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Sperm quality and fertility in rats after prenatal exposure to low doses of TCDD: A three-generation study

Marciana Sanabria[a,*], Maira Smaniotto Cucielo[a], Marina Trevizan Guerra[a], Cibele dos Santos Borges[a], Thais Petrochelli Banzato[a], Juliana Elaine Perobelli[a], Gabriel Adan Araújo Leite[a], Janete Aparecida Anselmo-Franci[b], Wilma De Grava Kempinas[a]

[a] Laboratory of Reproductive and Developmental Biology and Toxicology, Department of Morphology, Institute of Biosciences of Botucatu, UNESP—Univ Estadual Paulista, Botucatu, SP, Brazil
[b] Department of Morphology, Stomatology and Physiology, School of Dentistry, USP—University of São Paulo, Ribeirão Preto, São Paulo, Brazil

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ABSTRACT

Exposure to Tetrachlorodibenzo-*p*-dioxin (TCDD) in male rats promotes, decreased sperm concentration, alterations in motility and in sperm transit time. We evaluated the effect transgenerational of *in utero* exposure to low doses TCDD in the sperm quality. Pregnant rats (F0) were exposed to 0.1; 0.5 and 1.0 μg of TCDD, on gestational day 15, coincides with the end of most organogenesis in the fetus. Adult male offspring (F1, F2 and F3 generation) were investigated for fertility after artificial insemination *in utero*. After collection of the uterus and ovaries, the numbers of corpora lutea and implants were determined. TCDD provoked alterations in sperm morphology and diminution in serum testosterone levels and sperm transit time in the cauda epididymis. The fertility significantly decreased in all the generations, at least at one dose. In conclusion, TCDD exposure decreases rat sperm quality and fertility in adult male offspring and this effects persist into the next generation.

1. Introduction

Dioxins are persistent organic pollutants interfering with endocrine systems and can cause reproductive and developmental disorders [1]. The compound 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), one of the most potent environmental contaminants, is formed not only as an unwanted byproduct in the manufacturing of chlorinated hydrocarbons, but also in incineration processes, paper and pulp bleaching, and emissions from steel foundries and motor vehicles [2]. The lipophilicity and low metabolism rate of TCDD leads to its accumulation and persistence in adipose tissue [3].

The major source of human exposure to dioxins is through the diet [4], almost exclusively through consumption of animal foods including meat, fish and dairy products [5]. The dioxins can also be transferred prenatally through the placenta and/or after birth via lactation [6], resulting to the development of chronic diseases in later life [7].

TCDD exposure during development alters the characteristics of different tissues, leading to effects in adulthood. It has been recognized that chemicals capable of reprogramming developing tissues can in principle also alter the characteristics of germ cells, leading to effects that span generations after the exposure [8]. Because these chemicals alter gene expression and phenotype in a heritable manner, yet do not alter the DNA sequence in the genome, such effects have been termed epigenetic [9].

Maternal exposure to low doses of TCDD has been reported to cause diverse changes in the reproductive system of male offspring, including reduced sperm count, decreases in the ventral prostate size and anogenital distance, feminized sexual behavior and impaired development of the prostate and testes [1,6,10–16], reduced weights of reproductive organs [15–18], delayed initiation of sexual maturation [16,19,20], decreased serum testosterone levels [10,16], oxidative stress in the epididymis [21], alterations in motility and in sperm transit time [6,10–16,22,23]. These low doses (25ng–1 μg of TCDD) are within the limit of tolerable daily intake (TDI) for humans, suggested by World Health Organization

Abbreviations: TCDD, Tetrachlorodibenzo-*p*-dioxin; GD, gestational day; LOAEL, lowest observed adverse effect level; LD50, median lethal dose; PND, post-natal day.

* Corresponding author at: Laboratory of Reproductive and Developmantal Biology and Toxicology, Department of Morphology, Institute of Biosciences of Botucatu, UNESP – Univ Estadual Paulista, 18618–970 Botucatu, SP, Brazil.
E-mail address: marcisanabria@gmail.com (M. Sanabria).

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(WHO) and EC Scientific Committee for Food (SCF), equivalent to 1–4 pg/kg/bw [24,25]. However, it is still uncertain how much the human population is exposed to dioxins [26,27].

Although numerous studies in laboratory rodents have investigated the impact of in utero and lactational exposure to TCDD on male offspring [10–12,16,28], showing that their exposure promotes dysfunctions of the male genital, its action on sperm quality and fertility in subsequent generations is not clear.

Therefore, considering the scarcity in the literature of works on the effects of TCDD in the reproductive health of male offspring in next generations and in attempt to understand more deeply the relation of effects of TCDD about the sperm quality, including fertility, the present study aimed to investigate the effects of developmental exposure to TCDD in the male genital tract, with emphasis on sperm quality and fertility, and whether these effects are transmitted to the next three generations.

2. Materials and methods

2.1. Animals

Adult female (60 days of age, weighing 230 g) and male (90 days of age, weighing 400 g) Wistar rats were supplied by Central Biotherium of State University of São Paulo (UNESP) and maintained in polypropylene cages (43 × 30 × 15 cm) with laboratory grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1 °C) and lighting conditions (12L, 12D photoperiod, lights switched off at 07:00 h) and had free access to food and water. The experimental protocol followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by the Biosciences Institute/UNESP Ethics Committee for Animal Research (Protocol number: 381).

2.2. Experimental design and treatment

Two non-gravid female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear was considered day zero of gestation (gestational day 0 – GD0). The pregnant females were randomly assigned to the experimental groups and housed individually in cages.

Pregnant rats (F0 generation) were allocated into four experimental groups (n = 7–10 rats/group): exposed groups that received low doses of TCDD (Sigma Chemical Co., St. Louis, Mo., code 48599), at 0.1 μg/kg, 0.5 μg/kg or 1.0 μg/kg, orally (gavage), single dose on GD15, which coincides with the end of most organogenesis in the fetus and because the hypothalamic-pituitary-testis axis is just beginning to function [10–12,15,16]. These doses were chosen based on TCDD's lowest observed adverse effect level (LOAEL) – 0.01 μg/kg/day, of reproductive function for rats, and median lethal dose (LD50) – 20–100 μg/kg, by oral route for rats [10,16,20]. Other studies have also demonstrated that maternal treatment with doses larger than 1.0 μg TCDD caused maternal toxicity and high mortality among the offspring [10]. The control group received corn oil (vehicle), following the same experimental protocol.

At PND 1 (with PND 0 being defined as the day of birth), litters were weighed and reduced to eight, maintaining preferentially the male offspring [29]. At adulthood (90 days old), part of the male offspring (F1) were mated with unexposed females to obtain the F2 generation whereas another portion of the male offspring (F2) were mated to obtain the F3 generation. All the procedures were performed at 90 (adulthood) to assess possible transgenerational effects of TCDD on sperm quality, as described below (Fig. 1).

2.3. Experiment 1

2.3.1. Euthanasia, body weight and organ weights of male offspring

Male rats (7–10, one per litter) were weighed, euthanized by CO_2 inhalation and then decapitated (between 9:00 and 11:30 AM). The right testis, epididymis and vas deferens, ventral prostate, seminal vesicle (without the coagulating gland and full secretion) were removed and their absolute weights were determined.

2.3.2. Serum hormone levels

After decapitation, blood was collected and serum was obtained by centrifugation (2400 rpm, 20 min at 4 °C) and stored at −20 °C until analysis. The concentrations of testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by the technique of double antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases–NIADDK, USA). All the samples were analyzed in the same assay to avoid inter-assay variability.

2.3.3. Daily sperm production per testis, sperm number and transit time in the epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) in the right testis were counted as described previously [30], with adaptations adopted by Fernandes et al. [31]. Briefly, the right testis, decapsulated and weighed soon after collection, was homogenized in 5 mL of NaCl 0.9% containing Triton X100 0.05%, followed by sonication for 30 s. After a 10-fold dilution, one sample was transferred to Neubauer chambers (four fields per animal), and mature spermatids were counted. The number of mature spermatids in the testis was calculated using the following formula: *sperm number in the testis x10^6 = M x 0.05 x FD1 x FD2*, where: *M* = mean sperm count in the four fields of Neubauer chamber; *FD1 (first dilution)* = parenchyma + solution of NaCl 0.9% containing Triton X100 0.05% – weight of the pipe; and *FD2 (second dilution)* = 10. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium. In the same manner, caput/corpus and cauda epididymis portions were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis, where: FD1 = volume of NaCl 0.9% containing Triton X100 0.5%; FD2 = 20. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by DSP.

2.4. Experiment 2

2.4.1. Intrauterine artificial insemination

Because rats produce and ejaculate an excess of qualitatively normal sperm, artificial *in utero* insemination of a fixed, a critical number of sperm has been suggested as a means of augmenting the sensitivity of a toxicant-induced decrease in sperm quality in the rat [32]. According to this technique, a fixed number of sperm collected in the cauda epididymis is inseminated directly into the uterus to permit evaluation of sperm quality, without the interference of other factors such as alterations in the sexual behavior pattern and number of sperm available for ejaculation [33].

Females (n = 80; 60 days old, weighing 230 g) in estrus induced by LHRH (Luteinizing Releasing Hormone) agonist (Sigma Chemical Co., St Louis, Missouri), were paired with sexually experienced vasectomized males for 1 h. Receptive females (that exhibited lordosis) were selected for insemination. The isolation and preparation of distal cauda sperm for insemination were similar to previous descriptions [34,35]. When insemination was complete, the abdominal musculature was sutured. Females of the F1 and

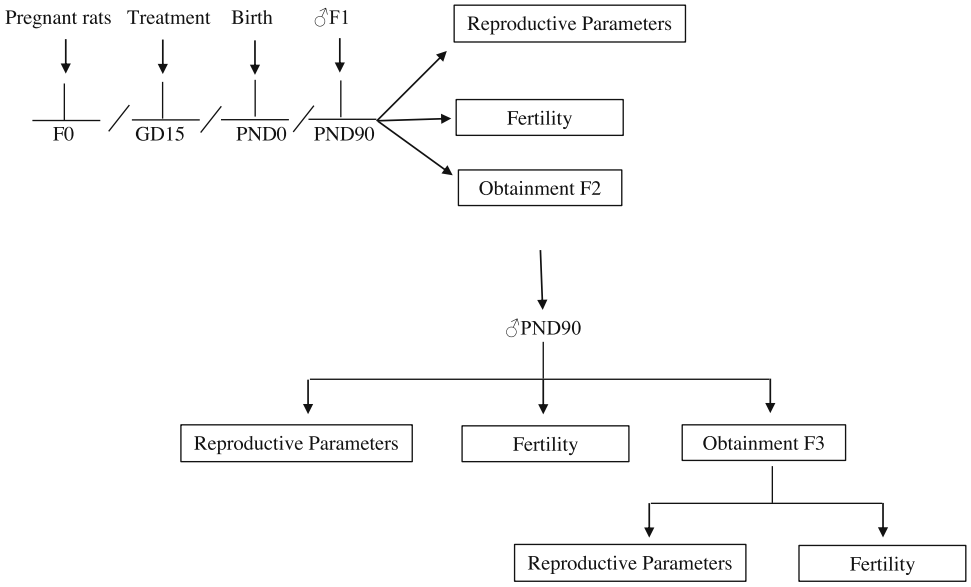

Fig. 1. Experimental design. GD – gestational day, PND – post-natal day.

F2 generations were killed 20 days later to evaluate fertility. Since there were no changes in relation to resorptions per implantation sites in the F1 and F2 generations, the females of the F3 generation were killed 9 days later to evaluate fertility.

2.4.2. Fertility evaluation

The females were killed by decapitation. After collection of the uterus and ovaries, the numbers of corpora lutea, implants and reabsorptions were determined. From these results the following parameters were calculated: proportions of implant per corpus luteum and proportions of resorptions per implantation site.

2.4.3. Sperm motility and morphology

Sperm motility was evaluated from sperm used for artificial insemination. The sperm sample was collected and immediately diluted in 2 mL of modified HTF medium (Human Tubular Fluid, Irvine Scientific®), pre-warmed at 34 °C. An aliquot of 10 μL of the diluted sperm was placed in a Makler chamber (Irvine, Israel) and analyzed under a phase-contrast microscope (Leica DMLS) at 400x magnification. One hundred sperm were evaluated per animal and classified for motility into: type A: motile, with progressive trajectory; type B: motile, with non-progressive trajectory; and type C: immotile [36].

The cauda sperm suspensions used for the artificial insemination were diluted 1:10 with 10% saline formol. To analyze the sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min after which 200 spermatozoa per animal were evaluated for head and/or flagellar defects by phase-contrast microscopy (x400, total magnification) [37].

2.5. Statistical analysis

For comparison of results among the experimental groups, statistical tests for analysis of variance (ANOVA) were utilized with the *a posteriori* Tukey-Kramer test or the nonparametric Kruskal–Wallis test with the *a posteriori* Dunn test or Qui-Square test, according to the characteristics of each variable. Differences were considered significant when $p < 0.05$. The statistical analyses were performed by the software GraphPad InStat (version 3.02).

3. Results

3.1. Experiment 1

Neither of the experimental groups presented a significant alteration in body weights between the control and treated groups under different doses of TCDD in adult male offspring of both generations. The absolute weights of reproductive organs also did not differ, as shown in Table 1.

Serum testosterone levels were significantly diminished in generation F1 at the highest dose (1.0 μg of TCDD), but this alteration was not maintained in generation F2. In relation to the F3 generation, there was a sharp but non-significant diminution as shown in Fig. 2. The serum levels of FSH and LH did not present significant alterations among the experimental groups in all the generations (Fig. 2).

The sperm transit time was also similar between the control and treated groups, but in generation F1 there was a reduction (acceleration) of transit time in the cauda epididymis of animals exposed *in utero* to 1.0 μg of TCDD in comparison to the control group, but the difference was not statistically significant ($p = 0.07$) as displayed in Fig. 3. This reduction in transit time (acceleration) was not observed in the subsequent generations (Fig. 3).

As to the number of mature spermatids in the testicle and DSP, there was no statistically significant difference between the four experimental groups, in all the generations, as shown in Table 2. In the epididymis the number of spermatozoids in the head regions was also similar between the control and treated groups, as were the sperm reserves in the cauda region in both generations (Table 2).

3.2. Experiment 2

The number and proportion of reabsorptions by implantation sites (%), did not differ between the experimental groups in generations F1 or F2. In generation F3 this analysis was not performed, since the laparotomy was carried out on DG9 (Table 3).

In generation F1 the proportion of implants per corpus luteum (I/CL) was reduced statistically at the doses of 0.5 (9.37%, I/CL = 61.95%) and 1.0 μg (21.95%, I/CL = 58.70%) of TCDD when compared to the control group (I/CL = 75.21%), as shown in Table 3. In generation F2, this reduction was statistically significant at the dose

Table 1
Final body weight and absolute reproductive organ weights of adult male offspring control and TCDD-exposed groups in the three generations.

Parameters	Control	Experimental Groups		
		0.1 μg/kg	0.5 μg/kg	1.0 μg/kg
F0 generation				
Final Body weight (g)	404.28 ± 17.22	409.86 ± 14.92	422.16 ± 11.96	398.84 ± 23.19
Absolute organ weights				
Testis (g)	1.60 ± 0.02	1.61 ± 0.04	1.62 ± 0.03	1.61 ± 0.03
Epididymis (mg)	591.54 ± 18.65	589.67 ± 15.13	576.74 ± 14.53	564.14 ± 16.91
Ventral prostate (mg)	372.11 ± 42.46	421.31 ± 17.43	430.56 ± 29.64	387.36 ± 29.97
Vas deferens (mg)	82.16 ± 3.71	79.77 ± 3.91	83.12 ± 4.24	85.66 ± 5.27
Seminal vesicle, with secretion (g)	1.04 ± 0.11	1.08 ± 0.10	1.18 ± 0.11	1.03 ± 0.11
Seminal vesicle (mg)	496.12 ± 62.83	453.56 ± 35.65	519.44 ± 40.18	481.54 ± 44.82
N	9	9	10	10
F1 generation				
Final Body weight (g)	451.93 ± 23.23	454.32 ± 20.75	458.18 ± 10.42	459.96 ± 22.45
Absolute organ weights				
Testis (g)	1.77 ± 0.05	1.63 ± 0.09	1.67 ± 0.02	1.78 ± 0.05
Epididymis (mg)	606.28 ± 21.54	609.85 ± 22.90	598.85 ± 15.88	635.07 ± 27.36
Ventral prostate (mg)	439.70 ± 37.97	540.23 ± 35.17	488.70 ± 48.63	575.83 ± 24.17
Vas deferens (mg)	90.61 ± 4.40	89.58 ± 5.97	86.49 ± 2.48	97.23 ± 2.42
Seminal vesicle, with secretion (g)	1.22 ± 0.12	1.21 ± 0.12	1.12 ± 0.10	1.32 ± 0.06
Seminal vesicle (mg)	543.37 ± 32.63	573.86 ± 40.83	601.47 ± 31.29	691.49 ± 56.72
N	9	8	10	7
F2 generation				
Final Body weight (g)	373.93 ± 10.93	398.86 ± 20.52	396.80 ± 13.52	390.90 ± 11.50
Absolute organ weights				
Testis (g)	1.57 ± 0.03	1.69 ± 0.04	1.55 ± 0.06	1.64 ± 0.04
Epididymis (mg)	557.75 ± 17.92	577.12 ± 9.99	526.99 ± 14.71	519.86 ± 19.66
Ventral prostate (mg)	402.08 ± 26.53	374.29 ± 46.30	377.99 ± 27.11	348.12 ± 19.63
Vas deferens (mg)	82.71 ± 4.14	84.02 ± 3.25	87.68 ± 6.47	76.98 ± 4.57
Seminal vesicle, with secretion (g)	1.16 ± 0.05	1.82 ± 0.09	1.20 ± 0.05	1.61 ± 0.06
Seminal vesicle (mg)	480.53 ± 31.49	521.53 ± 50.14	490.31 ± 20.49	430.26 ± 43.05
N	9	7	8	6

Values expressed as mean ± SEM. ANOVA test with an a posteriori Tukey test. $p > 0.05$.

Table 2
Sperm counts in the adult male offspring of control and TCDD-exposed groups in the three generations.

Parameters	Control	Experimental groups		
		0.1 μg/kg	0.5 μg/kg	1.0 μg/kg
F0 generation				
Sperm number in the testis ($x10^6$)	190.10 ± 12.61	198.75 ± 10.88	200.05 ± 13.69	201.81 ± 18.21
Daily sperm production ($x10^6$/testis/day)	31.16 ± 2.07	32.58 ± 1.78	32.79 ± 2.44	33.08 ± 2.99
Sperm number in the caput/corpus epididymis ($x10^6$)	115.36 ± 6.01	108.98 ± 6.53	112.89 ± 5.40	121.31 ± 7.69
Sperm number in the cauda epididymis ($x10^6$)	245.18 ± 13.32	237.34 ± 14.10	239.66 ± 10.02	207.67 ± 11.65
N	7	8	8	7
F1 generation				
Sperm number in the testis ($x10^6$)	223.63 ± 8.46	219.26 ± 10.94	203.47 ± 10.30	225.63 ± 18.34
Daily sperm production ($x10^6$/testis/day)	33.66 ± 1.39	35.94 ± 1.79	33.36 ± 1.69	36.99 ± 3.01
Sperm number in the caput/corpus epididymis ($x10^6$)	118.66 ± 7.02	128.60 ± 7.561	134.66 ± 6.50	138.41 ± 11.93
Sperm number in the cauda epididymis ($x10^6$)	251.55 ± 35.86	284.52 ± 16.27	236.30 ± 23.05	275.71 ± 28.85
N	9	7	10	7
F2 generation				
Sperm number in the testis ($x10^6$)	198.93 ± 9.61	208.72 ± 5.46	197.85 ± 8.34	216.50 ± 10.11
Daily sperm production ($x10^6$/testis/day)	32.61 ± 1.58	34.22 ± 0.90	32.44 ± 1.37	35.49 ± 1.66
Sperm number in the caput/corpus epididymis ($x10^6$)	108.58 ± 7.15	110.10 ± 9.38	114.83 ± 10.65	116.29 ± 9.50
Sperm number in the cauda epididymis ($x10^6$)	220.44 ± 10.33	279.87 ± 12.63	212.52 ± 13.74	227.79 ± 16.97
N	9	7	8	7

Values expressed as mean ± SEM. ANOVA test with an a posteriori Tukey test. $p > 0.05$.

of 0.1 μg/kg of TCDD (33.59%, I/CL = 41.11%) in relation to the control group (I/CL = 61.90%). At the dose of 0.5 μg/kg of TCDD this reduction was approximately 18.48% (I/CL = 50.46%), but not significant. Although the group receiving the highest dose also did not present a statistically significant reduction, there was a diminution of 29.65% (I/CL = 43.55%) ($p = 0.06$) when compared to the control group, as shown in Table 3.

However, in generation F3 this reduction was statistically significant in all the experimental groups in relation to control group (I/CL = 82.35%), being approximately 30.47% (I/CL = 50.67%); 31.29% (I/CL = 56.58%) and 61.36% (I/CL = 31.82%) at the doses of 0.1; 0.5 and 1.0 μg/kg of TCDD, respectively (Table 3).

The evaluation of sperm motility demonstrated that, among the experimental groups, in both generations, there was no statistically significant difference as to the number of Type A spermatozoids: mobile with progressive trajectory: Type B: mobile with non-progressive trajectory, and Type C: immobile (Fig. 4).

Table 3
Fertility parameters after *in utero* artificial insemination of adult male offspring control and TCDD-exposed groups in the three generations.

Parameters	Control	Experimental groups		
		0.1 μg/kg	0.5 μg/kg	1.0 μg/kg
F0 generation				
Body weight of dams (g) (mean ± SEM)	342.17 ± 10.48	320.11 ± 17.20	333.39 ± 17.07	321.91 ± 12.68
N	9	7	9	7
Implantation sites				
Total (N)	91	58	70	54
Implants per corpora lutea (%)	75.21	68.24	*61.95	*58.70
Number of implants (mean ± SEM)	10.22 ± 1.02	8.29 ± 1.76	7.67 ± 1.09	7.71 ± 1.27
Number of corpora lutea (mean ± SEM)	13.44 ± 0.80	12.14 ± 0.74	12.56 ± 0.44	12.43 ± 0.37
Resorptions				
Total (N)	29	19	24	17
Per implantation sites (%)	68.13	67.24	65.71	68.52
Number of resorption (mean ± SEM)	3.22 ± 0.60	2.71 ± 0.97	2.67 ± 0.88	2.43 ± 0.81
Fetal body weight (mean ± SEM)	2.86 ± 0.08	3.00 ± 0.06	2.99 ± 0.14	3.22 ± 0.32
F1 generation				
Body weight of dams (g) (mean ± SEM)	313.18 ± 11.77	317.98 ± 15.71	314.66 ± 12.18	333.08 ± 6.30
N	6	7	8	5
Implantation sites				
Total (N)	39	37	55	27
Implants per corpora lutea (%)	61.90	*41.11	50.46	**43.55
Number of implants (mean ± SEM)	6.50 ± 1.54	5.14 ± 0.80	6.11 ± 1.11	5.40 ± 1.15
Number of corpora lutea (mean± SEM)	10.50 ± 0.89	12.86 ± 0.40	12.11 ± 0.92	12.40 ± 0.51
Resorptions				
Total (N)	6	10	13	10
Per implantation sites (%)	84.62	72.97	76.36	62.96
Number of resorption (mean ± SEM)	1.00 ± 0.36	1.42 ± 0.30	1.44 ± 0.67	2.00 ± 0.55
Fetal body weight (mean ± SEM)	3.51 ± 0.28	3.62 ± 0.34	3.76 ± 0.28	3.44 ± 0.43
F2 generation				
Body weight of dams (g) (mean ± SEM)	243.70 ± 8.80	242.24 ± 7.29	229.83 ± 9.16	230.82 ± 14.09
N	6	7	8	6
Implantation sites				
Total (N)	56	38	43	21
Implants per corpora lutea (%)	82.35	*50.67	*56.58	*31.82
Number of implants (mean ± SEM)	9.33 ± 1.36	5.43 ± 0.92	5.63 ± 1.24	*3.50 ± 0.92
Number of corpora lutea (mean± SEM)	11.33 ± 0.80	10.71 ± 0.36	10.63 ± 0.38	11.00 ± 0.58

N, number. Proportions (%) were compared by the Chi-square. Values expressed as mean ± SEM were compared by ANOVA test with an a posteriori Tukey test.
* $p < 0.05$.
** $p = 0.06$.

Table 4
Sperm morphology in the adult male offspring of the control and TCDD-exposed groups in the three generations.

Parameters	Control	Experimental groups		
		0.1 μg/kg	0.5 μg/kg	1.0 μg/kg
F0 generation				
Normal sperm (%)	98.36	97.60	97.09*	96.89*
N	7	10	11	9
F1 generation				
Normal sperm (%)	98.22	98.28	97.90	97.31
N	9	9	10	8
F2 generation				
Normal sperm (%)	98.77	98.54	98.90	97.94
N	9	7	10	8

Data expressed as Qui-square test.
* $p < 0.05$.

In relation to sperm morphology, the percentage of morphologically normal spermatozoids was significantly diminished in the treated groups with 0.5 μg/kg and 1.0 μg/kg of TCDD (97.09% and 96.89%, respectively) in relation to control group (98.36%) in generation F1, as shown in Table 4. Yet generations F2 and F3 showed no significant statistical differences between the control and treated groups at different TCDD doses (Table 4).

4. Discussion

Several chemical environmental contaminants have been identified as having anti-androgenic and/or estrogenic activity, thus acting as endocrine disruptors [38,39]. Laboratory experiments have shown that exposure *in utero* to endocrine disruptors can provoke the appearance of chronic diseases and compromise reproductive function throughout the life of the individual [40–42].

TCDD, a congener of dioxin, is considered the most toxic environmental contaminant [38], is capable of inducing several adverse effects on human health [26,43,44], exerts estrogenic, anti-estrogenic and anti-androgenic activities, depending on dose and time of exposure [45], and is able to perturb the normal development of the hypothalamus, leading to alterations in sexual differentiation [46]. It is known that this compound can be transferred to developing organisms through the placenta or by lactation [6], although we have not measured TCDD in milk.

In the present study, we investigated the effects of developmental exposure to TCDD in the male genital system, with emphasis on epididymal functions and sperm quality at adulthood and whether these effects are transmitted to the next generations. In adult male offspring in F1 generation, the TCDD promoted alterations in sperm transit time and sperm morphology, as well as diminutions in serum testosterone levels. The numbers of implantations per corpus luteum after intrauterine artificial insemination were decreased in the three generations, showing that the fertility of

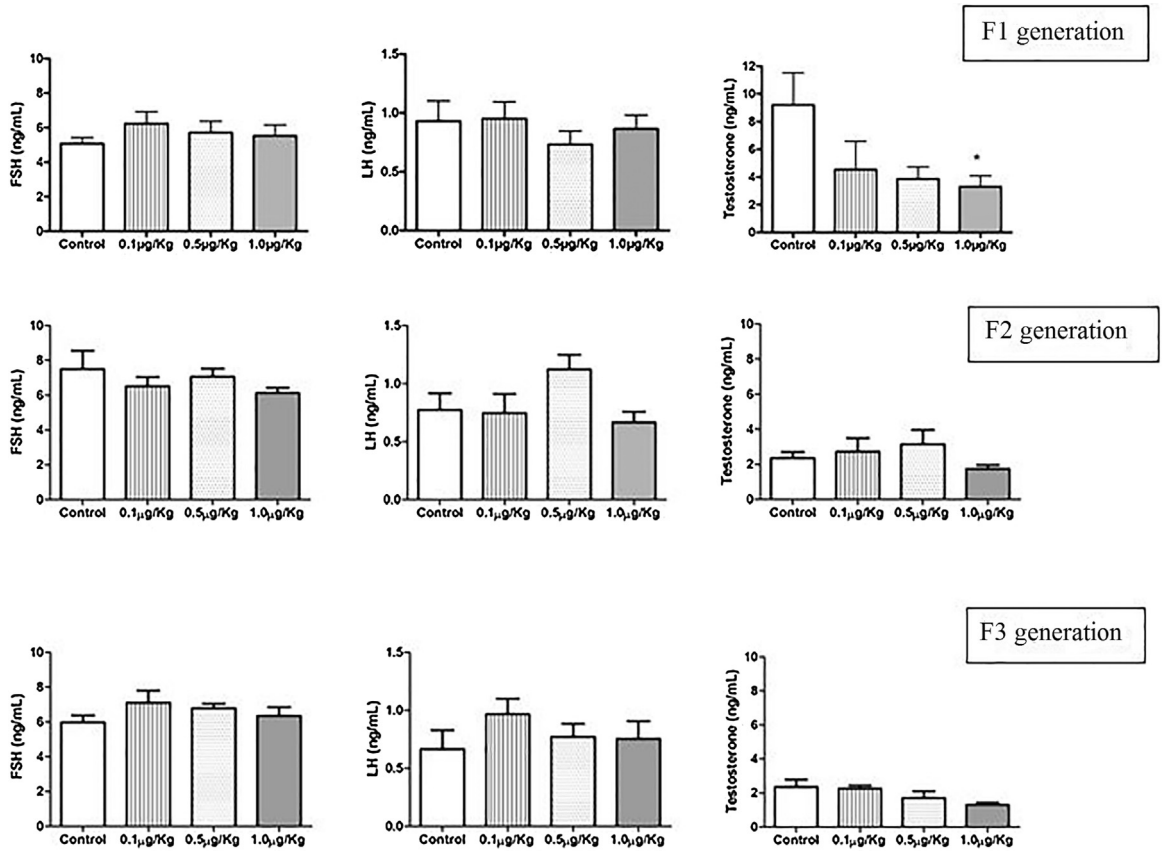

Fig. 2. Serum sexual hormone concentrations (ng/mL) in the adult male offspring of the control and TCDD-exposed groups in the three generations. Data expressed as mean ± SEM. ANOVA test with an a posteriori Tukey test. *$p < 0.05$.

these animals was compromised, especially at the higher doses of TCDD (0.5 and 1.0 μg) indicating a possible dose-response relationship.

The body weights of male offspring in adulthood (DPN90) and the weights of the reproductive organs remained similar, suggesting that TCDD did not compromise the general health status of these animals, corroborating the results of Ohsako et al. [17] obtained from pregnant Holtzman rats treated with TCDD at a dose of 12.5, 50, 200 or 800 ng/kg, under an experimental design similar to that employed in the present work.

In a study carried out by Faqi et al. [6], a 50% reduction of testosterone was observed in adult male offspring whose mothers had been exposed (s.c.) at 300 ng/kg prior to mating and during the period of pregnancy and lactation, followed by a weekly maintenance dose of 60 ng/kg (300/60 ng of TCDD), results similar to those found by [10,11] corroborating the data from the present study that presented expressive effects on serum testosterone levels in generations F1 and F3. The concentrations of FSH and LH in the present study were similar between the control and TCDD-treated animals in the three generations, corroborating data from the literature [10,14,17,23].

There was a reduction in serum testosterone and no changes in LH and FSH levels. It is known that the Leydig cells, *in vivo*, are programmed to release testosterone not only as a function of LH stimulation but also in response to various local paracrine factors most likely related to Sertoli cell function [47]. This would imply that Leydig cells are not exposed through the testicular interstitial fluid to LH pulses as occurs in circulation, and therefore, it would not be possible to 'read' these pulses, indicating no immediate relationship between LH peaks and testosterone response [48].

Mammalian spermatozoa, when they leave the testes, are functionally immature cells that necessitate a maturation process during the passage through the epididymis, to acquire the capacity to recognize and fertilize the oocyte [49]. During transit through the epididymis, many morphological, physiological and biochemical characteristics of the spermatozoa are modified, as part of the maturation process [50]; thus, some alteration during this process can provoke qualitative damage in this gamete [51].

Faqi et al. [6] reported that *in utero* and lactational exposure to TCDD (an initial dose of 25–300 ng/kg followed by 5–60 ng/kg of TCDD throughout mating, pregnancy and lactation) diminished the transit time of sperm through the epididymis in Wistar rats, corroborating the data of the present study, which presented a reduction (acceleration), although non-significant, in the transit time through the cauda epididymis of the animals exposed to 1.0 μg of TCDD. This rapid passage of spermatozoids through the epididymis promotes minimal exposure of the gametes to the epididymal microenvironment, which is normally associated with processes of post-testicular maturation [52]; however, this was observed only in generation F1. The present study also reaffirms the important role of testosterone in epididymal function during the maturation and fertile-capacity acquisition of spermatozoids, since the serum testosterone levels were also altered, confirming that the acceleration in sperm transit time is dependent on androgen [53].

The number of spermatids present in the testicle and the DSP are important indicators of male fertility potential. Faqi et al. [6] observed a 50% reduction of DSP in adult male offspring exposed *in utero* to the TCDD concentrations of 25, 60 or 300 ng/kg. In relation to the number of spermatozoids in the epididymis, Ohsako et al. [18] verified a diminution in adult male offspring exposed *in utero* to 1.0 μg/kg of TCDD, similar to results reported in other studies

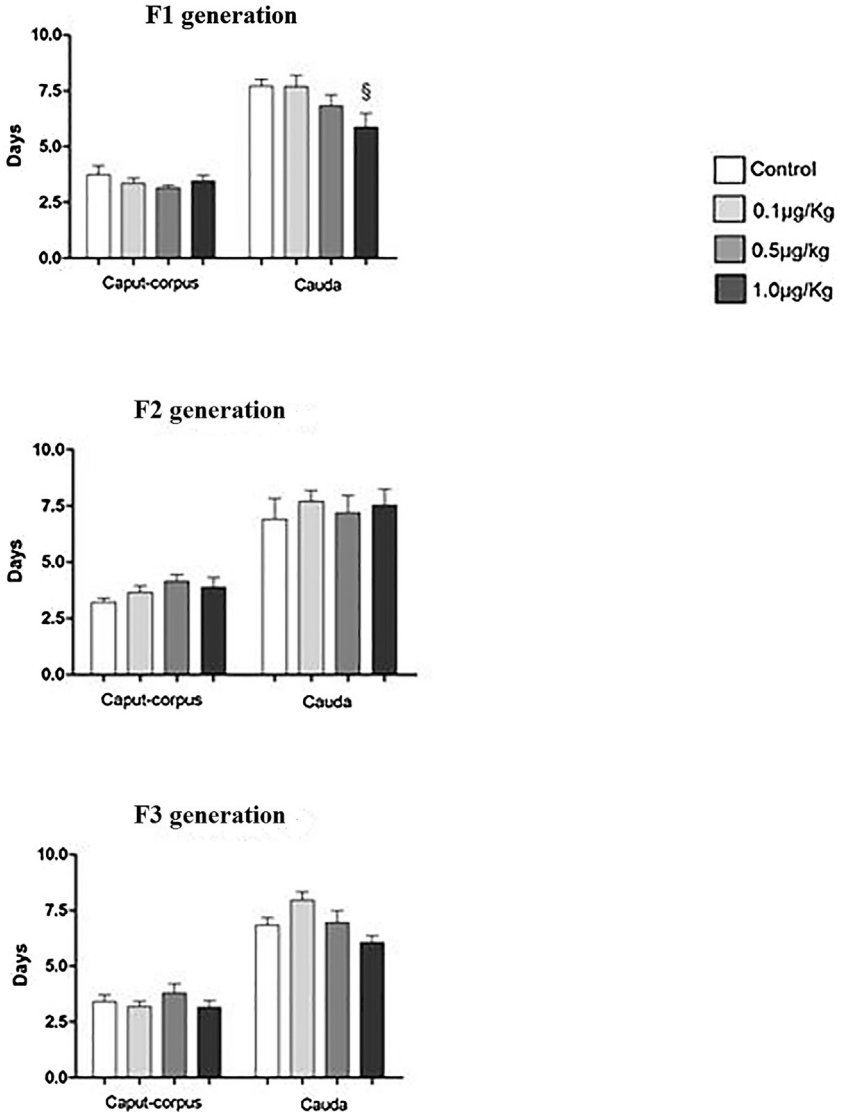

Fig. 3. Sperm transit time in caput-corpus and cauda epididymis in the adult male offspring of the control and TCDD-exposed groups in the three generations. Data expressed as mean ± SEM. ANOVA test with an a posteriori Tukey test. §p = 0.07.

[12,14,23]. In contrast to the previously cited studies, none of the three TCDD doses tested in the present work (0.1; 0.5 and 1.0 μg/kg) presented effects on sperm parameters such as the production and storage of spermatozoids.

Intrauterine artificial insemination was employed to evaluate sperm quality, given that this technique excludes the influence of sexual behavior and the number of gametes available for ejaculation in a fertility test [33]. In the present work, after this procedure, the number of reabsorptions and the proportion of reabsorptions per implantation site (directly related to post-implantation losses) were similar between the experimental groups in both generations. Corroborating our result, Bell et al. [54] observed an absence of toxic effects in relation to the number of reabsorptions in female rats treated with TCDD on DG15, at the doses of 50, 200 and 1000 ng/kg, indicating that TCDD does not induce an increase in fetal mortality and consequently in post-implantation losses.

After intrauterine artificial insemination was performed, the animals treated with 0.5 μg/kg and 1.0 μg/kg of TCDD showed a diminution of implants per corpus luteum in relation to controls in generation F1. In the F2 generation, this alteration was observed only at the dose of 0.1 μg/kg, although at the highest dose there was also a non-significant reduction. However, in generation F3 this reduction occurred in all the TCDD-treated groups when compared with the controls, similar to results reported by Gray et al. [15] in male Long Evans Hooded rats exposed to 1.0 μg/kg of TCDD *in utero*, in which they observed a fertility reduction of approximately 50%, measured by the number of implantations per female mated, suggesting that TCDD diminished the fertility capacity of spermatozoids and/or caused death in the first stages of development. Nevertheless, this result was not observed in other studies [6,10,55].

Murray et al. [56] reported diminished fertility only in generations F2 and F3 of Sprague-Dawley rats exposed to 0.01 μg TCDD/kg/day in the diet, however, in this dosage did not observe any in fertility effect in male offspring in generation F1, contrary to the observations in the present study, given that this reduction occurred in all generations, under at least one TCDD dose tested. These differences may be due the bioavailability of TCDD to the fetus differ depending upon whether TCDD was administered as a single dose ("pulse"), as in the current study, or by long-term low doses exposure, resulting in steady state conditions. However, both experimental conditions, reduces fertility throughout life in exposed rat progeny to low doses of TCDD [16,56].

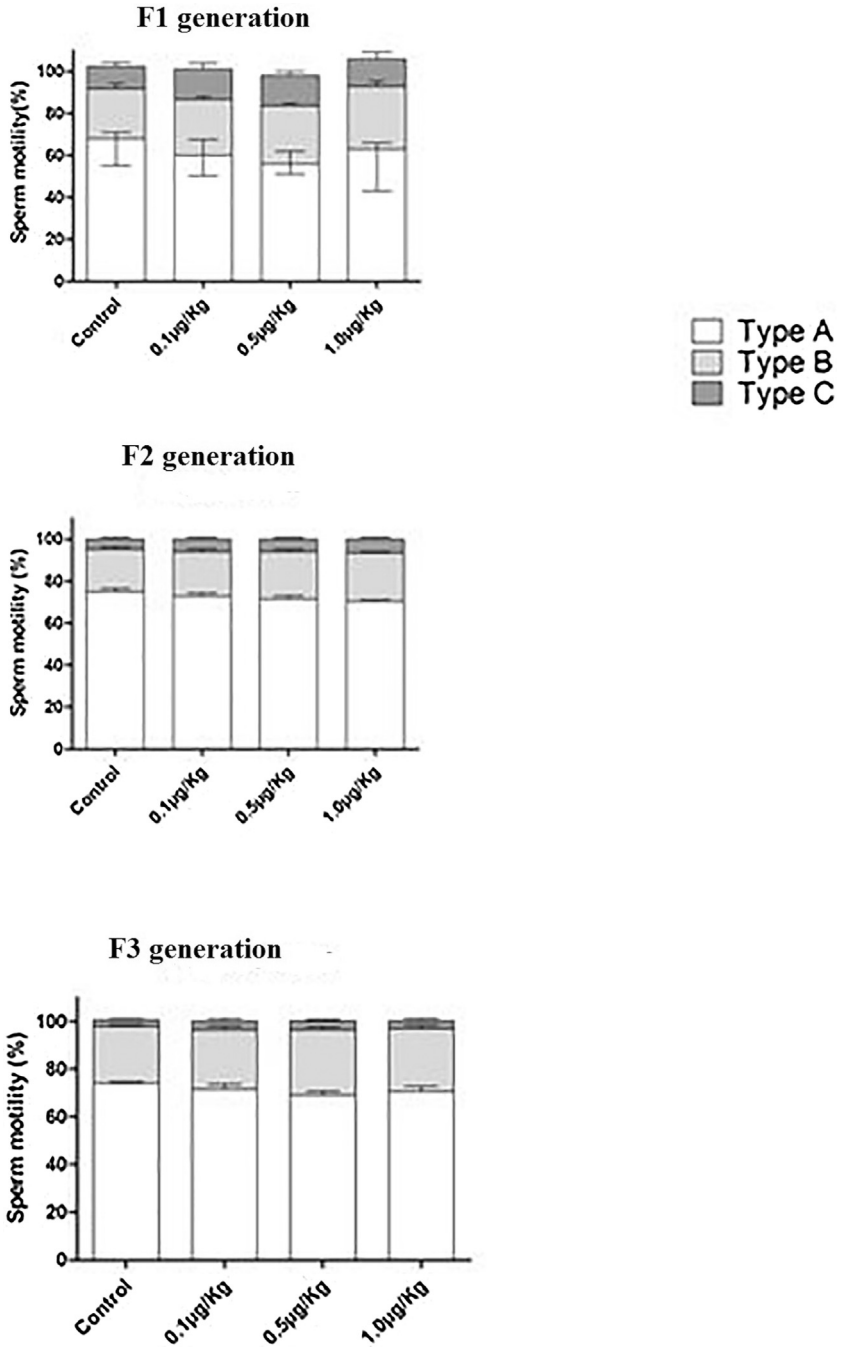

Fig. 4. Sperm motility in the adult male offspring of the control and TCDD-exposed groups in the three generations. Data expressed as median. Kruskall-Wallis test with an a posteriori Dunn test. $p > 0.05$.

However, in a study carried out by Bruner-Tran et al. [57] with adult female mice exposed *in utero* to 10 μg/kg TCDD also exhibited reduced fertility in three-generation study, corroborating our results. Widely reported in the literature is a dose-response relationship in the reproductive function of rats exposed to TCDD doses during the intrauterine period [16,17,55,56,58], which may be related to its effects on the I/CL and consequently on the fertility of the animals from the present study.

Sperm motility is one of the most important parameters utilized in the qualitative evaluation of sperm from semen samples obtained *in vitro* [59] and *in vivo* [60,61]. Bell et al. [54] verified that exposure of pregnant Wistar rats at DG15 to 1.0 μg/kg of TCDD did not provoke alterations in sperm motility in their adult male offspring, corroborating the data of the present work.

Another important parameter in the evaluation of male fertility is the analysis of sperm morphology [62]. An increase in the percentage of spermatozoids presenting morphological abnormalities can be considered evidence that the toxicant in the study had access to germ cells. By being one of the less variable sperm parameters among normal individuals, morphology has been utilized as indicative of a spermatotoxic event [63]. The results from the literature show that *in utero* exposure to TCDD did not provoke alterations in this parameter [12]; in contrast, Faqi et al. [6] after exposure initial loading dose of 25, 60, or 300 ng TCDD/kg body wt, followed by a weekly maintenance dose of 5, 12, or 60 ng TCCD/kg

body wt, subcutaneously 2 weeks prior to mating and throughout mating, pregnancy, and lactation, were observed significant effects in the percentage of morphologically abnormal sperm confirming the results of our present study. This parameter is used to enhance the identification of sperm toxicity and indicates that the given toxicant has gained an access to the germinal cells [64]. The similarities between the effects observed in the present study and those conducted by other investigators may be related to the mode of TCDD exposure. Bruner-Tran et al. [57], in a study of mice exposed in utero to 10 μg/kg TCDD, in three generations of study also observed significant alterations in these endpoints, confirming our results, demonstrating transgenerational impact inherited through the paternal germ line.

As previously reported, the F3 generation had no direct exposure to TCDD. A gestating female (F0 generation) exposure to an environmental compound results in the F1 generation embryo and F2 generation germ-line being directly exposed, such that the F3 generation is the first not directly exposed to the environmental compound [65]. Any mechanism describing F3 toxicity must therefore account for the heritability of the effects [9]. One possible mechanism would be chromatin modifications that are replicated in the DNA, e.g. changes in DNA methylation, histone modification, non-coding DNA, or some other covalent or non-covalent effect that ultimately causes an alteration in the expression of genes that are critical for reproduction. The biological phenomenon involved in this reproductive toxicology deals with embryonic gonadal development and germ-line differentiation, or postnatally the gametogenesis process and germ cell development. The ability of an environmental compound, (e.g. TCDD) to promote this reprogramming of the germ-line appears to be the causal factor in the epigenetic transgenerational phenotype [65]. Therefore, TCDD exposure can alter cell differentiation and promote transgenerational effects [8].

The half-life of TCDD is parameter important about results from animal studies. The half-life of dioxin ranges from 5.8 to 14.1 years in humans and is influenced by body composition, with higher body fat associated with a longer half-life [66–68]. By comparison, the half-life ranges from 10 to 15 days in mice [69] and is approximately 3 weeks in rats [70]. This may explain the high variation of literature available about differences in sensitivity to TCDD in the different species (e.g., rats vs. mice). The half-life of TCDD associated a difference in dosing protocol, as well as a strain difference, may (at least partly) explain the differences in sensitivity to TCDD, therefore, in the differences in the results found in mice when compared to the rats [71].

Taking into account that the reproductive potential of rats is higher compared to other animals including men, the impairment of fertility capacity showed in the present study, after prenatal and lactacional exposure to TCDD, can indicate risks for human fertility, although the mechanism by which this occurs is not understood and need to be more deeply investigated.

In conclusion the results of this study indicate that exposure to TCDD, under these experimental conditions, negatively interferes in the rat epididymal processes, harming sperm quality and ability of animals to generate offspring and this effects persist into the next generation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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