin/globulin; TAG. triacylglyceride; CHOL. cholesterol; HDL. high density lipoprotein; BUN. blood urea nitrogen; AST. aspartate transaminase; ALT. alanine transaminase; ALP. alkaline phosphatase.

Values are expressed as means ± SD.

* Significantly different from the control (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$).

^{abc} Significant differences between oil-treated groups at $P < 0.05$.

In contrast, spermatid counts showed the contrary effect. All groups, with the exception of the 1.5 µg/kg bw/d group, triggered a significant decrease in spermatid number, while the 1.5 µg/kg bw/d group showed a tendency to decrease only with respect to the control group (Table 5).

3.6. Sperm parameters: motility, viability and morphology

The effects of DIES treatment to rats during gestation and lactation were also observed in the sperm motility results. The number of progressive motile sperm (PMS) was lower in the DIES-treated groups. Significant differences with respect to the control-vehicle were found in most groups, with the exception at 1.5 µg/kg bw/d, a group that shown only a decreasing tendency (Fig. 1). Differences between the control and the control-vehicle groups were not noticed. Therefore, the observed differences could be a result of the DIES effect. On the other hand, regarding the number of non-progressive motile sperms (NPMS), the 0.75 µg/kg bw/d group showed a higher percentage. Differences between the control-vehicle and 0.75 µg/kg bw/d groups were also observed (Fig. 1). In turn, although a tendency to increase the number of non-motile sperms (NMS) in the DIES-treated groups was observed, only the 1.5 µg/kg bw/d group showed a significant increase. However, no significant differences were found between the control and the control-vehicle group. Thus, probably the vehicle (commercial oil) did not affect this parameter (Fig. 1).

Likewise, viability and morphology of epididymal spermatozoa were examined (Figs. 2–4). A significant decrease on viability in three higher DIES doses was noted. On the other hand, the morphology was affected by both oil and DIES. Fig. 3 depicts a percentage of normal morphology of sperms in the different groups. A significant effect of DIES was found when treated groups were compared to the control-vehicle group. An exception was the 1.5 µg/kg bw/d group, which only showed a tendency to decrease percentage of normal forms. Moreover, differences on morphology were observed in all oil-treated groups with respect to the control group, which is a result of the vehicle effect (Figs. 3 and 4). The percentages of different abnormalities on sperm

morphology are summarized in Table 6, being tail abnormalities the most common in oil-treated groups regarding control group.

3.7. Histopathological evaluation

No histopathological abnormalities were found in liver, testis, epididymis and seminal vesicle sections in all groups (data not shown). However, dose-dependent chronic prostatitis was found in prostate sections (Fig. 5). The control and the control-vehicle groups did not present infiltrations (Grade 0) (Fig. 5A), while the 0.75 and 1.5 µg/kg bw/d groups showed a slight infiltration in prostate (Grade 1) (Fig. 5B). In turn, at 3.12 and 6.25 µg/kg bw/d a high presence of infiltrations was noted, being of grade 2 and 3, respectively (Fig. 5C and D & E, respectively).

4. Discussion

While adult exposure to EDCs is important, the effects on the fetus are of great concern taking into account that developing organisms are extremely sensitive to alterations by chemicals with hormone-like activity (Bommarito et al., 2017; Braun, 2017). Prenatal exposure to DES was the first documented example, where exposure of the fetus resulted in long-term changes in the offspring that were not apparent until usually after the onset of puberty (Newbold, 2008; Titus et al., 2019). The DES metabolite, DIES, has also been classified as an EDC, with possible toxic effects on the development (Stefanidou et al., 2009). Nowadays, there is no clinical uses of this chemical, but recent studies have shown that there are still traces of DIES in milk and dairy products (D'Orazio et al., 2016; Socas-Rodríguez et al., 2017). However, no studies about the perinatal exposure effects of DIES into the offspring are available.

In the current study, pregnant rats were exposed to oral doses of DIES from GD 6 to PND 21. To correct the effect of the vehicle, a control group was added. At the end of the treatment, body weight and AGD of male pups were recorded. Furthermore, some male rats were evaluated

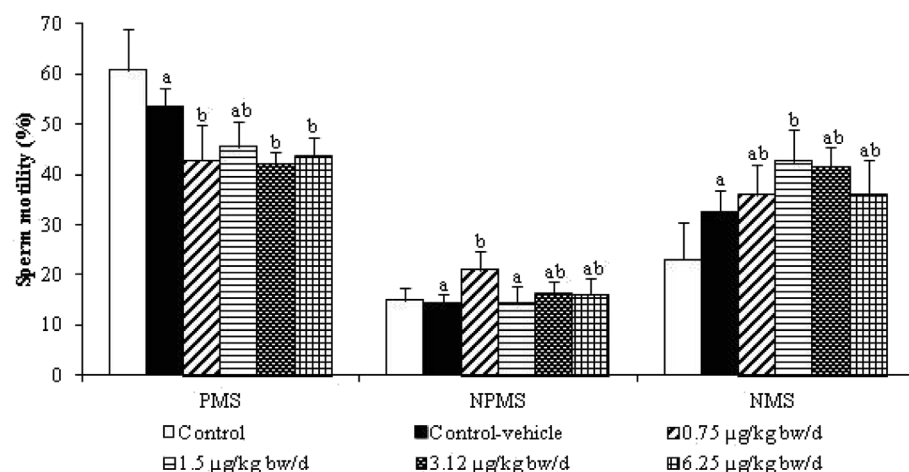
Table 5Spermatozoa counts ($\times 10^8$ /g epididymis) and spermatids counts ($\times 10^7$ /g testicle) in male offspring of DIES-treated dams during gestation and lactation.

	Control	Control-vehicle	0.75 µg/kg bw/d	1.5 µg/kg bw/d	3.12 µg/kg bw/d	6.25 µg/kg bw/d
Spermatozoa counts	2.43 ± 8.07	6.63 ± 2.32 [*]	5.06 ± 1.60	4.56 ± 1.99	5.65 ± 2.10	6.62 ± 3.23 [*]
^a Spermatids counts	3.91 ± 6.35	2.39 ± 9.38 [*]	1.84 ± 4.88 ^{**}	2.97 ± 6.53	1.61 ± 6.62 ^{***}	2.14 ± 7.41 [*]

Values are expressed as means ± SD.

* Significantly different from the control (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$).

^a Spermatid stages: 9–19.



ssFig. 1. Sperm motility of male offspring of DIES-treated dams during gestation and lactation, where PMS means progressively motile sperm; NPMS means non-progressively motile sperm and NMS means non-motile sperm. Different superscripts (^{ab}) indicate significant differences between oil-treated groups in each parameter respectively at $P < 0.05$.

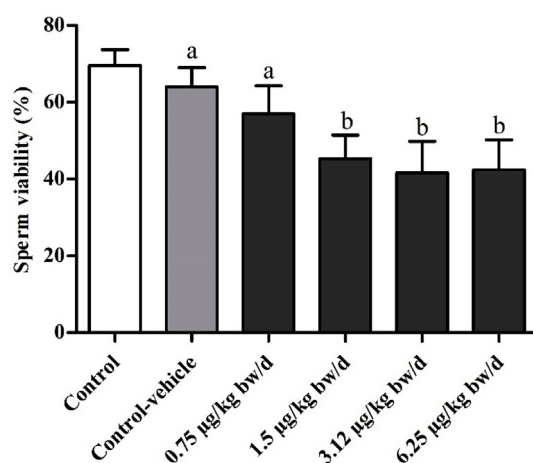


Fig. 2. Sperm viability of male offspring of DIES-treated dams during gestation and lactation. Values are expressed as percentage of viable sperms. Different superscripts (^{ab}) indicate significant differences between oil-treated groups in each parameter respectively at $P < 0.05$.

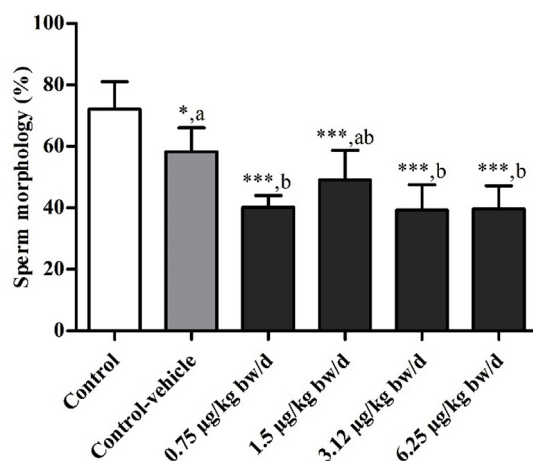


Fig. 3. Sperm morphology of male offspring of DIES-treated dams during gestation and lactation. Values are expressed as percentage of spermatozoa with normal morphology. Asterisks (*) indicate significant differences from the control group (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$). Different superscripts (^{ab}) indicate significant differences between oil-treated groups in each parameter respectively at $P < 0.05$.

at PND 90 to ensure sexual maturity alterations after perinatal exposure, being assessed the general health status of the rats. In turn, various sperm parameters were measured, while a histopathological evaluation in sexual organs was conducted.

The first signs of DIES toxicity were already seen during the treatment. All pregnant animals treated at the highest doses of DIES showed vaginal hemorrhages during pregnancy, resulting in miscarriages. Furthermore, pregnant rats treated at 12.5 µg/kg bw/d showed a 75% of miscarriages. EDCs have the potential to interfere with endogenous hormone action and to affect the implantation process during pregnancy (Krieg et al., 2016). In the present investigation, the maximum dose to assess perinatal DIES effects on the offspring avoiding miscarriages was found at 6.25 µg/kg bw/d.

After DIES treatment, no significant changes in body weight in PND 21 or PND 90 animals were found between DIES-treated groups with respect to control and control-vehicle groups. However, results showed that the sunflower oil vehicle had significant effects in the anogenital distance values of the PND 21 pups, as well in reproductive organs weight, in AST, ALT and ALP serum concentrations, and also in some sperm parameters of the PND 90 male rats. The effect of oil vehicle in various physiological and reproductive parameters was also previously reported (Cardoso et al., 2014; Garcia et al., 2017; Yamasaki et al., 2001).

AGD is a typical parameter used to assess development of the external genitalia, being an important marker of the EDCs effects. In the PND 21 pups, a significant effect of the vehicle was found in the AGD values, showing all male pups from the sunflower oil-treated groups significant decreased AGD values compared to those in the control group. Although a significant decrease of the AGD was found in PND 21 animals, a similar effect was not observed in PND 90 animals. However, when the DIES effect was assessed, we found that, on PND 21, only animals in the 3.12 µg/kg bw/d group showed a significant decrease in the relative AGD values with respect to the control-vehicle group. These results suggest that sunflower oil can mask the effects of the treatment on PND 21 animals. Even so, results indicated that in PND 90 animals, DIES treatment affected AGD values. These animals showed a tendency to increase the AGD values in DIES-treated groups, increases that were significant at 0.75, 3.12 and 6.25 µg/kg bw/d groups. These data are not in accordance with PND 21 results. This difference could suggest that measuring AGD is more appropriate for pups than for mature rats. Nevertheless, more information is still necessary to clarify the inconsistent results.

All PND 90 animals in the oil-treated groups showed significant increased testis, prostate and seminal vesicle absolute and relative weight, and epididymis relative weight in relation to those in the control group. It is known that oil vehicle is able to influence *in vitro* reproductive tests and *in vivo* studies, interfering in reproductive

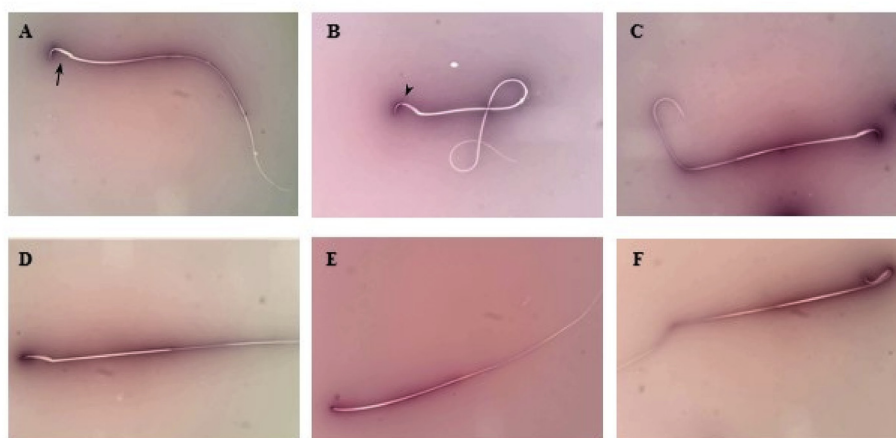


Fig. 4. Different sperm abnormalities in male offspring of DIES-treated dams during gestation and lactation. Magnification: 400 $\times$. (A) normal morphology, arrow shows a viable sperm; (B&C) tail abnormalities, arrow head shows a non-viable sperm (B); (D&E) head abnormalities and (F) neck abnormalities.

Table 6

Percentage of abnormal morphologic forms of sperm (%) in male offspring of DIES-treated dams during gestation and lactation.

	Control	Control-vehicle	0.75 $\mu\text{g/kg bw/d}$	1.5 $\mu\text{g/kg bw/d}$	3.12 $\mu\text{g/kg bw/d}$	6.25 $\mu\text{g/kg bw/d}$
Head abnormalities	45.50 $\pm$ 13.60	31.05 $\pm$ 8.53*	37.33 $\pm$ 11.33	36.76 $\pm$ 10.67	36.03 $\pm$ 5.55	35.47 $\pm$ 9.12
Neck abnormalities	25.24 $\pm$ 4.87	17.47 $\pm$ 5.48*	17.38 $\pm$ 5.19*	21.02 $\pm$ 4.47	17.42 $\pm$ 3.11*	20.34 $\pm$ 3.34
Tail abnormalities	22.62 $\pm$ 6.87	51.47 $\pm$ 7.75***	46.58 $\pm$ 7.25***	44.51 $\pm$ 12.31***	45.10 $\pm$ 6.91***	44.14 $\pm$ 9.07***

Values are expressed as means $\pm$ SD.

* Significantly different from the control (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$).

toxicological studies, also affecting the assessed reproductive parameters (Cardoso et al., 2014; Yamasaki et al., 2001). Moreover, liver relative weight was affected in almost all oil-treated groups, showing those animals a significant weight increase with respect to those in the control group. However, no significant related differences between DIES-treated animal and the control-vehicle group were noticed in any of the reproductive organ weights values. This indicates that perinatal DIES exposure had no significant effects on the organ weights of the offspring. Therefore, the differences could be due to the variability of the own rat groups.

In the biochemical parameters, a significant decrease in the total protein levels was noted in all animals in the oil-treated groups with respect to those in the control group. Recent studies have reported a negative effect of sunflower oil in the liver of rats treated with this oil, inducing fibrosis, ultrastructural alterations and high oxidation levels (Pierce et al., 2016; Varela-Lopez et al., 2018). In turn, all DIES-treated animals showed increased albumin levels with respect to those in the control-vehicle group. ALP levels were also increased at 6.25 $\mu\text{g/kg bw/d}$ with regard to the control-vehicle group. These results suggest a significant effect of the perinatal exposure to DIES into the liver function of the offspring at PND 90.

The perinatal endocrine toxicity of DIES on the reproductive system of PND 90 male rats was assessed by testis and epididymis parameters, and the histology of sexual organs. The World Health Organization (WHO, 2010) classifies patients according to descriptive analyses of sperm number, motility and morphology, being the most common form of male infertility a low sperm count (oligozoospermia) (Kitamura et al., 2015; Malo, 2005; Malo et al., 2006). Furthermore, one method to evaluate the production of sperm is the quantification of testicular spermatid number (Nardi et al., 2017). This parameter allows evaluating agents with possible toxic effects on spermatogenesis as some authors have reported (Ullah et al., 2018).

In the current study, a significant effect of the vehicle was found, showing control PND 90 animals an increased number of spermatids in relation to those from oil-treated groups. An increased number of testis

spermatid counts could indicate -if accompanied by histopathological positive results-that there is spermatid retention in testes, which is one of the specific histopathology endpoints of endocrine disruption (Hood, 2011). No significant spermatid retention in the testes of any of the PND 90 animals was noted. Moreover, our results did not reveal significant effects of DIES perinatal treatment on spermatid counts with respect to the control-vehicle group.

Epididymal spermatozoa counts show both, production of sperm by the testes, and ability of the epididymis to store sperm. In this investigation, no significant differences in the spermatozoa number were observed between DIES-treated animals and those at control-vehicle group. Only an effect of the vehicle was noted. Previous studies assessing the effects of the perinatal DIES exposure on the reproductive system of the male offspring are not available. However, it has been shown an effect of DES exposure during sensitive early life stages on the male reproductive system (Reed and Fenton, 2013; Toyama et al., 2001).

Epididymal sperm motility, viability and morphologic were evaluated after one complete cycle of spermatogenesis. Significant decreased levels of progressive motile sperms (PMS) were observed in almost all the PND 90 DIES-treated animals with respect to the control-vehicle group, showing a significant effect of DIES perinatal exposure on the sperm motility. In turn, a significant decrease of sperm viability was noticed in the 1.5, 3.12 and 6.25 $\mu\text{g/kg bw/d}$ groups in comparison with the control-vehicle group. On the other hand, a significant increase of the sperm abnormalities was found with regard to the control group. Although no previous studies in relation with those results were available, Goyal et al. (2003) showed that a neonatal exposure of male rats to DES altered sperm motility patterns, sperm fertility, and sexual behavior. Based on the current results, the most sensitive effects of perinatal DIES exposure correspond to the sperm parameters.

Histopathological analysis of the testis, epididymis and accessory sex organs revealed a dose-dependent chronic prostatitis (prostate inflammation) of animals in the DIES-treated groups. Prostatitis, which affects 9% of men of all ages, can be very painful and distressing. A

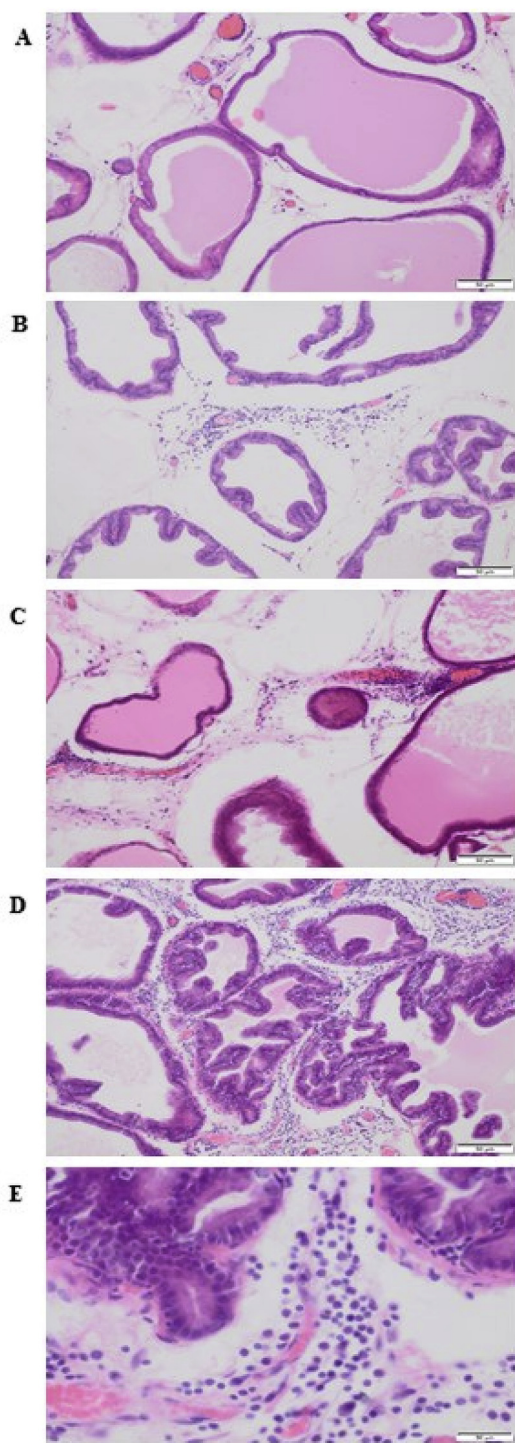


Fig. 5. Prostate histology with prostatitis of male offspring of DIES-treated dams during gestation and lactation. (A) Control rat at $\times 100$ (Grade 0); (B) 1.5 $\mu\text{g/kg bw/d}$ group at $\times 100$ (Grade 1 of severity); (C) 3.12 $\mu\text{g/kg bw/d}$ group at $\times 100$ (Grade 2 of severity); (D) 6.25 $\mu\text{g/kg bw/d}$ group at $\times 100$ (Grade 3 of severity) and (E) detail of infiltrations at $\times 400$ (Grade 3 of severity).

number of studies have suggested the potential relation between inflammation and its association with prostate cancer development (Cowin et al., 2008; Ellem et al., 2009; McNaughton-Collins et al., 2007). Normal development of the prostate gland and testes are greatly dependent on the regulation of both locally acting and circulating hormones. For this reason, interactions with EDCs during lifetime is

responsible for an increased predisposition toward developing endocrine-related cancers later in life (Sweeney et al., 2015). Rodent studies have shown effects of the estrogenic exposure during development in fetal and neonatal life, resulting in prostatic inflammation later in life (Ellem et al., 2009; Prins et al., 2006). EDCs have been also linked with prostatitis (Sanchez de Badajoz et al., 2017; Scarano et al., 2018). The current results have shown that perinatal DIES exposure results in a chronic inflammation of the prostate of the animals at PND 90. During fetal and neonatal life, the reproductive tract development is hormonally regulated, with lacking compensatory homeostatic mechanisms to prevent adverse effects of EDCs (Mallozzi et al., 2016; Ünüvar and Büyükgözü, 2012).

In summary, the results of this investigation have shown that exposure during gestation and lactation periods to DIES, can induce toxic effects on the reproductive system of male offspring, which could hinder the fertilization capacity of these animals.

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