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Sexual differentiation and reproductive development of female rat offspring after paternal exposure to the anti-tumor pharmaceutical cisplatin

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ABSTRACT

Cisplatin (CP) is used to treat a number of cancers, including testicular cancer. Studies indicate that CP-treatment can impair spermatogenesis in humans and rodents by germ cell DNA binding, through different modes of action. CP-paternal exposure resulted in adverse effects in F1 male offspring. In this study, F1 female offspring was assessed for reproductive development after CP-paternal exposure. *Peri*-pubertal male rats, treated with 1 mg/Kg/day of CP or vehicle for 3 weeks, were mated with unexposed females. F1 female offspring of CP-treated fathers showed a decrease in fetal ovary germ cells, in estrous cycle length and FSH levels, and an increase in the percentage of antral follicles in adults. Based on our previous results and the findings of the present work we concluded that CP-paternal exposure leads to adverse effects on rat male and female reproductive development, raising concern, in humans, for children born to men exposed to CP.

1. Introduction

Cancer is a leading cause of death worldwide. Cisplatin (CP) is a chemical complex with platinum widely used as an anti-tumor pharmaceutical in the treatment of ovarian, head and neck, bladder, colorectal, lung and testicular cancer [1–7]. Advances in multimodal treatment (surgery, chemotherapy and radiotherapy) have increased survival, particularly when performed soon after diagnosis [8].

Testicular cancer, specially germ cell tumors, is one of the most common types of cancer affecting young men, between 15 and 35 years old [9]. Despite the therapeutic success of CP in this type of cancer [6,7], it can cause several adverse effects, including nephrotoxicity, neurotoxicity, ototoxicity and abnormal spermatogenesis [10,11].

The ability of CP to inhibit tumor growth is a result of several factors, including diffusion through the cell membrane, accumulation in the cells and the binding to tumor cell DNA, generating adducts [12]. The formation of covalent bonds with DNA affects fundamental cellular processes, inducing tumor cell apoptosis [13]. However, it can also bind to DNA of germ cells, resulting in azoospermia or oligospermia in men [14]. In rodents, the treatment with CP resulted in formation of CP-DNA adducts in the testis [15,16].

Studies have shown that rodents with damaged DNA spermatozoa, such as CP-DNA adducts, can maintain their fertility capacity. However, there are evidences that, if not corrected by the repair system, these damages can be transmitted to the offspring, resulting in adverse effects in the progeny development [17,18]. These adverse effects may be resulted from alterations of genomic and epigenetic regulation during development, as observed in a study with cyclophosphamide, another chemotherapy agent that forms adduct with the spermatozoa DNA [19].

Another well-characterized mode of action for CP is the disruption of the redox balance of tissues leading to oxidative stress and reproductive toxicity [20–22]. The oxidative stress induced by CP mainly results from the combination of the drug with glutathione

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(GSH) tripeptide in the cytoplasm [23]. This interaction results in the formation of a complex that is converted into a highly reactive thiol compound into the cells. This compound can bind to essential proteins, leading to apoptosis [24–27]. Moreover, studies have reported that CP is capable of reducing the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) [28], which contributes to the alteration of the oxidant-antioxidant balance and promotes oxidative stress. Adult male rats exposed to 7 mg/Kg of CP showed oxidative damage via induction of lipid peroxidation and reduction of the antioxidant defense system potency in testis, followed by a decrease in sperm concentration and motility as well as an increase in sperm abnormalities and testicular damage as evidenced by histopathology [20].

In a previous study from our laboratory, *peri*-pubertal male rats treated with 1 mg/Kg/day of CP for 3 weeks were evaluated as to reproductive parameters at post-puberty (PND 66) and adult age (PND 140, after a recovery period of 74 days) [29]. The post-pubertal male rats showed changes in reproductive parameters, such as decrease in sperm production and reserves, sperm with progressive movement, tubular diameter, intratesticular testosterone levels and fertility potential, as well as increased pre-implantation losses, number of TUNEL-positive seminiferous tubules and immotile sperm. Moreover, we observed changes in sexual behavior and testicular histology as seminiferous tubules with few layers of germ cells, vacuolization in Sertoli cells, acidophilic cells with pyknotic nuclei and flaking of immature germ cells. In the adulthood these changes were recovered, except for sperm motility and damage to testicular histology [29].

In another study, performed in the same experimental conditions, short and long-term reproductive effects of paternal exposure to CP were investigated in the male F1 generation. For this, the pre-pubertal treated rats were mated with unexposed females at the end of 3 weeks of CP exposure and at adulthood, i.e, 74 days after suspension of treatment. Impaired postnatal growth, delay in the age of testicular descent, decrease in seminal gland weight, delay in sperm transit through the epididymis and increase in sperm number in the caput/corpus epididymis were observed in the offspring obtained after mating the young males exposed to CP. Testicular histopathological changes were also observed and persisted in the F1 generation of rats mated at adulthood. All the other parameters were comparable with the control group [17].

Given that, the present study aimed to investigate the reproductive development and fertility of the female offspring of young male rats, soon after the exposure to CP.

2. Materials and methods

2.1. Animals

Male (45 days old, n = 38) and female (66 days old, n = 38) *Wistar* rats were supplied by the Central Biotherium of UNESP – Univ Estadual Paulista and maintained in the Small Mammal Biotherium at the Department of Morphology, Institute of Biosciences, UNESP, Botucatu, SP, Brazil, under controlled conditions (23 ± 2 °C, 30% air humidity, 12:12-h photoperiod) with food and water delivered *ad libitum*. The animals were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory-grade pine shavings as bedding. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no 426-CEUA).

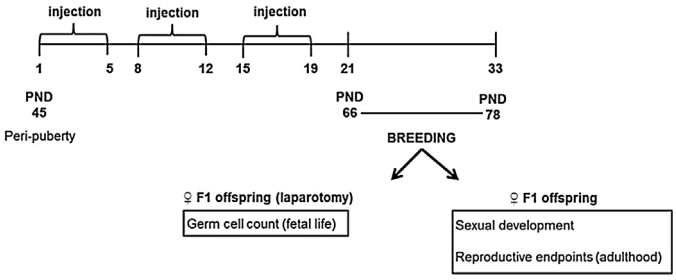

Fig. 1. Experimental design and treatment. PND = postnatal day.

2.2. Experimental design and treatment

The male rats were randomly divided into two experimental groups: control group (n = 20) that received 0.9% saline solution (vehicle) and the CP-treated group (n = 18) which received 1 mg/Kg/day of CP (cisdiammineplatinum (II) dichloride, Sigma Chemical Co., P4394, St. Louis, USA) diluted in vehicle, by intraperitoneal injection. Dose and treatment regimen were selected based on clinical use of the drug on human chemotherapy for testicular cancer [30]. The clinical dose of CP usually used in human (20 mg/m^2) was converted to rat doses by adjusting for body weight/surface area ratio [31], and a third of the rat dose was selected based on our prior study [29]. The drug was administered 5 days per week, for 3 weeks, during *peri*-puberty, from postnatal day 45 (PND 45) until PND 63 (Fig. 1). Body weight was recorded throughout the experiment.

The study was conducted into two experiments. In experiment 1, males from control (n = 10) and CP-treated (n = 8) groups were mated in separated cages with one naïve female per male for the evaluation of the F1 offspring fetal ovaries and blood platinum (Pt) levels of male rats from experimental groups. In experiment 2, another males (n = 10/group) treated with the same dose and time of exposure of the first experiment were mated in separated cages with another naïve females (one female per male) and were allowed to deliver the F1 offspring, whose postnatal female reproductive parameters were analyzed. In both experiments, pregnancy was detected by vaginal smears analysis after mating, and the presence of spermatozoa was considered day 0 of gestation (GD 0). The pregnant females were weighted each two days from GD 0 until GD 20.

2.3. Experiment 1

2.3.1. Pt serum levels

On PND 78, following the mating period, paternal rats from control (n = 10) or CP-treated (n = 8) groups were euthanized by CO_2 inhalation followed by decapitation and the blood was collected from the ruptured cervical vessels into trace metals free tube containing K_2EDTA anticoagulant (BD Vacutainer®) for the determination of blood Pt levels, according to Brouwers et al.[32]. Measurement was performed using inductively coupled plasma–mass spectrometry (ICP–MS) (ELAN DRC II, Perkin Elmer, Norwalk, CT).

2.3.2. Fetal ovaries

Only 5 parental (P0) females, from the 8 originally mated, became pregnant and were euthanized in GD 20 by CO_2 inhalation followed by decapitation and the ovaries from the fetuses (n = 5/group) were collected. The gonads were fixed in Karnovsky (2.5% glutaraldehyde, 8% paraformaldehyde), embedded in historesin, sectioned at 5 μm and stained with hematoxylin and eosin. Germ cells were counted in 3 sections per animal with 50 μm of

Table 1
Litter size, sex ratio and absolute and relative anogenital distance of the F1 offspring from the control or CP-treated groups in PND 1.

Parameters	Control	CP
Mated females (n)	10	10
Females that delivered viable litters (n)	10	7
Number of pups[a]	11.80 ± 0.55	11.57 ± 0.81
Female[a]	6.10 ± 0.43	5.57 ± 0.43
Male[a]	5.70 ± 0.40	6.00 ± 0.69
Sex ratio (%)[a]	108.30(80.36–146.70)	100.00(85.71–120.00)
Absolute anogenital distance (mm)		
Female	2.44 ± 0.03 (n = 58)	2.37 ± 0.04 (n = 39)
Male	4.42 ± 0.05 (n = 59)	4.37 ± 0.04 (n = 42)
Relative anogenital distance (mm/g$^{1/3}$)		
Female	1.30 ± 0.02	1.29 ± 0.02
Male	2.31 ± 0.02	2.34 ± 0.02

[a] Sample size (n) expressed in litters. Values are expressed as mean ± SEM (Student *t*-test), with exception for the sex ratio, expressed as median (Q1–Q3), Mann-Whitney test. $p > 0.05$ for all comparisons.

Table 2
Mean body weight of the F1 male and female offspring from the control or CP-treated groups on postnatal days (PND) 1, 8, 13 and 22.

Parameters	Control	CP
Body weight on PND 1 (g)		
Female	6.63 ± 0.06 (n = 57)	6.21 ± 0.08*** (n = 39)
Male	7.05 ± 0.07 (n = 59)	6.54 ± 0.12** (n = 42)
Female and male	6.85 ± 0.05 (n = 117)	6.38 ± 0.08*** (n = 81)
Body weight on PND 8 (g)		
Female	18.06 ± 0.22 (n = 57)	17.54 ± 0.35 (n = 39)
Male	18.76 ± 0.32 (n = 23)	17.72 ± 0.45 (n = 16)
Female and male	18.27 ± 0.18 (n = 80)	17.59 ± 0.28* (n = 55)
Body weight on PND 13 (g)		
Female	27.13 ± 0.38	25.85 ± 0.60
Male	27.94 ± 0.58	26.45 ± 0.69*
Female and male	27.36 ± 0.32	26.02 ± 0.47**
Body weight on PND 22 (g)		
Female	46.96 ± 0.76	48.09 ± 0.99
Male	49.09 ± 1.33	48.57 ± 0.85
Female and male	47.57 ± 0.67	48.23 ± 0.74

Values are expressed as mean ± SEM (Student *t*-test). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. On PND 1 the litters were reduced to 8 pups per litter after registering the body weight.

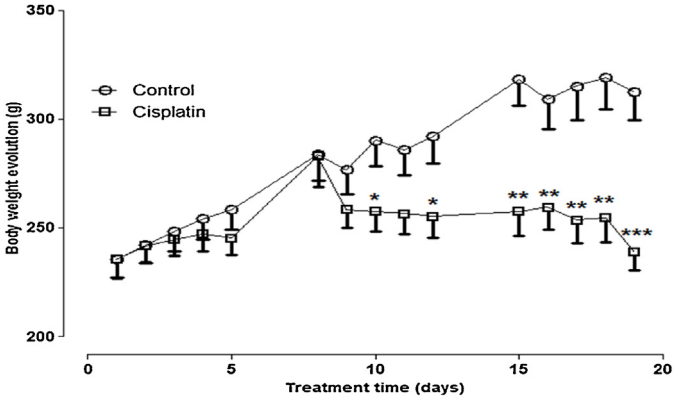

Fig. 2. Body weight gain (g) of P0 male rats from control (n = 10) or CP-treated (n = 8) groups during treatment period. Values are expressed as mean ± SEM. Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

distance among them and the results were expressed as germ cell number/area (germ cell number/mm^2).

2.4. Experiment 2

After the mating period, pregnant rats were weighed on alternate days from GD 0 until GD 20. The day of F1 pupsís delivery was recorded in the females mated with control or CP-treated rats.

2.4.1. Anogenital distance

On PND 1 all the pups (male and female) were weighed and the anogenital distance (AGD, the distance from the anus to the genital papilla) measured with a Vernier caliper (Mitutoyo, 530-320, Japan). For this, the mother was allocated in a separated cage and each pup was gently held and the distance from the anus to the genital papilla was scored and normalized by dividing AGD to the cube root of body weight [33]. As AGD in males is approximately double that of females [34], all the pups were classified as male or female based on the AGD values. The sex ratio (number of female pups/number of male pups × 100) and number of pups per litter (litter size) were scored and, afterwards, the offspring were equalized by reducing each litter to eight pups per mother, maintaining preferentially female pups. These pups were returned to the mother and the remaining pups were killed by decaptation.

On PND 22, the offspring were weaned, weighed and reduced to four females per litter. The male and female pups that were not used for the study were euthanized by CO_2 inhalation followed by decapitation.

2.4.2. Postnatal growth

Both female and male pups from experimental groups that remained after the offspring size equalization were weighed in PND 1, 8, 13 and 22 for the evaluation of body weight gain of the litters until weaning.

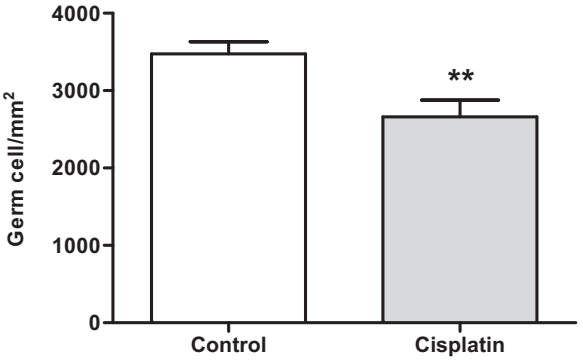

Fig. 3. Number of germ cells per ovarian area (mm^2) of F1 female fetuses from control (n = 5) or CP-treated (n = 5) groups. Values are expressed as mean ± SEM. Student's *t*-test. $^{**}p < 0.01$.

2.4.3. Puberty onset

From PND 30, four F1 females per litter were evaluated daily for the complete vaginal opening (VO), an external marker of puberty completion in rats [35].

2.4.4. First estrus and estrous cyclicity

After VO, the vaginal lavage was collected daily for the determination of the occurrence of the first estrus, characterized by the predominance of cornified squamous epithelial cells [36]. From PND 60 and over a period of 15 days, vaginal lavage of adult F1 female offspring from both control and CP-treated groups (n = 4 females per litter) was collected for the assessment of the estrous cyclicity as described by Marcondes et al. [37] with some adaptations: each day at approximately the same time in the light cycle, 10 μL of saline solution was gently introduced into the rat vagina with an automatic pipettor and the fluid aspired was placed on clean histological slides for analysis under a light microscope (10x magnification). The estrous cycle phases were classified based on the proportion of cellular types from the vaginal smear: proestrus (predominance of nucleated epithelial cells), estrus (predominance of anucleated cornified cells), metestrus (the same proportion among leukocytes, cornified, and nucleated epithelial cells) and diestrus (predominance of leukocytes). Data from each animal were used to calculate the frequencies of the phases (number of days in each phase), cycle length (number of days from the first day of a cycle phase to the first day of the next same phase) and number of cycles of the female F1 offspring from control and CP-treated groups.

2.4.5. Collection of blood and organs

During the first estrus after PDN 75, one female offspring per litter from females mated to control males (n = 10) or females mated to CP-treated males (n = 7), was weighed and euthanized by CO_2 inhalation followed by decapitation. Blood was collected from the ruptured cervical vessels for the determination of sex hormonal levels as described below. Liver, kidney, pituitary, ovaries, uterus (with fluid) and adrenal glands were removed and their weight (absolute and relative to body weight) were recorded. The reproductive organs were fixed in Bouinís solution (75% saturated picric acid solution, 25% formaldehyde and 5% acetic acid) for subsequent histological and immunohistochemical analysis.

2.4.6. Histological analysis of reproductive organs

After collected and fixed in Bouinís solution, ovary and right uterine horn from adult F1 offspring (n = 5/group) were processed and embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin. The ovaries were scanned for histomorphometric analysis with Panoramic Viewer software (3DHISTECH Ltd.) and the uterine horns were analyzed using a stereoscopic microscope (Leica MZ 125 Stereomicroscope) equipped with the Leica Application Suite Version 3.7.0 software.

The ovarian structures (corpora lutea and follicles) were counted in one section taken from the middle of the ovary per animal. Classification of the follicles was based on the different stages of follicular development, the morphology of the follicles and the number of layers of granulosa cells according to Borgeest et al. [38] and Talsness et al. [39]. Follicles were classified as primordial when the oocytes were surrounded by a single layer of squamous cells, while primary follicles were surrounded by a single layer of cuboidal epithelial cells. Preantral follicles were characterized by oocytes surrounded by more than one layer of granulose cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Follicles were considered atretic if they presented pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane. These follicles and corpora lutea are expressed as percentages of the total number of ovarian structures [40].

Three sections of the right uterine horns per animal were used to measure the following parameters: total uterine area (μm^2), myometrial, endometrial and luminal epithelium areas (μm^2), thickness and width index. The areas and perimeters of each region were measured by tracing around the images of the sections with a cursor, and the values were used to calculate the thickness, as described by Mosquete et al. [41], with adaptations. Endometrial and luminal epithelium width index were measured by deriving the width from their respective areas.

Heights of the luminal epithelium and endometrium (μm) were measured in five different regions of three sections per animal.

2.4.7. Immunohistochemistry

After collected and fixed in Bouinís solution, the left uterine horns from adult F1 offspring (n = 5/group) were dehydrated in ethanol, embedded in paraplast and serial transverse sections (5 μm) were mounted on silanized slides for immunohistochemical detection of the estrogen alpha receptor (ERα) and progesterone receptor (PR) proteins expression, using the polymer system (MACH 4 Universal HRP polymer Detection, Biocare, CA, USA) as described by Mariani et al. [42], with adaptation.

The slides were deparaffinized in xylene and rehydrated in decreased concentrations of ethanol followed by antigen recovery with citrate buffer (0.01 M, pH 6.0) in a Pascal Pressure Chamber (DakoCytomation Denmark A/S) at 120 °C for 5 min. Afterwards, the sections were allowed to cool down to room temperature, rinsed three times in phosphate-buffered saline (PBS), incubated in 3% of H_2O_2 diluted in PBS for 10 min to block endogenous peroxidase activity, rinsed again, incubated in 1% nonfat milk for 60 min and finally incubated overnight at 4 °C with mouse monoclonal anti-ERα (6F11, 1:50 dilution, Biocare Madical, USA) or rabbit polyclonal anti-PR (sc-538, 1:50 dilution, Santa Cruz Biotechnology, USA).

Following three 5 min washes at PBS and incubation at mouse and rabbit probe and HRP polymer for 10 min each at room temperature, the peroxidase activity was revealed using the 3,3′-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma–Aldrich Co., USA) for 10 min at room temperature. The slides were counterstained with Harris haematoxylin, dehydrated in ethanol, cleared with xylene and mounted with Permount.

The PR expression in the uterine luminal epithelium was qualitatively assessed, while ERα expression was semi-quantitatively assessed by manual scoring, based on the intensity of the DAB reaction product. The staining intensity was graded on a relative intensity scale of (0) no visible staining, (1) low intensity of staining (medium brown) and (2) high intensity of staining (black or dark brown, apparently well localized and specific staining), adapted

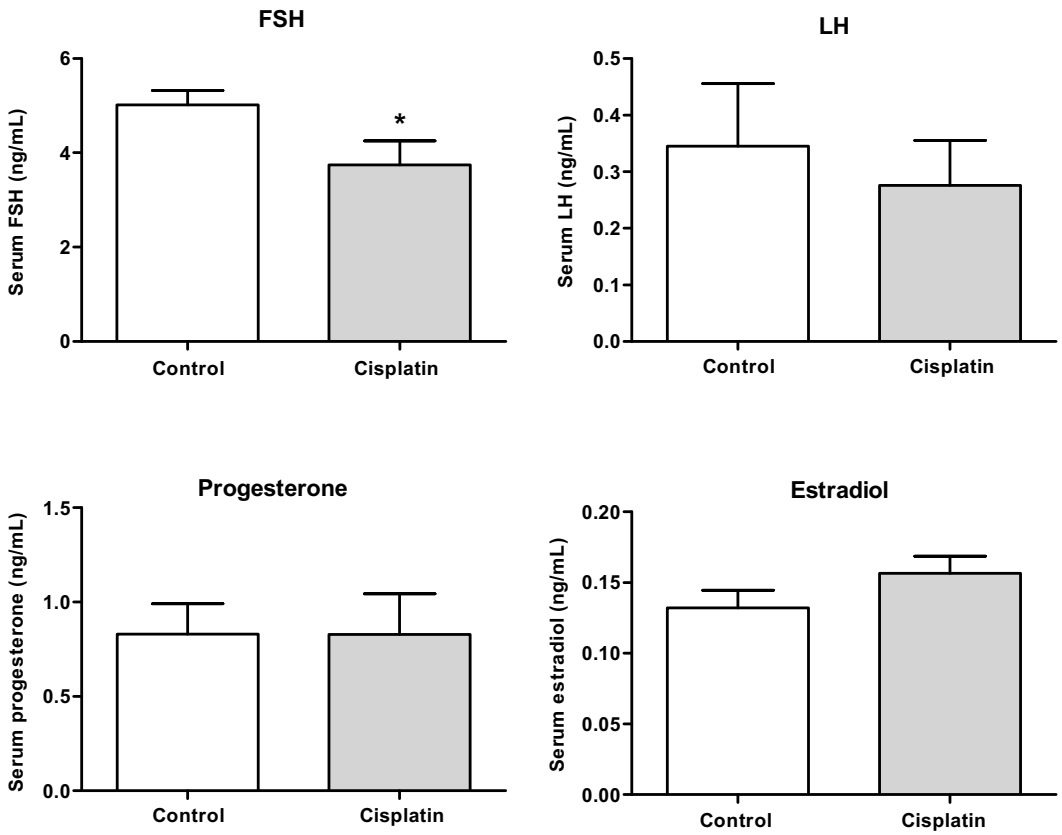

Fig. 4. Serum hormonal levels (ng/mL) of estradiol, progesterone, FSH and LH of adult F1 female offspring from control (n = 10) or CP-treated (n = 7) groups on the first estrous after PND 75. Values are expressed as mean ± SEM. Student's *t*-test. $^{*}p < 0.05$.

from Petrusz et al. [43]. One transverse section of each sample was observed in the light microscope Leica DMLB and ten different areas (40x magnification) were selected in sequence to avoid overlapping localization. These areas were scanned in the image analyzer Leica Q-win software version 3.1 for Windows TM for cell counts, which was performed by a single rater.

To measure the percentage of stained cells that expressed ERα and PR, the number of cells from each intensity was divided by the total number of counted cells [44].

2.4.8. Serum hormone levels

The blood collected from the adult F1 females euthanized in the first estrus after PDN 75 was centrifuged at 4 °C for 20 min at 2400 rpm and the serum collected was stored at −20 °C until analysis. Serum concentrations of estradiol, progesterone (PR), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by using a double-antibody radioimmunoassay (RIA). Plasma FSH and LH concentrations were performed using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIDDK, USA), while estradiol and progesterone concentrations were determined using MAIA kits provided by Biochem Immunosystems (Bologna, Italy). The intra-assay coefficient of variation was 3.4% for LH, 2.8% for FSH, 4.3% for estradiol and 7.5% for progesterone. All samples were dosed in duplicate and, when needed, at different dilutions. To avoid inter-assay variation, all samples of the same experiment were assayed in the same RIA.

2.4.9. Sexual behavior

Adult F1 female offspring from control (n = 10) or CP-treated groups (n = 7), one F1 female per litter, were assessed in the mating test. For this, after nine days of acclimation in the inverted dark cycle and during the first proestrus phase after PND 84, the females were introduced into the cage of a sexually mature male which were allowed to mount ten times on the female. During each mount, the presence or absence of lordosis was recorded and the results were expressed as lordosis quotient (LQ; number of lordosis/ten mounts × 100), based on Guerra et al. [36].

2.4.10. Fertility

Immediately after the sexual behavior test, the same F1 females (one/litter) were evaluated for fertility. For this, they were maintained with the same male used in the behavior test for 4 h and, after that, vaginal smears were collected, examined and presence of spermatozoa was considered GD 0.

On GD 20, pregnant females were euthanized by CO_2 inhalation followed by decapitation for the laparotomy procedure. The uterus and ovaries were collected and the number of corpora lutea, implantation sites (implants), early or late resorptions (depending on absence or presence of distinguishable features such as the head or limbs, respectively), live fetuses and fetal weights were recorded for subsequent determination of the following fertility parameters: pregnancy rate: number of pregnant females/number of inseminated females × 100; implantation rate (efficiency of implantation): implantation sites/corpora lutea × 100; pre-implantation loss rate: number of corpora lutea – number of implantations/number of corpora lutea × 100; post-implantation loss rate: number of implantations – number of live fetuses/number of implantations × 100; sex ratio: number of female fetuses/number of male fetuses × 100 [45].

2.5. Statistical analysis

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. Student's *t*-test was used for

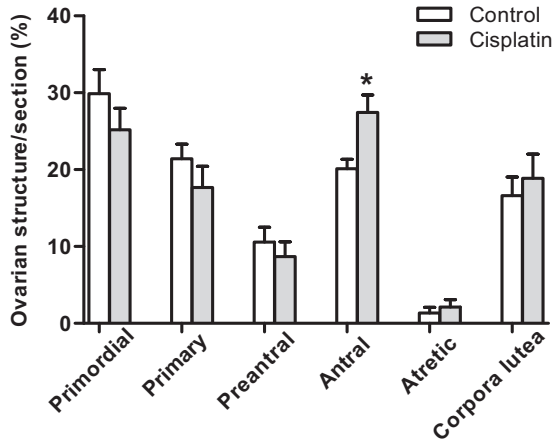

Fig. 5. Ovarian structures (follicles and corpora lutea) percentages from adult F1 female offspring of control (n = 7) or CP-treated (n = 7) groups. Values expressed as median (Q1–Q3). Mann-Whitney test. *$p < 0.05$.

comparison of parametric variables. Nonparametric variables were compared by Mann-Whitney test. Chi-square test was utilized for comparison of the gestational rate between groups. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by GraphPad InStat (version 5).

3. Results

3.1. Experiment 1

3.1.1. Effects in parental generation (PO)

From day 10 until the end of treatment there was a significant reduction in the body weight gain of the male rats treated with CP (Fig. 2). The serum levels of Pt (μg/L, mean ± SEM, Mann-Whitney test) in males euthanized on PND 78 were significantly increased ($p < 0.001$) in CP-treated group (2,411.00 ± 146.50) when compared to control group (0.30 ± 0.25).

The gestational rate of the females mated to CP-treated males was significantly reduced (62.5%, 5 pregnant in 8 inseminated rats) when compared to control group (100%, $p < 0.05$, Chi-square test).

3.1.2. Fetal gonad analysis

There was a significant decrease in the number of germ cells per ovarian area of female fetuses from females mated to CP-treated males when compared to the control males (Fig. 3). Heretofore, "treated offspring" are defined as "offspring from CP-treated males mated to untreated females" and "control offspring" are defined as "offspring from vehicle-treated males mated to untreated females".

3.2. Experiment 2

3.2.1. Paternal analysis

All sperm-positive females mated with males of the control group (n = 10) became pregnant, whereas in the treated group, the gestational rate was 80% (8 pregnant in 10 inseminated rats, $p > 0.05$). These sperm-positive females from both groups delivered pups on GD 21 or GD 22, except one female from CP-treated group that gave birth to six stillborn fetuses on GD 23. In addition, this female presented 10 uterine resorption sites visualized by laparotomy.

During pregnancy, no statistical difference was observed in the body weight gain (GD 20 weight subtracted from the GD 0 weight) of the pregnant females mated with males from experimental groups ($p > 0.05$; data not shown).

Table 3
Age of complete vaginal opening (VO) and first estrus of the F1 female offspring from the control or CP-treated groups.

Parameters	Control (n = 40)	CP (n = 28)
VO (days)	35.63 ± 0.57	35.04 ± 0.53
Body weight on VO day (g)	114.00 ± 2.89	111.20 ± 3.14
First estrus (days)	37.40 ± 0.71	37.30 ± 0.60
Body weight on first estrus day (g)	123.00 ± 3.63	123.70 ± 3.17

Values are expressed as mean ± SEM. Student *t*-test. $p > 0.05$ for all comparisons.

Table 4
Assessment of estrous cycle regularity in the adult F1 female offspring from control or CP-treated groups.

Parameters	Control (n = 40)	CP (n = 28)
Frequency of proestrus (days)	3.35 ± 0.12	2.96 ± 0.13*
Frequency of estrus (days)	3.87 ± 0.10	3.54 ± 0.16*
Frequency of metestrus (days)	4.60 ± 0.30	5.18 ± 0.33
Frequency of diestrus (days)	3.12 ± 0.31	3.25 ± 0.37
Number of cycles	2.82 ± 0.06	2.79 ± 0.13
Estrous cycle length (days)	4.21 ± 0.07	3.79 ± 0.13**

Values are expressed as mean ± SEM. Student *t*-test. **$p < 0.01$.

Table 5
Relative and absolute organ weights of adult F1 female offspring from control or CP-treated groups on the first estrus after PND 75.

Parameters	Control (n = 10)	CP (n = 7)
Body weight (g)	225.20 ± 6.98	215.10 ± 12.19
Absolute weights		
Uterus (mg)	407.80 ± 18.54	363.50 ± 25.26
Ovaries (mg)	88.06 ± 5.01	86.10 ± 5.99
Pituitary (mg)	12.65 ± 1.61	9.31 ± 0.87
Liver (g)	10.30 ± 0.16	9.49 ± 0.56
Kidneys (g)	1.73 ± 0.03	1.63 ± 0.09
Adrenal glands (mg)	7.50 ± 0.29	8.43 ± 0.74
Relative weights		
Uterus (mg/100 g)	182.20 ± 8.68	168.50 ± 5.20
Ovaries (mg/100 g)	39.63 ± 3.07	40.00 ± 1.40
Pituitary (mg/100 g)	5.60 ± 0.61	4.30 ± 0.26
Liver (g/100 g)	4.59 ± 0.13	4.43 ± 0.16
Kidneys (g/100 g)	0.77 ± 0.25	0.76 ± 0.01
Adrenal glands (mg/100 g)	3.35 ± 0.13	3.91 ± 0.26

Values are expressed as mean ± SEM. Student *t*-test. $p > 0.05$.

3.2.2. Reproductive analysis of the adult female offspring

The absolute and relative AGD of the offspring, as well as the litter size and sex ratio were similar between experimental groups ($p > 0.05$; Table 1). On PND 1, 8 and 13, there was a statistically significant decrease in the mean body weight of the litters (females and males) from CP-treated rats ($p < 0.0001$, $p = 0.0382$ and $p = 0.0095$, respectively) (Table 2). The mean body weights of the male and female pups were significantly reduced on PND 1 ($p < 0.0001$ and $p = 0.001$, respectively). On PND 13, only the male pups showed significant decrease in body weight ($p = 0.0385$).

Evaluation of the age of VO and first estrus showed that paternal treatment with CP did not interfere with these parameters ($p > 0.05$; Table 3). However, there was a statistically significant decrease in the estrus cycle length and in the frequencies of proestrus and estrus in the offspring of CP-treated group (Table 4).

The absolute and relative organ weights of the female offspring euthanized on the first estrus after PDN 75 were similar between groups ($p > 0.05$; Table 5). In contrast, assessment of the hormonal levels revealed that FSH serum concentrations were significantly reduced in CP-treated group, but not on estradiol, LH or progesterone (Fig. 4).

Histological analysis of the ovary and uterus of the adult female offspring showed no evidence of structural alterations after CP paternal treatment. There were also no intergroup differences in

Table 6
Area (μm^2), thickness and width index and height (μm) of different uterine regions in adult F1 female offspring from control (n = 5) or CP-treated (n = 5) groups.

Parameters	Control (n = 5)	CP (n = 5)
Area $\times 10^{-4}$ (μm^2)		
Uterus	679.20 ± 31.74	756.1 ± 42.57
Endometrium	304.70 ± 19.53	323.70 ± 20.16
Myometrium	179.70 ± 11.59	199.20 ± 12.07
Luminal epithelium	26.26 ± 1.67	25.95 ± 4.23
Thickness index		
Endometrium	329.80 ± 11.36	340.20 ± 19.43
Myometrium	194.40 ± 7.45	209.80 ± 13.05
Luminal epithelium	28.33 ± 1.11	26.54 ± 1.89
Width index ($\times 10^3$)		
Endometrium	0.70 ± 0.04	0.63 ± 0.01
Luminal epithelium	2.63 ± 0.23	2.36 ± 0.11
Height (μm)		
Endometrium	724.50 ± 59.66	935.80 ± 14.08
Luminal epithelium	38.00 ± 1.30	35.44 ± 0.65

Mann-Whitney test. Values expressed as mean ± SEM. $p > 0.05$ for all comparisons.

Table 7
Percentages of luminal epithelial cells immunostained for ERα in adult F1 female offspring from control (n = 5) or CP-treated (n = 5) groups.

Staining intensity	Control (n = 5)	CP (n = 5)
0 (%)	8.56 (6.53 – 12.56)	10.44 (9.02 – 12.83)
1 (%)	29.47 (23.48 – 34.97)	44.21 (19.02 – 52.76)
2 (%)	61.96 (57.21 – 65.25)	46.05 (36.86 – 69.16)

Values expressed as median (Q1–Q3). Mann-Whitney test. $p > 0.05$.

the results of uterine histomorphometry ($p > 0.05$; Table 6). On the other hand, assessment of the ovarian structures (follicles and corpora lutea) showed a statistically significant increase in the percentage of antral follicles (Fig. 5) in the CP-treated group.

Immunohistochemical analysis of the right uterine horns revealed no statistically significant difference in the ERα and PR proteins expression pattern on the luminal epithelium in CP-treated group when compared to the control group (Figs. 6 and 7). Semi-quantitative analysis of the ERα expression showed no statistical difference between groups ($p > 0.05$; Table 7).

In the first proestrus after PND 84, no statistical difference between the female offspring treated *vs.* control groups was observed in sexual behavior test, calculated by lordosis quotient (data not shown). Fertility measures were not statistically significant in female offspring from CP-treated fathers compared to controls ($p > 0.05$; Table 8).

4. Discussion

Cisplatin is a pharmaceutical widely used in the treatment of several cancers, including germ cell tumor [6,7], affecting men aged 15–35 years old. The most commonly used CP doses are between 20 mg/m^2 or 40 mg/m^2 daily, administered five consecutive days a week, for 2–5 weeks, by intravenous injection [30,46–48]. Despite its high activity and wide use, studies in human and rats have demonstrated that the therapeutical success can be accompanied by side effects, including nephrotoxicity, neurotoxicity, ototoxicity, and abnormal spermatogenesis [10,11,49]. In rats, a single intraperitoneal injection of 7 mg/kg of CP resulted in oxidative damages and reduction in the antioxidant defense system in the testis tissue, leading to reproductive toxicity [50].

It is known that the main mechanism of action of CP to inhibit tumor cells growth is through the formation of platinum-DNA adducts, leading to apoptosis in germ cells, azoospermia or oligospermia in men [14,51]. In rodents, treatment with a single dose of 6 mg/kg of CP resulted in DNA adduct formation in the testis,

Table 8
Parameters of sexual behavior and fertility of adult F1 female offspring of control or CP-treated groups.

Parameters	Control (n = 10)	CP (n = 7)
Lordosis coefficient[a]	100.00 (90.00 – 100.00)	100.00 (10.00 – 100.00)
Gestational rate (%)[b]	100	100
Implantation rate (%)[a]	94.44 (93.33 – 100.00)	92.86 (84.62 – 100.00)
Fetal weight (g)[c]	3.40 ± 0.06	3.60 ± 0.14
Placenta weight (g)[c]	0.53 ± 0.15	0.51 ± 0.01
Number of live fetuses[c]	13.60 ± 0.43	11.14 ± 1.37
Number of corpora lutea[c]	15.20 ± 0.61	14.57 ± 0.78
Number of implantations[c]	14.50 ± 0.48	12.86 ± 0.70
Number of resorptions[c]	0.90 ± 0.35	1.86 ± 0.86
Sex ratio (%)[a]	116.70 (49.17 – 170.00)	114.30 (55.56 – 225.00)
Pre-implantation loss (%)[a]	5.56 (0.00 – 6.67)	7.14 (0.00 – 15.38)
Post-implantation loss (%)[a]	3.33 (0.00 – 13.57)	9.09 (0.00 – 27.27)

Sample size (n) expressed in litters. [a]Values expressed as median (Q1–Q3). [b]Chi-square test. [c]Mann-Whitney test.[3] Values expressed as mean ± SEM. Student *t*-test. $p > 0.05$ for all comparisons.

which was detected in the nuclei by immunohistochemistry for up to 9 days following treatment [16]. In another study with rats, in which the formation of DNA adducts was quantified by ELISA in the testis following the treatment with a single intraperitoneal dose of 8 mg/kg of CP, 40–50% of the adducts present at day 2 persisted until days 7 and 14 [15]. Atessahin et al. [52] reported that administration of a single dose of 7 mg/kg of CP in mice resulted in significant reduction of spermatozoa concentration and motility as well as the increased rates of sperm abnormalities.

Currently, there are several studies in the literature demonstrating direct effects of CP exposure on male and female genital systems [53–55]. However, few of them have reported the effects of paternal exposure to the drug on the reproductive system and fertility of the offspring. In a previous work [17] we demonstrated, in the male offspring of male rats exposed to CP at *peri*-puberty, delay in postnatal growth and testicular descent, decrease in seminal gland weight and adverse effects in the spermatogenesis. In the present study, carried out to evaluate the female offspring of male rats exposed to CP in the same experimental conditions, reproductive dysfunctions were also observed. Although DNA integrity was not evaluated, we observed a significant increase in Pt levels in blood that may have resulted in spermatozoa DNA-adduct formation and increased germ cell apoptosis.

In Experiments 1 and 2, the gestational rates were reduced in the females mated with the treated males, compared with the control group, corroborating results from a previous study [29]. Increase in pre- and post-implantation losses were reported by Seethalakshmi et al. [56] in male rats exposed to 0.5 mg/Kg of CP for 9 weeks.

In the present work one female from CP-treated group delivered stillborn pups on GD 23 and the remaining litter were resorbed. Delivery delay and litter mortality were also observed in previous works in females mated to CP-exposed males [17,53]. Further studies should be performed to elucidate this indirect effect of paternal exposure to CP.

Interestingly, in the present work the litter size as well as the absolute and relative anogenital distances of male and female F1 offspring at PND 1 were comparable between groups. However, the postnatal growth of offspring was affected by paternal exposure to CP, as observed by a significant decrease in the mean body weight of the litters.

Puberty in mammals is timed by the increase in gonadotropin-releasing-hormone (GnRH) secretion [57], which promotes gonadal steroidogenesis. In rats, the age of vaginal opening and first estrus are markers of age of puberty onset in females [35]. The estrous cycle in these animals begins immediately after the vaginal opening, which occurs around PND 32 and PND 36 [58]. In the present study, no changes were observed in the age of vaginal opening and

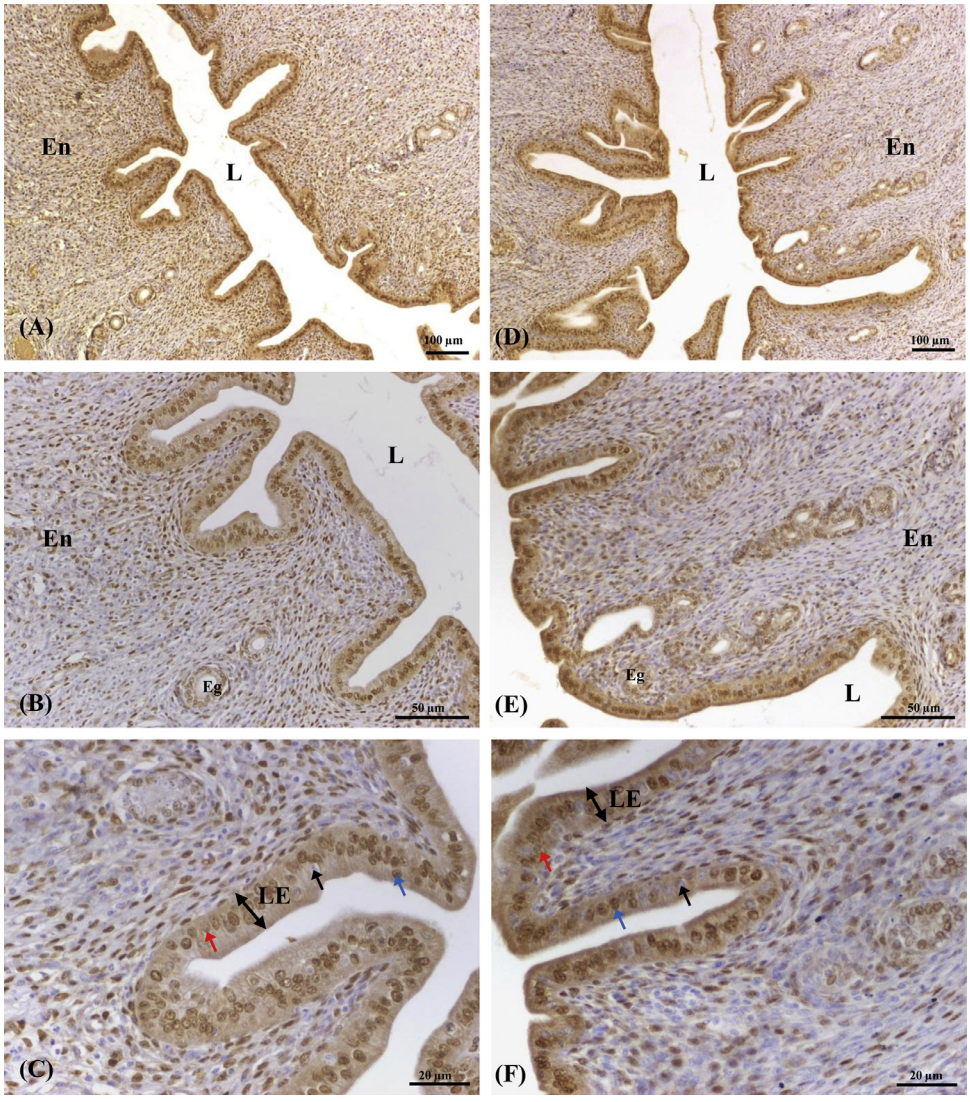

Fig. 6. Immunostaining of ERα in adult F1 female offspring uterus (at estrus phase) from control (A, B and C) or CP-treated groups (D, E and F). L = lumen; En = endometrium; Eg = endometrial gland; LE = luminal epithelium; Black arrows = not stained cells; Blue arrows = high intensity of staining; Red arrows = low intensity of staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

first estrus of F1 female offspring, indicating that puberty onset was not affected by paternal exposure to CP. FSH is a hormone that stimulates follicular development in post-pubertal mammals and represents an important indicative of follicular reserve in the ovaries. However, it is only in the last stages of maturation that follicles are under control of this hormone [59]. The secretion of FSH, in turn, is regulated by the levels of estradiol, which is synthesized by the granulosa cells of the follicles [59]. In the present study, adult F1 female offspring of CP-treated group showed a significant decrease in the serum concentration of FSH. Despite the fact that no gross structural damage was found in the ovaries and uterus of the female offspring, the histomorphometric assessment of the ovarian structures in adult female offspring revealed a significant increase of antral follicles in CP-treated group. There were no significant intergroup differences in circulating estrogen levels, but emerging evidence suggests that local production of estradiol is an important factor in the regulation of the differentiation and function of granulosa cells of developing follicles. Uterine receptors for estrogen and progesterone were not altered by paternal CP treatment, as revealed by immunohistochemistry.

In the present study, the length of the estrous cycles and the frequencies of estrus and proestrus phases were significantly reduced in the adult F1 offspring of CP-treated group when compared to control group. Although we do not have an explanation for this result, based on the endpoints herein evaluated, we hypothesize a dysregulation in the estrous cyclicity in the offspring of CP-exposed males.

No significant changes were detected in the weights of vital and reproductive organs in female progeny from CP-treated fathers consistent with most of the findings of Favareto et al. [17]. In addition, there were no significant differences in sexual behavior and fertility in the female offspring after paternal CP treatment. However, the number of litters evaluated in the present study may have limited the findings of adverse effects in the F1 female offspring.

In female mammals, the reproductive lifespan is determined by the pool of primordial follicles established during embryonic development, which is essential for reproductive success since represents the total population of available germ cells during their entire reproductive life [60,61]. The analysis of fetal ovaries revealed a decrease in the number of germ cells per ovarian area in the F1 offspring of CP-treated group when compared to control group. However, in the adult females the ovarian structures were not affected by paternal CP, with an exception for the increased incidence of antral follicles. Adler and Tarras [62] reported that

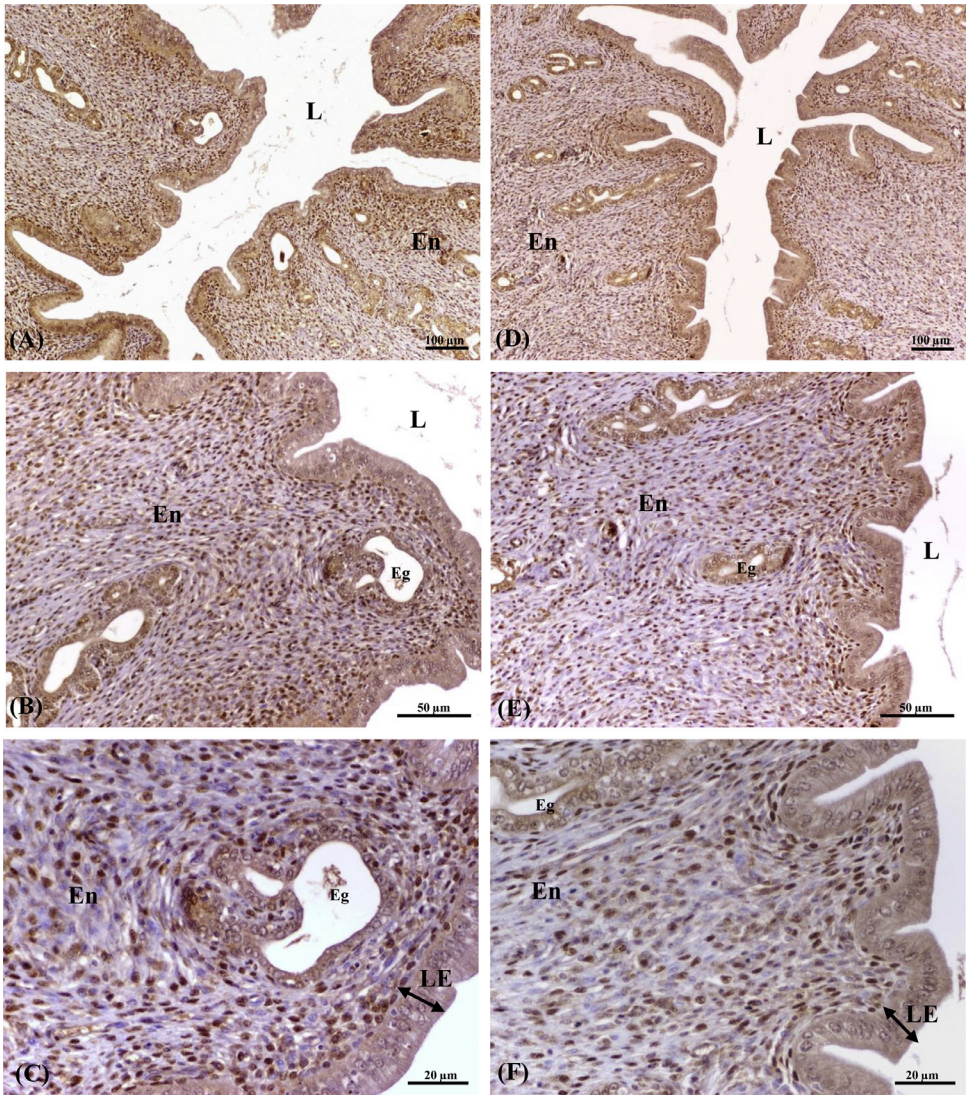

Fig. 7. Immunostaining of PR in adult F1 female offspring uterus (at estrus phase) from control (A, B and C) or CP-treated groups (D, E and F). L = lumen; En = endometrium; Eg = endometrial gland; LE = luminal epithelium.

the exposure of mice to CP resulted in aberrations in germ cells during the premeiotic phase of DNA synthesis. It is becoming established that sperm DNA damage can result in adverse effects in the offspring [18]. Thus, in the present study, although sperm DNA damage was not investigated, we postulate that these results were mediated by epigenetic mechanisms [19,63]. However, one limitation of our study is that only one ovary section was analyzed per animal.

Studies show that alterations in sperm methylation patterns are associated with infertility and transgenerational effects in human and rodents [64–66]. Chan et al. [67] reported that rats treated for 9 weeks with bleomycin-etoposide-cisplatin (BEP), a cocktail used for testicular cancer, showed epigenetic alterations of methylation patterns in spermatozoa DNA. In addition, studies in resistant tumor cells to CP treatment showed that the formation of DNA-platinum adducts is accompanied by the activation of cellular self-defense system by activating or silencing a variety of different genes that are required for cytotoxicity, leading to epigenetic and/or genetic alterations [68,69]. In the present study, the male rats treated with CP maintained their reproductive capacity to sire the offspring. Thus, based on aforementioned studies and given the findings on female offspring in the present study, and the findings on male offspring from our previous study [17], we suggest that possible alterations in spermatozoa DNA methylation patterns may be involved in the reproductive developmental effects observed in the offspring after paternal exposure to CP.

Although we cannot rule out the mechanisms responsible for the effects observed in the present study, and due to the relatively small number of litters assessed, our study is pioneer showing morphofunctional adverse effects in the female offspring of young male rats exposed to CP. Moreover, the results of the present work in conjunction with a previous work revealed that the male offspring may be more vulnerable to reproductive toxicity than the female offspring after paternal exposure to CP.

In summary, our results in rats raise concern for the children born to human males being treated with CP. Future research needs include analysis of DNA integrity of the sperm of CP-treated males, spermatozoa DNA methylation patterns, multi-dose studies, and shorter dose intervals to determine the critical window of male exposure in order to minimize the adverse effects of paternal exposure to CP on the offspring.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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