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Protective effect of resveratrol on urogenital sinus and prostate development in rats exposed *in utero* to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)

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ABSTRACT

This study evaluated the protective effects of resveratrol on the prostate development of rats exposed to TCDD. Pregnant rats received TCDD (1 μg/kg) at GD15 and/or RES (20 mg/kg/day) from GD10 to PND21. Newborn and adult males from Control, TCDD, TCDD + RES and RES groups were euthanized and the prostate was excised. On PND1, there was a reduction in the number of prostatic buds, AR-positive mesenchymal cells and proliferation index in epithelial and mesenchymal cells in TCDD group, but restored by RES. AhR immunoreactivity was greater in TCDD group than the other groups. On PND90, there was higher frequency of functional hyperplasia in the distal area of the prostate acini in TCDD group, but restored by RES. AhRR expression was higher in the TCDD while NRF2 was higher in the TCDD + RES compared to the other groups. Resveratrol was able to reduce the adverse effects of TCDD on prostate development and its long-term repercussions.

1. Introduction

Dioxins are environmental molecules with highly contaminant effects that are almost mainly generated as byproducts of industrial processes, including incineration of medical waste and plastics, chlorine bleaching of paper, and the manufacture of some pesticides, herbicides, and fungicides [1–8]. Small amounts of these contaminants are also found in areas where natural combustion and geological processes occur [9–11]. These are lipophilic compounds that are resistant to biological and environmental degradation, making them persistent in the environment, which leads to bioaccumulation and the biomagnification of dioxins in the food chain [12]. Thus, human exposure is mainly through the consumption of contaminated high-fat foods such as milk, cheese, meat, some fish and breast milk [13–17].

2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD), the dioxin that acting as a xenobiotic agent can produce toxicity and cancer [8,18], is the most toxic among dioxins. It exerts its effects by high-affinity binding to a specific cellular protein known as aryl hydrocarbon receptor (AhR), an intracellular ligand-activated transcription factor ubiquitously expressed in many tissues and cell lines [19–21]. The high affinity binding properties of TCDD to the aryl hydrocarbon receptor (AhR) have linked the toxic and carcinogenic effects of the dioxin to this receptor [22]. In the absence of ligand, AhR is in the cytoplasm complexed with HSP90, X-associated protein 2 (XAP2), and HSP90 cochaperone p23 [23]. Upon ligand binding, the AhR complex translocates into the nucleus and dissociates to form a heterodimer with a AhR Nuclear Translocator (ARNT), which recognizes an enhancer DNA element known as dioxin responsive element (DRE) sequence located in the promoter region of several genes [22,23]. However, if aryl-hydrocarbon receptor repressor (AhRR) interacts with the AhR/ARNT-TCDD complex, the transcription is inhibited [24,25]. In human and animal studies, TCDD exposure has been linked to several biochemical and toxicological effects, including immunosuppression [26], impaired neurodevelopment [27,28], tumor promotion [22], teratogenesis, thyroid dysfunction [27,28] and reproductive/developmental abnormalities [22].

In utero TCDD exposure resulted in decreased anogenital distance in male rat offspring, a delay in the descent of the testis, a reduction in testicular parenchyma weight, a reduction in spermatogenesis, a decreased response to androgen stimulation in the adult prostate, and a reduction in accessory glands weight [29–33]. In Holtzman rats, one of the most sensitive effects of *in utero* and lactational (400 ng/kg – before

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mating; 80 ng/kg – during mating, pregnancy and lactation) exposure to TCDD was a reduction in ventral prostate weight [34]. In pregnant Sprague-Dawley rats, TCDD were administered on gestation day 15 (GD15) in three different doses (0.5-1.0–2.0 μg/kg) and there was a reduction in the prostate, seminal vesicle, epididymis and testis weights when higher doses of TCDD (1.0 and 2.0 μg/kg) were administered [35]. Also, TCDD can cause abnormal prostate development in rats and mice [36].

In C57BL/6 mice, a single maternal dose (5 μg/kg on GD 13) reduced ventral, dorsolateral, and anterior prostate weights, it altered androgen-dependent gene expression, and inhibited ductal morphogenesis and branching. Furthermore, the ventral surface of the fetal urogenital sinus (UGS) was devoid of epithelial projections, showing that TCDD interferes with prostatic budding by acting in the elongation stage of the buds [37], since AhR signaling is more abundant in this UGS specific region [38]. Moreover, *in utero* and lactational exposure to TCDD (5 μg/kg on GD 13) resulted in prostate pre-neoplasic lesions in senescent C57BL/6 J mice, which are not naturally susceptible to developing prostate cancer [39]. Thus, AhR activation by TCDD can increase the risk of prostate cancer in adulthood in rodent models susceptible to prostatic carcinogenesis [40].

Resveratrol (RES or 3,5,4′-trihydroxystilbene) is a natural polyphenol found in at least 72 plant species (in 32 genera and 12 families), such as peanuts, cranberries, blueberries, and especially in grape skins and red wine [41,42]. This natural compound, an AhR antagonist, has antitumor, platelet antiaggregation, anti-inflammatory, antiallergic, antioxidant and estrogenic activities [43,44]. In addition, Resveratrol acts on three carcinogenesis stages (initiation, promotion and progression) through the modulation of several signal transduction pathways, such as those that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis [41,42].

The anticancer property of Resveratrol has been supported by studies indicating that it inhibits proliferation of a wide variety of human tumor cells *in vitro*, such HL60, Hepa 1c1c7, BP*C1 and others [45–47]. Thus, these data have led to numerous preclinical animal studies to evaluate the potential of Resveratrol for cancer chemoprevention and chemotherapy [48].

There are few studies relating practical interventions that prevent and/or reverse the toxic effects of TCDD and its congeners. However, since its toxic effects are AhR-mediated, the effective blocking TCDD-AhR interactions, which would be an effective way to inhibit TCDD toxicity. Thus, resveratrol is a polyphenolic compound with an AhR antagonist activity and that has anti-inflammatory, antioxidant and anti-cancer properties that might to inhibit TCDD adverse actions [49–53]. Some *in vivo* studies have shown that resveratrol can mitigate the toxic effects induced by *in utero* TCDD exposure in male and female reproductive organs, including reduction in breast cancer susceptibility [54–56].

The aim of this study was to evaluate the possible chemoprotective activity of resveratrol during gestation and lactation on prostate development in rats exposed *in utero* to TCDD and to clarify whether resveratrol could prevent possible changes related to exposure to TCDD in adulthood.

2. Materials and methods

2.1. Animals and experimental conditions

Animals were handled in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation (COBEA), and the experiment was approved by the Committee for Ethics in Animal Experimentation of the Institute of Biosciences, UNESP, Botucatu-SP, Brazil (Protocol no. 477-CEUA).

Outbred male and female Wistar rats were obtained from colonies maintained under specific pathogen-free conditions in the Central Bioterium of Botucatu (Botucatu-SP, Brazil). All animals were kept in polypropylene cages with autoclaved white pine shavings, which was used as bedding material, and maintained under controlled environmental conditions (temperature: 22 ± 2 ∘C; relative humidity: 55 ± 20%; 12/12-h light–dark cycle; and continuous air ventilated). All animals received *ad libitum* access to Nuvilab CR-1 commercial chow (Nuvital, PR, Brazil) and glass bottles containing filtered drinking water. The polypropylene cages were cleaned manually and dried at room temperature to avoid exposure to high temperatures.

2.2. Experimental design

After a 2-week acclimation period, 12-week-old female Wistar rats were mated with 12-week-old male Wistar rats by placing 2 females in a cage with 1 male. Mating was realized during the dark period of the cycle and gestational day 0 (GD0) was determined by the presence of sperm on the vaginal smears of female rats in estrus (sexually receptive) cycle. The amount of food and water consumed by the dams and the litters were weekly recorded.

On GD15, pregnant Wistar rats (n = 40) were orally treated with 1 μg/kg body weight of TCDD (4-8599, Supelco Analytical, Bellefonte, PA, USA) or corn oil (vehicle). TCDD (10 μg/mL of toluene) was solubilized in corn oil with the final concentration of 0.2 μg/mL. From GD10 to post-natal day 21 (PND21), dams (n = 40) received oral resveratrol (R5010, Sigma-Aldrich, St. Louis, MO, USA) by gavage (20 mg/kg body weight) [57] or ethanol 8% (vehicle). Resveratrol was dissolved in ethanol 8% following the manufacturer's recommendation (datasheet Sigma-Aldrich Co. LLC) [58]. Mothers were exposed to resveratrol until weaning due to long half-life of TCDD in biological organisms and because of its passage through cord blood and breast milk [59; 60]. Four groups (n = 10 dams/group) were constituted as follows: C, Control group (corn oil + ethanol 8%), TCDD (1 μg/kg TCDD + ethanol 8%), TCDD + RES (1 μg/kg TCDD + 20 mg/kg Resveratrol) and RES (corn oil + 20 mg/kg Resveratrol).

After birth, the litter size was standardized to 8 pups (the gender ratio was kept as close to 1:1 as possible) and the anogenital distance was measured for each litter in the groups. In litters of more than 10 pups, the extra animals were removed randomly. Litters with less than 8 pups were not considered for the experiment. Males and females chosen and ignored in this study were selected randomly. Newborns from different litters were not moved to balance litter sizes to maintain litter independence. In addition, after birth, we selected 2 males/litter for each group, which were assessed in two stages: PND1 to assess the immediate effects and PND90 to assess the latter effects. Females from this protocol were used to another experiment.

Newborn rats (PND1) were decapitated (n = 20 males/group; 2/litter), and blood was collected and the urethral pelvic region of each animal was collected for morphological and immunohistochemical analysis of the immediate effects of TCDD and/or resveratrol on prenatal prostate development and morphogenesis. After decapitation, at PND90, blood was collected for testosterone dosage, and the ventral prostate (n = 10 males/group; 1/litter) was collected for the analysis of effects of maternal TCDD and/or resveratrol on the adult prostate. In addition, fragments of the ventral lobe (n = 4/group) were frozen and stored at −80 ˚C for Western Blot analysis.

2.3. Serum hormonal assays

At PND1 (5 pools from 10 animals/group) and PND90 (n = 10 samples/group), blood samples from the ruptured cervical vessels were collected in a tube (additive free) at the time of euthanasia (between 8:30 and 11:30 am). The serum was separated by centrifugation (3000 rpm, 20 min, at 4 ˚C). Testosterone levels were determined by double-antibody radioimmunoassay, using specific kits provided by MP Biomedicals (Orangeburgh, NY, USA) at the Laboratory of Neuroendocrinology of Reproduction of School of Dentistry of Ribeirão Preto, University of Sao Paulo (USP). All the samples were dosed in the

same assay, to avoid inter-assay errors. The lowest detection limit was 0.064 ng/mL, with a 4% intra-assay variation.

2.4. Histology and stereological analysis

Fragments of the urogenital sinus (PND1) and the ventral prostate (PND90) were removed and fixed by immersion in methacarn [59] for 3 h and embedded in Paraplast®. The histological sections (5 µm) sections were produced in rotary microtome, collected in silanized slides and stored until ready to use. After microtomy, the sections were stained with hematoxylin-eosin (HE) for morphological and stereological analysis. The slides were analyzed and microscopic fields scanned using an image analyzer system (Axio-Vision) connected to a Zeiss AxioLabA1 light microscope.

At PND1, the urogenital sinuses (prostatic urethra) from 5 different animals were chosen randomly in all the groups (n = 5/group). These fragments of the prostatic urethra were vertically included in Paraplast® and serial sections were obtained and stained with HE to count the number of buds and for urogenital sinus morphology analyzes. All prostatic buds that emerged from the urethra were considered, regardless of the region they occupied in relation to the urethra. To count the number of buds, transversal sections from the entire length of the urethra were analyzed and only proximal parts of the prostatic buds were counted, as they emerged from the urethra.

A stereological analysis was performed in the ventral prostate, at PND90, as described by Weibel [60]. Then, 10 histological fields were analyzed per ventral prostate from 5 different animals chosen randomly in all the groups (n = 5 animals/group; 50 histological fields/group). Five histological sections per animal were observed and two histological fields per section chosen randomly were digitalized. These sections were provided from the distal part of the ventral prostate (approximately 50 µm far one each other). Thus, the relative proportion of the lumen, epithelium and stroma components were obtained for each group.

2.5. Immunohistochemistry

Urogenital sinus and ventral prostate sections were subjected to antigen retrieval in a humid environment at 100 °C in Tris/EDTA, pH9.0 for 30 min. After washing in distilled water, the sections were blocked for endogenous peroxidase (hydrogen peroxide at 3% in methanol) for 15 min. Then, the UGS and ventral prostate sections were covered with 3% BSA plus non-immunogenic goat serum (1%) for blocking unspecific bindings. After these, it was performed immunohistochemical reactions for cell proliferation marker (Ki-67 - clone ab16667 - Abcam®, Inc., USA), androgen receptor (AR – clone sc816 - Santa Cruz® Biotechnology, Inc., USA), alpha-smooth muscle actin (clone ab5694 - Abcam®, Inc., USA) and Aryl hydrocarbon receptor (AhR – clone ab84833 - Abcam®, Inc., USA). After overnight incubation with the primary antibody at 4 °C, sections were incubated for 2 h with HRP secondary antibody (IgG goat-anti rabbit, ab97051, Abcam® Inc., USA). Chromogen color development was accomplished with 3,3′-diaminobenzidine tetrahydrochloride (DAB - Sigma-Aldrich Co®., USA). The slides were counterstained with Harris' hematoxylin. Negative controls for IHC signals were performed by incubating the sections with phosphate buffered saline (PBS) instead of primary antibodies.

2.6. Mesenchymal AR-positive cells and cell proliferation index

Cell proliferation index was determined by dividing the number of Ki-67- positive cells (PND1: prostatic buds; PND90 epithelial cells from the acini) by the number of cells analyzed and finally the value multiplied by 100 for each animal in the different experimental groups. At the same way, mesenchymal AR-positive cells index on PND1 was determined by dividing the number of AR-positive cells by the number of cells analyzed and finally the value multiplied by 100 for each animal in the different groups. These parameters were analyzed in 25 microscopic fields per group chosen randomly (x40 objective lens for PND1 and x20 objective lens for PND90; 5 different animal/group; 5 histological fields per animal from different litters).

2.7. Western blotting (WB)

Ventral prostate (PND90) of the experimental animals (n = 4 samples/group) was rapidly removed and immediately frozen in liquid nitrogen and stored at −80 °C. For western blot assay samples were mechanically homogenized and processed as described by Peixoto et al. [61]. The nitrocellulose membranes were incubated, overnight at 4 °C, with the primary antibody to Androgen Receptor (AR - clone sc-816, Santa Cruz® Biotechnology, Inc., USA); aril hydrocarbon receptor repressor (AhRR - ab108518 - Abcam®, Inc., USA); nuclear factor (erythroid-derived 2)-like 2 (NRF2 - ab31163 - Abcam® Inc., USA); alpha-tubulin (clone sc398103 - Santa Cruz® Biotechnology, Inc., USA). The primary antibodies were diluted in 3% or 1% non-fat milk diluted in TBST (Tris-buffered saline, 0.1% Tween 20). After five washing cycles in TBST, membranes were incubated with specific HRP secondary antibody (IgG goat-anti rabbit, ab97051, Abcam® Inc., USA or IgG goat-anti mouse, ab97023, Abcam® Inc., USA) in 3% or 1% non-fat milk diluted in TBST for 2 h at room temperature. After five washing cycles in TBST, immunoreactive components were revealed by ECL Select chemiluminescent detection kit (GE Healthcare®). Protein expression was determined by semi-quantitative assays by densitometry of bands using Image *J* software (Version 1.33 u - National Institutes ofHealth, USA), normalized by α-tubulin density.

2.8. Statistical analysis

For all comparisons, the litter was used as the unit of measure into the experimental groups. Quantitative data were compared among the groups by Kruskal-Wallis test, followed by Dunn post test, using the software GraphPad Prism®. Furthermore, the data were expressed as mean ± SEM and the statistical difference among the groups were considered when $p \leq 0.05$.

3. Results

3.1. Gestational and lactational parameters

There were no differences in the maternal body weight gain and food and water consumption during pregnancy among groups, as well as the dam´s weight on the 20th day of gestation. After birth, the average number of pups per litter and the male/female ratio was examined and, in both parameters, they were similar among the groups (Data not shown).

During the lactational period, the maternal water and food consumption were also assessed. However, there was no statistically significant difference between these two parameters among the groups (Data not shown).

3.2. Post-natal day 1 (PND1)

3.2.1. Biometrical analysis and serum testosterone concentration

Biometric parameters of the each litter were evaluated on PND1. The body weight of males and females offspring was similar throughout the groups (Table 1). In addition, there was no significant difference in the anogenital distance and in the serum testosterone levels among the groups (Table 1).

3.2.2. Histological analysis of the urogenital sinus

The ventral part of the UGS consisted of prostatic buds or epithelial cellular cords that differentiate into prostatic ducts and acini during

Table 1
Quantitative parameters from different experimental groups on PND1.

Parameters	Group/Treatment			
	G1 Control	G2 TCDD	G3 TCDD + Res	G4 Res
Male weight (g)	6.90 ± 0.37	6.73 ± 0.21	6.87 ± 0.13	7.46 ± 0.24
Female weight (g)	6.486 ± 0.32	6.30 ± 0.20	6.00 ± 0.00	6.83 ± 0.21
Male AGD (mm)	4.34 ± 0.04	4.23 ± 0.06	4.27 ± 0.13	4.28 ± 0.08
Female AGD (mm)	2.35 ± 0.05	2.28 ± 0.06	2.30 ± 0.30	2.33 ± 0.11
Serum testosterone (ng/ml)	0.55 ± 0.19	0.46 ± 0.20	0.49 ± 0.23	0.59 ± 0.38

Values expressed as Mean ± SEM. AGD: anogenital distance.

development. As shown in Fig. 1, the prostatic buds developed around the initial part of urethra and within a mesenchyme that was rich in cells and poor in extracellular matrix in all groups. Furthermore, these epithelial buds developed distally to form prostatic lobes.

There was a reduction in the number of prostatic buds in the UGS from TCDD group compared to the Control group (Fig.1I). In addition, the TCDD + RES group showed a similar number of prostatic buds compared to the Control group. Histological sections HE-stained confirm these findings, where there were fewer prostatic buds in the UGS from TCDD group compared to the Control group, while this parameter was restored in TCDD + RES group (Fig. 1A–I).

3.2.3. Immunohistochemical analysis

As shown in Fig. 2A–D, androgen receptor (AR) immunostaining was intensive and frequent in the surrounding mesenchyme, and weak in the epithelial buds. In addition, there was a reduction in the number of labeled nuclei in the UGS from the TCDD group compared to other groups (Fig. 2E).

Immunoreactivity for α-actin was punctual and weak around the epithelial buds in the UGS from TCDD group (Fig.3B). In the TCDD + RES (Fig.3C) group, the staining was more intense and homogeneous, revealing a thin differentiating layer of smooth muscle cells around the buds, similar to the Control (Fig. 3A). In turn, there was a strong staining and a thick and organized layer of mesenchymal cells expressing α-actin in UGS from RES group (Fig. 3D).

Mesenchymal cell proliferation index in the UGS from TCDD group was lower compared to the Control group (Fig. 4B and E). It was reflected in epithelial cell proliferation, where the same data was

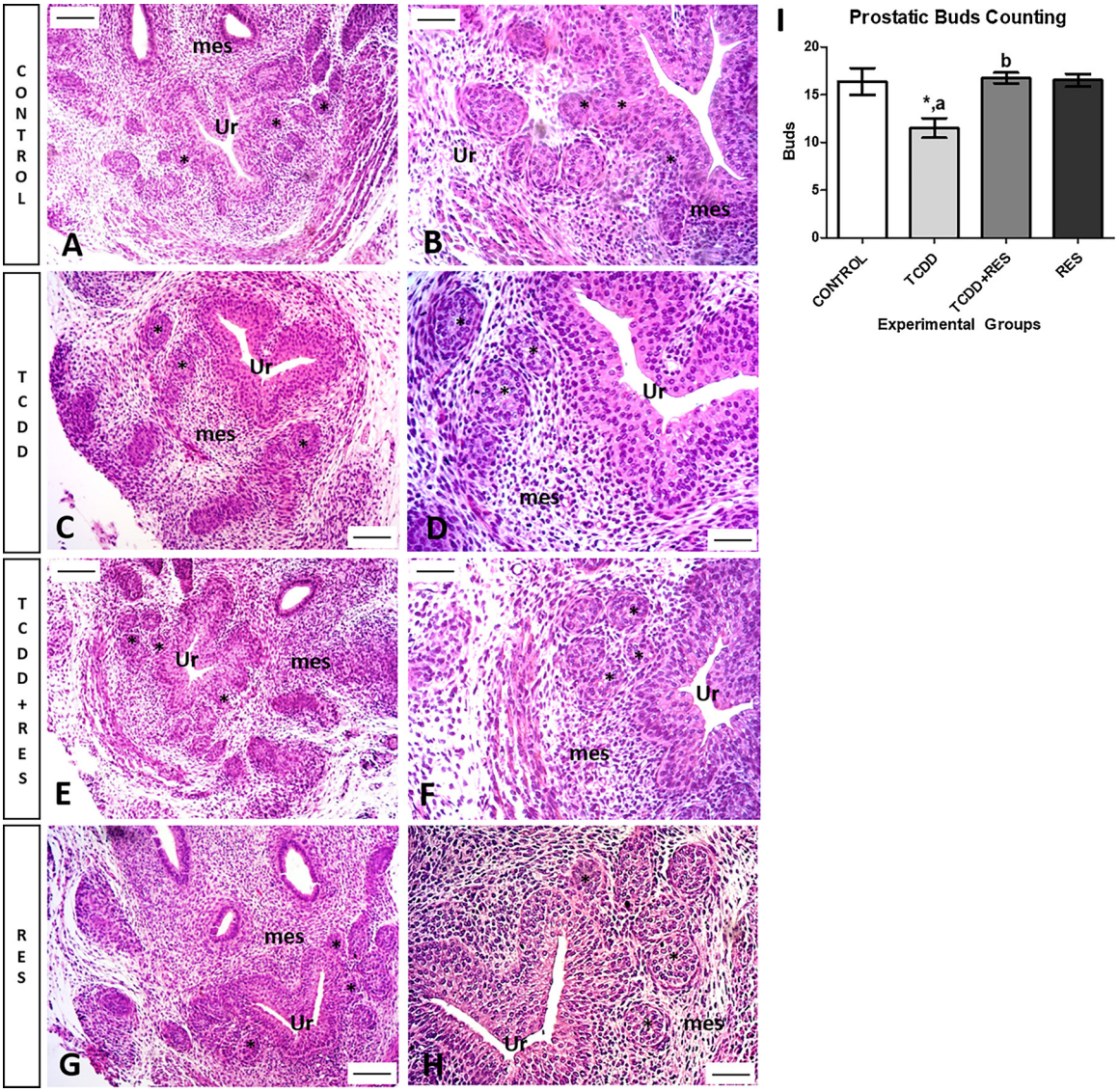

Fig. 1. Histological sections of UGS (part of pelvic of urethra) from animals on PND1 stained by hematoxylin-eosin. Abbreviations: *: prostatic buds; Ur: urethra; mes: mesenchyme. Bars: A, C, E, G (100 μm); B, D, F, H (50 μm); (I): Graphic representation of mean ± SEM of prostatic buds counting on PND1. The asterisk represents statistical difference related to the Control group; different letters mean statistical difference among the groups ($p \leq 0.05$).

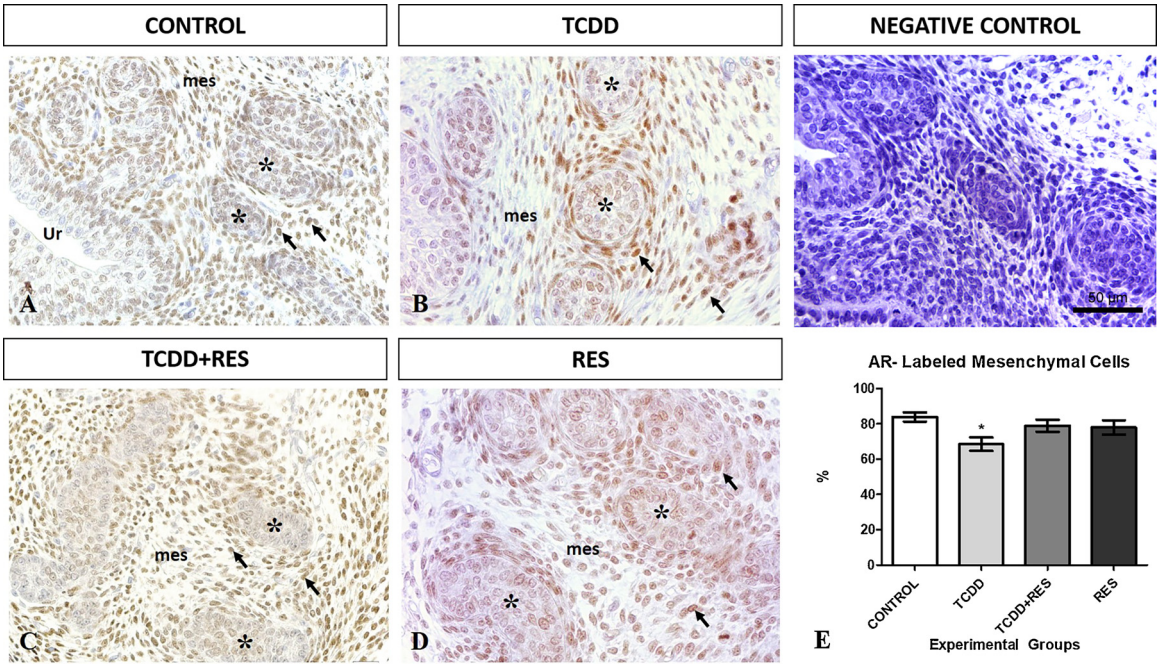

Fig. 2. Histological sections of UGS (part of pelvic of urethra) from animals on PND1 submitted to Androgen Receptor (AR) immunohistochemistry (A–D). Abbreviations: *: prostatic buds; Ur: urethra; mes: mesenchyme; arrows: AR-positive cells. Magnification: x400; (E): Graphic representation of mean ± SEM of AR-positive mesenchymal cells. The asterisk represents statistical difference related to the Control group ($p \leq 0.05$). Negative Control to IHC.

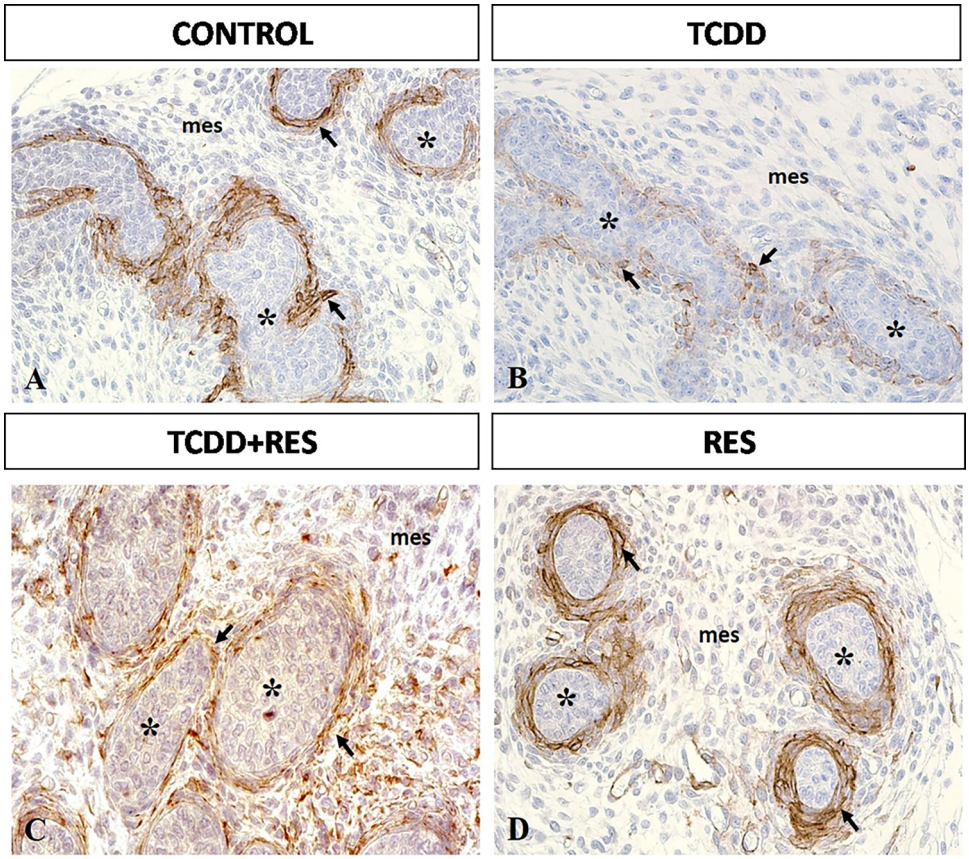

Fig. 3. Histological sections of UGS (part of pelvic of urethra) from animals on PND1 submitted to alpha-actin immunohistochemistry (A–D). Abbreviations: *: prostatic buds; Ur: urethra; mes: mesenchyme; arrows: positive labeling for alpha-actin. Magnification: x400.

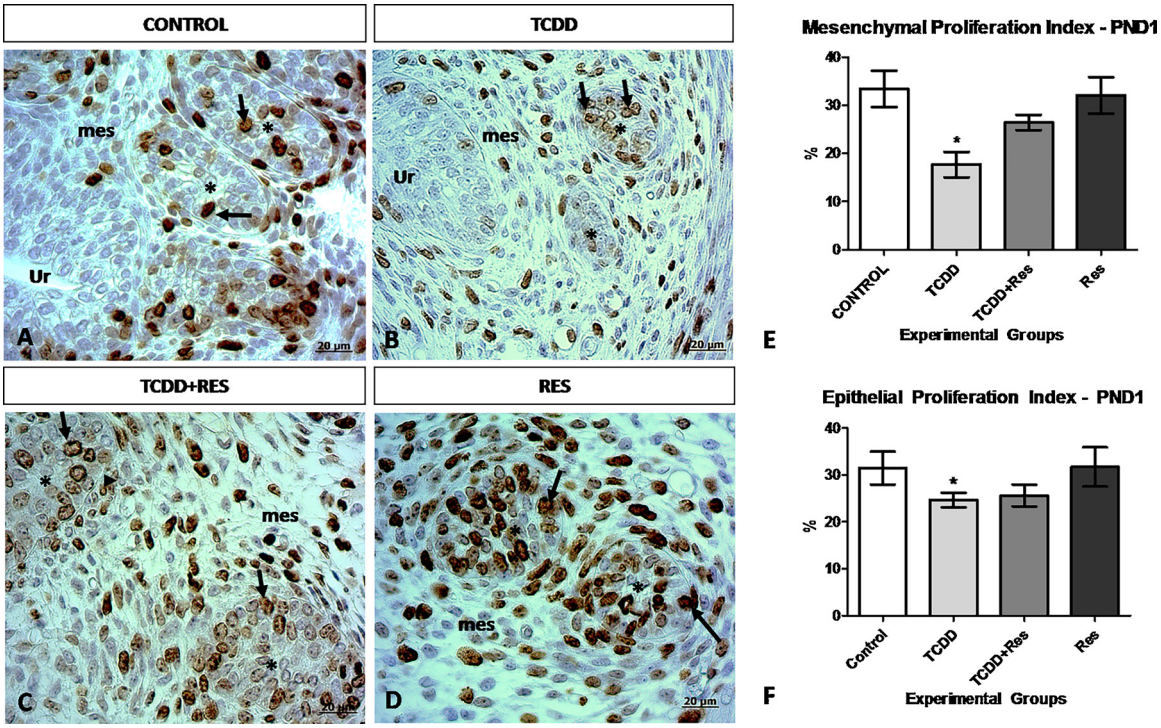

Fig. 4. Histological sections of UGS (part of pelvic of urethra) from animals on PND1 submitted to Ki67 immunohistochemistry (A–D). Abbreviations: *: prostatic buds; Ur: urethra; mes: mesenchyme; arrows: Ki-67 positive cells. Magnification: x400; (E): Graphic representation of mean ± SEM of Ki67-positive mesenchymal cells; (F): Graphic representation of mean ± SEM of Ki67-positive epithelial cells. The asterisk represents statistical difference related to the Control group ($p \leq 0.05$).

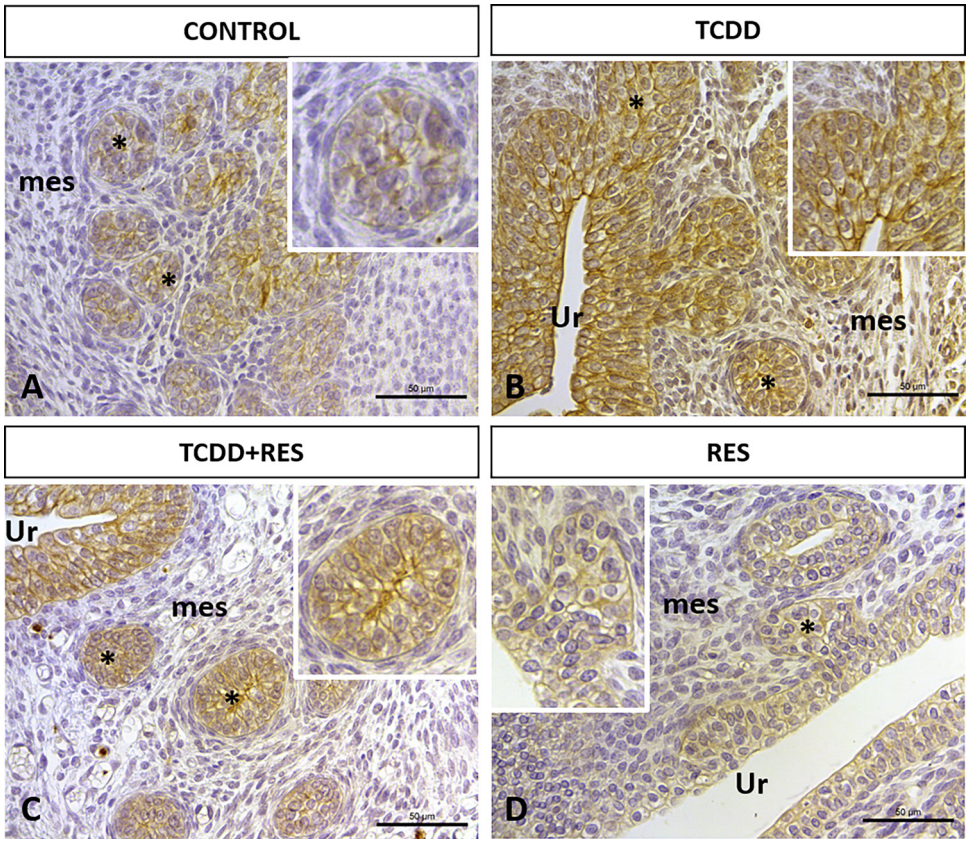

Fig. 5. Histological sections of UGS (part of pelvic of urethra) from animals on PND1 submitted to AhR immunohistochemistry (A–D). Abbreviations: *: prostatic buds; Ur: urethra; mes: mesenchyme; brown staining means positive cells to AhR. Magnification: x400.

Table 2
Quantitative parameters from all experimental groups on PND90.

Parameters	Group/Treatment (n)			
	G1 Controle (n = 10)	G2 TCDD (n = 10)	G3 TCDD + RES (n = 10)	G4 RES (n = 10)
BIOMETRICS PARAMETERS				
Body Weight (g)	361.47 ± 6.12	406.07 ± 6.98*	391.73 ± 8.95	391.39 ± 9.03
Ventral Prostate Weight (g)	0.38 ± 0.03	0.37 ± 0.01	0.37 ± 0.01	0.44 ± 0.03
Ventral Prostate Relative Weight (mg/g)	1.06 ± 0.08	0.91 ± 0.03	0.96 ± 0.04	1.14 ± 0.06
HORMONAL PARAMETER				
Serum Testosterone (ng/mL)	1.97 ± 0.32	2.27 ± 0.56	2.52 ± 0.72	1.68 ± 0.63

Values expressed as mean ± SEM. Statistical test: ANOVA followed by Kruskal-Wallis. The differences were considered significant when *p ≤ 0.05.

observed in the TCDD group compared to the Control group (Fig. 4B and F). The labeling index in TCDD + RES and RES groups were similar to the Control group and there was no difference between the Control and RES groups (Fig. 4E–F).

Aryl hydrocarbon receptor (AhR) immunostaining was predominantly present in the epithelium of urethra and prostatic buds of all groups, and a weak reactivity was observed in some areas of mesenchyme (Fig. 5A–D). AhR immunoreactivity was higher in the UGS from TCDD group (Fig. 5B), when compared to other groups. RES group showed a thick cytoplasmic stain in the prostatic epithelial bud cells similar to the Control group (Fig. 5A and D).

3.3. Postnatal day 90 (PND90)

3.3.1. Biometrical analysis and serum testosterone concentration

At the end of experiment, TCDD group presented higher body weight compared to the other groups (Table 2). However, ventral prostate absolute and relative weights were not altered by *in utero* TCDD exposure (Table 2).Also, there was no difference in the serum testosterone levels among all groups (Table 2).

3.3.2. Ventral prostate morphology and stereology

Ventral prostate morphology showed regularity in the glandular architecture, with the acini predominantly composed of simple and low columnar epithelium supported by a delicate fibromuscular stroma (Fig.6A–E). However, there was a predominance of smaller folded acini in TCDD group, which were localized on the periphery of the lobe (Fig. 6B–C). Stereology measures of the ventral prostate revealed no difference in epithelial, luminal and stromal compartments among the groups (Supplementary data).

3.3.3. Immunohistochemical analysis

The prostatic epithelium showed an increase in the number of ki67-positive cells and consequently in the cell proliferation index in the TCDD group compared to the other groups (Fig. 7A–E). In addition, Ki-67 labeling index was similar among TCDD + RES, RES and Control groups (Fig. 7A–E).

For androgen receptor (AR) immunostaining, there was no difference among the groups, in terms of localization and mean number of labeled epithelial nuclei (Data not shown).

3.3.4. Western blot

AhRR protein expression was higher in the ventral prostate from TCDD and TCDD + RES groups compared to the Control group (Fig.8A).On the other hand, AR expression was not different among the groups (Fig. 8B); and NRF2 expression revealed a higher expression in the TCDD + RES group compared to the Control and TCDD groups (Fig. 8C).

4. Discussion

Lin et al. [62] demonstrated that TCDD interferes with prostatic bud

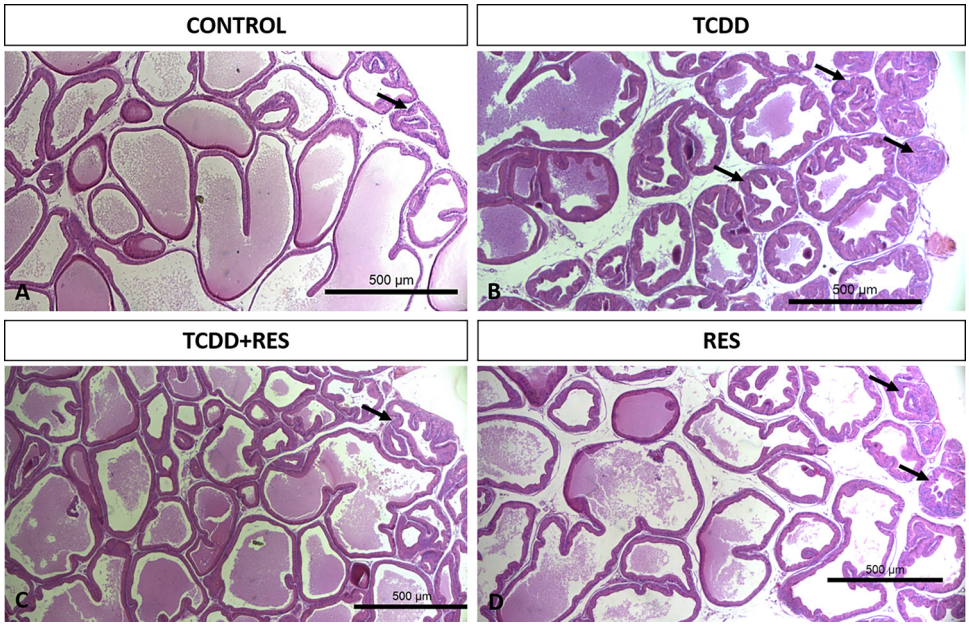

Fig. 6. Histological sections ventral prostate from animals on PND90 stained by hematoxylin-eosin. Arrows point to the folded epithelium of peripheral acinum.

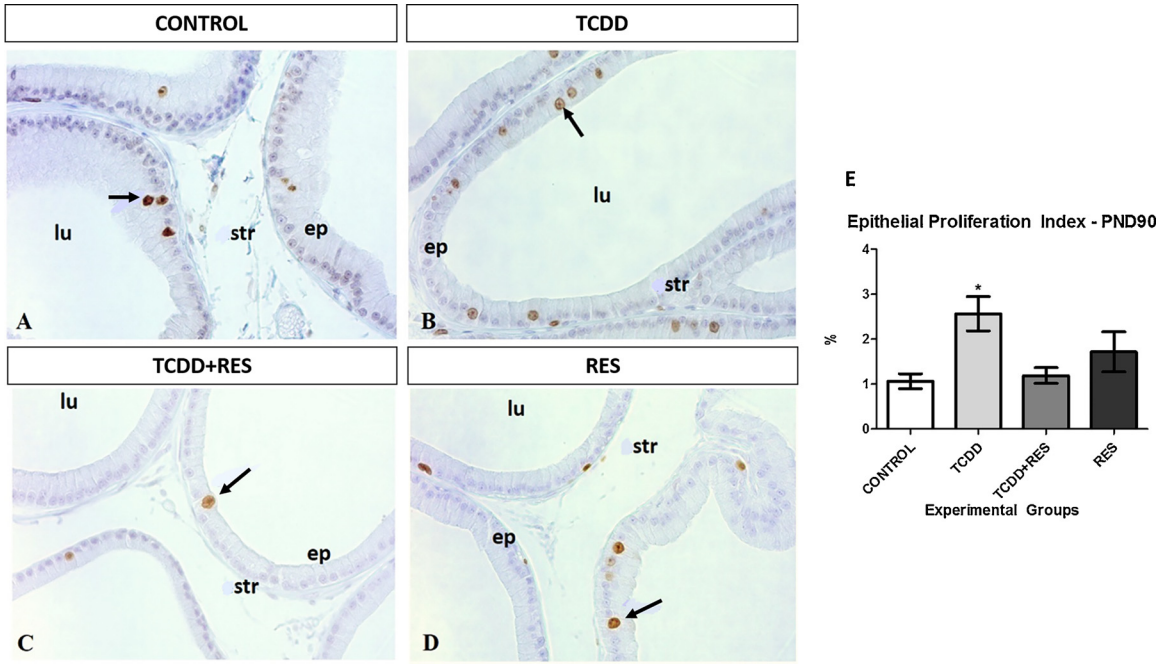

Fig. 7. Histological sections ventral prostate from experimental animals on PND90 submitted to Ki67 immunohistochemistry (A–D). Abbreviations: ep: epithelium ;str: stroma; lu: lumen; arrows: Ki67 positive cells. Magnification: x400; (E): Graphic representation of epithelial proliferation index (Ki67 positive cells). The asterisk represents statistical difference related to the Control group ($p \leq 0.05$).

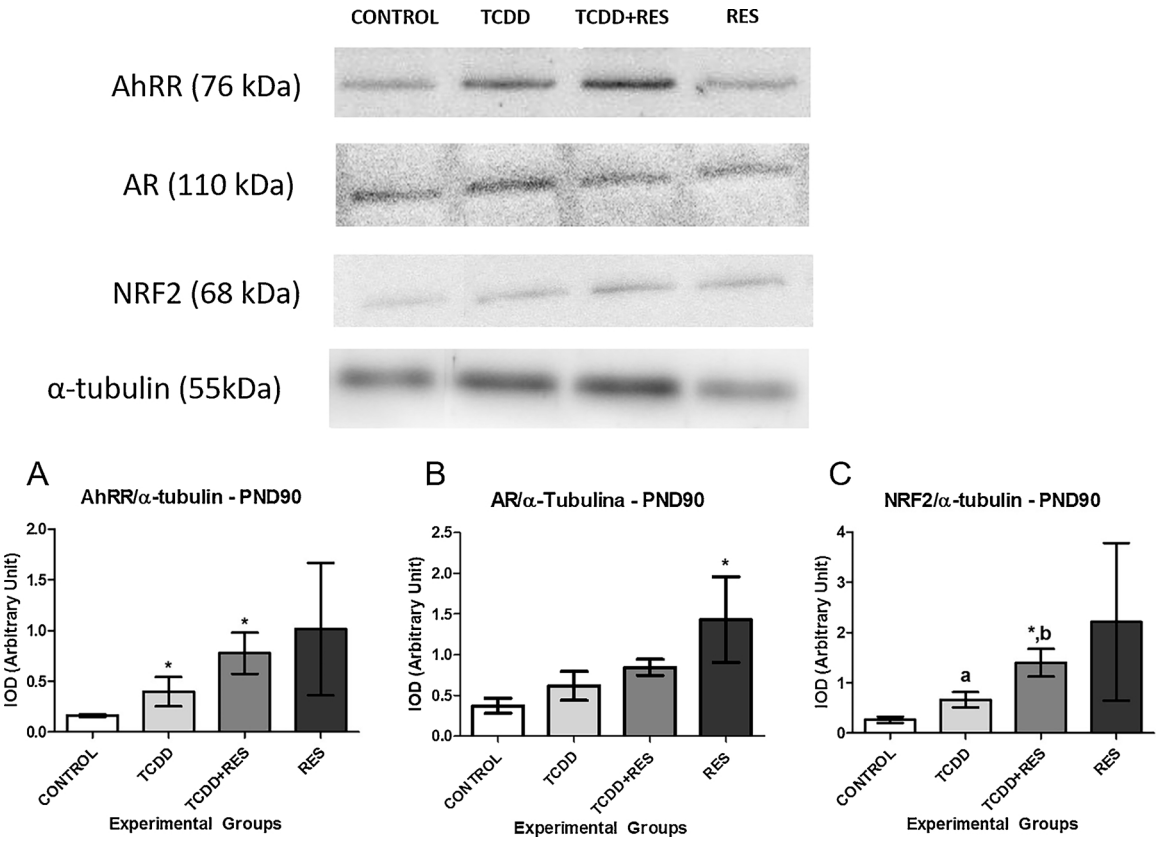

Fig. 8. Representative western blots for Aril hydrocarbon receptor repressor (AhRR), androgen receptor (AR), nuclear factor (erythroid-derived 2)-like 2 (NRF2) and alpha-tubulin from protein extracts from the ventral prostate (each band represents a pool with samples from three animals) at PND90. B) The graph represents the relative expression of integrated optical density for AhRR (A), AR (B) and NRF2 (C), normalized by alpha-tubulin expression and expressed as mean ± SD. The asterisk represents statistical difference related to the Control group; different letters mean statistical difference among the groups ($p \leq 0.05$).

patterns by acting directly on the UGS to stimulate AHR signaling. Our results on PND1 newborns showed that TCDD group had a higher cytoplasmic labeling for AhR in the epithelial buds than the other groups, showing that TCDD could induce a response through AhR activation. Moreover, the TCDD + RES group showed a slight labeling for this receptor, showing that the polyphenol was capable in inhibiting, at least partially, the binding and activity of AhR.

Urogenital sinus histology of newborn animals showed that TCDD exposure reduced the number of emerging UGS prostate buds, the epithelial and mesenchymal cell proliferation index and AR positive mesenchymal cells. These findings together suggest that TCDD exposure resulted in a hypoplastic response in the early stages of prostate development, which was also shown by a reduced number of prostatic buds and delayed cytodifferentiation in Holtzman rats by *in utero*/lactational TCDD exposure [63]. TCDD + RES group showed a similar morphological arrangement to the Control group, demonstrating a possible prostate development restoration by resveratrol treatment.

Prostatic morphogenesis is a process that is responsive to androgens and dependent of epithelium and mesenchyme interactions, and is modulated by paracrine growth factors [64]. According to Chen et al. [65] and Roman et al. [66], TCDD does not reduce testicular androgen production or consistently decreasing prostate DHT concentration and these findings are in agreement with our results. Therefore, it is possible that TCDD interferes with the production and/or release of stromal signals that stimulate budding, proliferation and/or cell differentiation [65,66].

It is known that anogenital distance is a marker for endocrine disruption during genital system development. Maternal TCDD exposure was responsible for reducing the anogenital distance in the litter of Sprague-Dawley rats exposed to 1 μg/kg body weight on GD15 [30,35]. However, our results showed no difference in male and the female anogenital distance among the groups. Perhaps this result is related to the fact that TCDD was not able to alter testosterone levels in our model.

Although the results did not definitely confirm a clear change in AR levels between groups, it was observed a reduction in AR-positive mesenchymal cells in the TCDD group than the other groups. According Vezina and coworkers [38] 'although AhR signaling modulates androgen signaling in other tissues [67], crosstalk between these steroid hormone receptors and AhR signaling cannot account for actions of TCDD on prostate morphogenesis'. Thus, although there is some modulation between the two pathways (AR and AhR), probably the changes observed in the prostate development are related to other paracrine factors [68].

Between PND3 and 5, the cells adjacent to the ducts form a periductal layer of smooth muscle cells, while intraductal cells differentiate into mature fibroblasts [69,70]. According to Roman et al. [63], uterine and lactational exposure to TCDD in *Holtzman* rats impaired differentiation of prostatic mesenchymal cells into smooth muscle cells, which reduced marking α-actin on PND1. Therefore, our results are in agreement with Roman et al. [63] and showed that the TCDD group had a delay in mesenchyme differentiation in smooth muscle around the prostatic buds, and resveratrol treatment contributed to reverse it.

On PND90, histological analyzes indicated that animals in the TCDD group showed a glandular architecture compound by an epithelium with many unfolding acini on the periphery of the lobe, indicating an increase in the functional hyperplasia. Functional hyperplasia in the ventral prostate can be found at the periphery of the lobe [71]. A mild degree of this hyperplasia is frequently present in adult rats, but its presence is rarely registered as a spontaneous finding [71]. In our study, epithelial proliferation index and histological functional hyperplasia areas increased at TCDD group but reactive and atypical hyperplasias were not found; in addition, there was a recovery in the expression of RA, which was decreased on DPN1, and now it appears similar between the groups. These results probably point to the ability of the prostate to recover after the inhibitory stimulus of development/growth is removed. The TCDD + Res group showed a glandular structure similar to the Control group, once again, showing the possible protective activity of resveratrol against TCDD during prostate development.

AhRR protein analysis showed a high prostatic expression in TCDD and TCDD + RES group on PND90 (late effect). This fact can indicate that a high expression of AhR in these groups signaled an increased expression of its repressor to produce a down-regulation in AhR expression, mainly in the TCDD + RES group. Thus, resveratrol consumption can increase the expression of AhRR, which possibly suppressed, at least partially, the effects caused by TCDD thought the AhR signaling pathway.

TCDD, through AhR activation, mediates toxicity by oxidative stress, which is a component that promotes many toxicological processes [72,73]. Many mechanisms were proposed to explain the oxidative stress caused by TCDD exposure, including the reduction of enzymatic antioxidants expression systems levels [74], and disruption in the levels of cytochrome P450 [75,76]. To assess whether oxidative stress/antioxidant pathways might be operative in the RES mediated reversal of TCDD effects, NRF2 levels were assessed by Western blot in the adult VP. The NF-E2 Related factor 2 (NRF2) is a transcription factor that activates genes containing the transcriptional control element known as antioxidant response element (ARE) [77], including phase II enzymes: NADPH quinone oxidoreductase (NQO1), glutathione peroxidase (GPx), ferritin and heme oxigenase-1 (HO-1) [78,79]. Thus, our results show that resveratrol can increase NRF2 expression in the TCDD + RES group in relation to TCDD and Control groups on PND90, demonstrating the potential that resveratrol has as an antioxidant, which could contribute to reducing the effects of TCDD. Probably, these long-term effects observed on PND90 are due to a long half-life of TCDD, which in humans has an estimated half-life of 7–9 years [80].

Although the effects of oxidative stress caused by TCDD in the prostate were not excessively harmful during the study period, other studies point to an increase in the susceptibility to breast carcinogenesis [54], alteration in the stereogenesis and in the antioxidant system of epididymal sperm [81]. Adittionally, further studies can be proposed for an evaluation of the effects of gestational exposure to TCDD in older adult animals and in models of prostatic carcinogenesis.

Our results showed that TCDD was able to disrupt the pattern of rat prostate development *via* AhR that alters the paracrine mechanisms of androgen receptor modulation and increases oxidative stress in the tissue. Probably, resveratrol plays an important role in reducing the activation of AhR and, consequently, the oxidative stress caused by TCDD. The adult prostate appears to recover from the hypoplastic inhibition of TCDD in early development by enhancing cell proliferation, primarily the distal part of the acini.

5. Conclusion

Thus, we can conclude that resveratrol administration was able to reduce the adverse effects of TCDD on prostate development in rats exposed to a single dose in the uterine environment.

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