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RESEARCH ARTICLE

Effects of 2,4-dichlorophenoxyacetic acid on the ventral prostate of rats during the peri-pubertal, pubertal and adult stage

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Abstract

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is used on a wide variety of terrestrial and aquatic broadleaf weeds. 2,4-D has been shown to produce a wide range of adverse effects on animal and human health. The aim of the current study was to evaluate the effects of pre- and postnatal exposure to 2,4-D on rat ventral prostate (VP). Pregnant rats were exposed daily to oral doses of 70 mg/kg/day of 2,4-D from 16 days of gestation up to 23 days after delivery. Then, the treated groups ($n = 8$) were fed with a 2,4-D added diet until sacrificed by decapitation on postnatal day (PND) 45, 60, or 90. Morphometric studies were performed and androgen receptor (AR) protein levels in the VP were determined. AR, insulin-like growth factor-I (IGF-1) and insulin-like growth factor-I receptor (IGF-1R) mRNA expression in the VP along with testosterone (T), dihydroxytestosterone (DHT), growth hormone (GH) and IGF-1 serum levels were also determined to ascertain whether these parameters were differentially affected. Results of this study showed that 2,4-D exposure during gestation and until adulthood altered development of the prostate gland in male rats, delaying it at early ages while increasing its size in adults, indicate that 2,4-D could behave as endocrine disruptors (EDs).

Keywords

2,4-Dichlorophenoxyacetic herbicide, developmental toxicity, hormone, androgen receptor

History

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Introduction

Differentiation of the prostate gland during embryogenesis and subsequent tissue growth during postnatal life is controlled by androgenic hormones synthesized in the testes (George et al., 1991). The two most important androgens are testosterone (T) and its metabolite, 5α-dihydrotestosterone (DHT). Both act through the same receptor and each of these androgens has its own specific role during male sexual differentiation (Knobil & Neill, 1994). Some androgenic effects, such as the promotion of spermatogenesis and the enhancement of muscle growth, are believed to be mediated by the testicular androgen testosterone. In other target tissues, including prostate, testosterone is converted to DHT by the enzyme steroid 5α-reductase (George et al., 1991). In addition to hormonal influences, studies have demonstrated that several growth factors, such as insulin-like growth factor-I (IGF-I), display important mitogenic effects on the prostate and are essential for the development of this gland (Ruan et al., 1999).

Epidemiological studies have indicated the influence of height, weight, dietary and lifestyle factors on IGF-I serum levels and several of its binding proteins (Baibas et al., 2003; Kaklamani et al., 1999; Sandhu et al., 2006; Schneider et al., 2006). Other factors, such as the environment and dietary contamination by chemicals should also be taken into account. The possibility exists that environmental contaminants could influence the IGF system. Thus, studies in animals and human subjects have demonstrated that environmental pollutants, such as benzopyrene, dioxins, dibenzofurans and hexachlorobenzene could alter the normal synthesis and/or secretion of IGF-I (Randi et al., 2006; Tannheimer et al., 1998; Wang et al., 2005).

Chlorophenoxy herbicides are widely used in agriculture and forestry for the control of broad-leaved weeds in pastures, cereal crops, as well as along public rights of way. 2,4-Dichlorophenoxyacetic acid (2,4-D) is used on a wide variety of terrestrial and aquatic broadleaf weeds. It has little effect on grasses (Shaner, 2014). Several studies have shown that doses of 50, 70 or 100 mg/kg body weight (bw)/day of 2,4-D produce a wide range of toxic effects on the embryo as well as on the reproductive and neural systems in animal (mostly rat) and human models (Barnekow et al., 2001; Charles et al., 2001; Rosso et al., 2000). Lerda & Rizzi (1991) studied the reproductive function of 32 male farm sprayers who were exposed to 2,4-D and found significant levels of asthenospermia, necrospermia and teratospermia in exposed workers compared with unexposed controls. Doses of 50 mg/kg bw/day of 2,4-D have been reported to increase ventral

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prostate (VP) weight in rats (Kim et al., 2002). Treatment of human prostate cancer cell cultures with 10 nM 2,4-D enhanced the androgenic activity of dihydroxytestosterone (DHT) on cell proliferation and transactivation (Kim et al., 2005).

There are few studies on developmental toxicology addressing the effects of 2,4-D on the prostate. Recently we have found that 2,4-D (70 mg/kg bw/day) induced an increase in reactive oxygen species (ROS) levels, lipid peroxidation and protein oxidation, thereby causing oxidative stress in VP. This, in turn, could provoke important deleterious changes in the development of the organ at different ages (Pochettino et al., 2013). The aim of the current study was to evaluate the effects of pre- and postnatal exposure of 2,4-D on rat VP. For this purpose, we carried out morphometric studies and measured AR protein levels in the prostate. AR, IGF-1 and IGF-1R mRNA expression in the VP along with T, DHT, growth hormone (GH) and IGF-1 serum levels were also determined to ascertain whether these parameters were differentially affected.

Materials and methods

Animals and exposure to 2,4-D

Nulliparous female rats of Wistar origin, between 90 and 110 days old and weighing approximately 180–210 g were obtained from the animal breeding colony of the Faculty of Pharmacy and Biochemistry, Rosario, Argentina. Stages of the reproductive cycle were monitored via daily cytological examination of vaginal smears. Females were mated individually with fertile males on the night of pro-estrus. This day was denoted as gestation day 0 (GD 0). At this time, pregnant females were individually housed in plastic breeding cages in a temperature-controlled nursery (22–24 °C) and maintained on a 12-h light/dark cycle. Food (Cargill pellets, Buenos Aires, Argentina) and water were available ad lib. All experimental protocols were performed according to the Regulation for the Care and Use of Laboratory Animals (File6109/012 E.C. Document267/02) approved by the Institutional Committee for Animal Use of the National University of Rosario, Argentina. On GD 16, the pregnant females were randomly divided into two groups, as follows:

2,4-D-treated groups. Dams treated with a daily oral dose (by diet) of about 70 mg of 2,4-D per kg body weight (bw) per day (70 mg/kg/day) from GD 16 until postnatal day (PND) 23. Selection of the 2,4-D dose was based on previous studies, which demonstrated behavioral changes (Bortolozzi et al., 1999), alterations in neurotransmitter levels in adult rats (Evangelista de Duffard et al., 1990) and in neonate rats (Ferri et al., 2000, 2003, 2007), when pups were exposed to the herbicide through mother's milk. The selected dose was lower than the no-observed-adverse-effect level (NOAEL) for chronic dietary 2,4-D toxicity in rats, which was established as 77 mg/kg/day (Munro et al., 1992). An alcoholic solution of 2,4-D was mixed with the food and allowed to dry before administration in the diet (Bortolozzi et al., 1999). According to previous work, the dietary intake of animals was adjusted to the most recent body weight and food consumption determinations (Stürtz et al., 2006).

Control groups. Dams were fed the same food (sprayed with alcohol and dried), as described for the treated groups but without the herbicide.

After parturition, each litter was reduced to eight male pups when possible on PND 1 to ensure good nutrition. Pups were weaned at PND 23.Next, the treated groups were fed the 2,4-D- diet until sacrifice at 45, 60 or 90 days of age (Figure 1). Animals were weighed, euthanized by decapitation between 10.00 and 12.00 h, and trunk blood was collected. Serum was separated by centrifugation at 4 °C for 15 min at 3500 rpm and stored at −80 °C for hormone level determination. The VP was dissected from the abdominal cavity of each animal. After weighing, a portion of the VP was fixed in 10% buffered formalin for paraffin embedding. The remaining tissue was immediately frozen in liquid nitrogen for further analysis.

Histopathological analysis

Fixed tissue samples were dehydrated in a graded ethanol series and embedded in paraffin wax. Sections of 3–5 μm thickness were cut with a Reichert-Jung Hn 40 microtome and stained with hematoxylin–eosin (H&E). Slides were examined under an Olympus Provis microscope (BX40, Olympus Optical Corp., Toyota, Japan) and images were captured digitally with the Olympus D-560 camera (Olympus Optical Corp.).

Digital VP images were examined with a digital image analysis program (ImageJ). Epithelial thickness and alveoli cell number per unit area were measured and averaged from four sections per rat (Mandarim-de-Lacerda, 1999; Ma et al., 2004).

Western blotting analysis for androgen receptor (AR)

Prostate samples were mechanically homogenized in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS) and 1 mM phenylmethylsulfonyl fluoride (PMSF) by means of a Polytron for 30 s at 4 °C. Following centrifugation of the homogenate, proteins were extracted from the supernatant and quantified by the Lowry method (Lowry et al., 1951). Aliquots of each sample containing equal amounts of protein were loaded onto 8% SDS-polyacrylamide gels for SDS-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis,

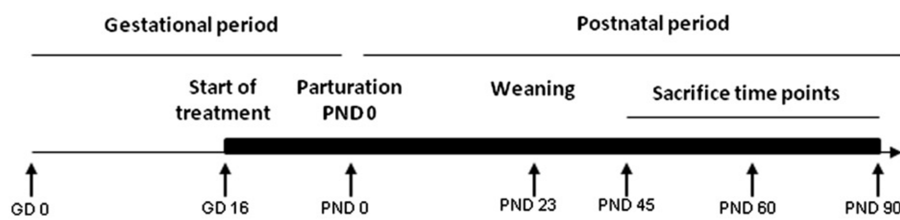

Figure 1. Treatment schedule. Pregnant rats were treated with 2,4-D (70 mg/kg/day) from gestational day (GD 16) until weaning. Then, the treated groups were fed with a 2,4-D added diet until sacrificed by decapitation on postnatal day (PND) 45, 60, or 90.

proteins were transferred onto a nitrocellulose membrane (Sigma). The blot was blocked with 10% nonfat dry milk in phosphate buffered saline/3% milk powder/0.1% triton X100 (PBSX) for 1 h, incubated overnight at 4 °C with 3% BSA in PBSX containing a 1:1000 dilution of the anti-AR (Sigma) or anti-βactin (Sigma) primary antibodies and washed twice for 20 min in PBSX. Blots were subsequently incubated with appropriate alkaline phosphatase-labeled secondary antibody for 1 h, and then visualized by incubating the membrane for 15 min in a solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. AR and β-actin protein expression were quantified by densitometric analysis of the bands as integrated optical density (IOD). AR expression was normalized to βactin values.

Hormone assays

GH was measured by double antibody radioimmunoassay (RIA) using materials generously provided by A. F. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA). Hormones were radio-iodinated using the Chloramine T method and purified by passage through Sephadex G75 (Rosato et al., 1992). Results were expressed in terms of the rat GH RP-2 standard preparations. Assay sensitivity was 0.5 $g\,l^{-1}$ serum and the inter- and intra-assay variation coefficients were <10%.

Rat IGF-I, testosterone and DHT concentrations in sera were measured by radioimmunoassay using commercial kits for total hormones (DSL-2900, DSL-4100, DSL-9600 double antibody radioimmunoassay, respectively; all from Diagnostic Products Corporation, Los Angeles, CA).

RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated from VP using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. RNA pellets were dissolved in RNase-free water and stored at −80 °C until analysis. Total RNA was quantified by OD 260 nm spectrophotometry (Beckman DU 640 Spectrophotometer). Integrity of purified RNA was determined by 2% agarose gel electrophoresis. cDNA was synthesized from 2 μg of total RNA using oligo (dt) primer (Biodynamics S.R.L, Argentina) and 200 U M-MLV reverse transcriptase (Promega, WI). Briefly, 5 × M-MLV Reaction Buffer, 0.4 mM dNTPs (Promega, WI); 21.5 U RNase Inhibitor (Promega, WI), 0.4 μM oligo (dt) primers; 2 mM $MgCl_2$ (Invitrogen) and RNase-free water for 50 μl of final volume. Retrotranscription cycling programs consisted of 5 min at 65 °C, 1 h at 40 °C followed by enzyme inactivation at 95 °C for 3 min. cDNA was stored at −80 °C until use. qPCR was performed with the ABI PRISM 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using 10 μl of a 1/200 dilution of cDNA, 0.4 μM of each primer (Invitrogen, Argentina) (Table 1) and 25 μl of FastStart Universal SYBR Green Master (ROX) (Roche Applied Science) in a final volume of 50 μl. The reaction mixture was run online at 50 °C for 2 min and 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with an extension phase of 1 cycle at 95 °C for 1 min, 60 °C for 1 min and 95 °C for 1 min.

Table 1. Description of primers used in this study.

Primers	Type	Primer sequence	Length (bp)
AR	Forward	5′-TAGCAGGGCAGATCCTGTCT-3′	197
AR	Reverse	5′-CCACCGAATTCCCTTATCCT-3′	
IGF-1	Forward	5′-TCCGCTGAAGCCTACAAAGT-3′	197
IGF-1	Reverse	5′-GGGAGGCTCCTCCTACATTC-3′	
IGF-1R	Forward	5′-GACAGTGAATGAGGCTGCAA-3′	200
IGF-1R	Reverse	5′-TCTCCACCTCTGGCCTTAGA-3′	
GADPH	Forward	5′-TGCCAAGGCTGTGGGCAAGG-3′	100
GADPH	Reverse	5′-GCTTCACCACCTTCTTGATG-3′	

Primer sequences were designed according to cDNA sequence from Genbank® (Table 1).

Samples were deemed positive at any given cycle when the value of the emitted fluorescence was greater than the threshold value calculated by the instrument's software (Sequence Detector Ver. 1.9.1). The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value (i.e. usually 15 times greater than the standard deviation of the baseline), is given as the mean value. Relative expression of each mRNA was calculated by the ΔCt method (where ΔCt is the value obtained by subtracting the Ct value of GADPH mRNA from the Ct value of the target mRNA), specifically, the amount of target mRNA relative to GADPH mRNA is expressed as $2^{-\Delta\Delta Ct}$. Data are expressed as the ratio of the target mRNA to GADPH mRNA. Each PCR run included a no-template control and a sample without reverse transcriptase.

Statistical analyses

Data are presented as mean ± standard error (SE) of each group. All statistical comparisons were performed between the control and treated groups for each period of study: 45, 60 and 90 PND. Comparisons were analyzed by Student's t test. Differences of $p < 0.05$ were considered significant. Litters with $n = 8$ per each treated or control group were evaluated in every case.

Results

No differences were observed in food and water consumption between control and treated groups. Maternal exposure to 70 mg/kg/day 2,4-D had no effect on body weight gain during gestation or lactation, on the number of pups born or on postnatal mortality. In agreement with our previous study, 2,4-D reduced slightly the pup weight gain (8–12%) (Bortolozzi et al., 1999).

Absolute and relative VP weight decreased significantly in the treated groups when compared with controls (47.2% and 39.7% at PND 45 and 54.9% and 33.6% at 60 PND, respectively). However, absolute and relative VP weight increased at PND 90 (19.1% and 26.1%, respectively) (Table 2).

Effects of 2,4-D on the histology of rat prostate

As shown in Figure 2(A, C and E), the alveoli of control prostates were lined with a layer of tall columnar epithelial

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cells with a high cytoplasm/nuclear ratio. The luminal epithelial cells showed a significant reduction in cytoplasmic area after 2,4-D treatment in the VP at PND 45 and 90 (Figure 2B and F). The increase in luminal volume was accounted for by a significant ($p < 0.01$) decrease in the average cell number per unit area, reaching 29.5% of control rats (Table 3).

Table 2. Body, absolute and relative prostate weight of male rats, controls and 2,4-D treated with 70 mg/kg/day.

Age	Body weight (g)	Absolute prostate weight (g)	Relative prostate weight (g/g bw)
45 PND			
Control	139.83 ± 2.61	0.091 ± 0.005	0.066 ± 0.003
2,4-D	121.36 ± 3.72**	0.048 ± 0.006***	0.039 ± 0.003**
60 PND			
Control	198.73 ± 5.62	0.253 ± 0.012	0.122 ± 0.004
2,4-D	176.51 ± 6.63*	0.114 ± 0.004***	0.081 ± 0.008***
90 PND			
Control	310.53 ± 4.77	0.301 ± 0.013	0.096 ± 0.003
2,4-D	284.30 ± 5.72**	0.343 ± 0.016	0.121 ± 0.005*

2,4-D treated versus controls: each value is the mean ± SEM (n=8).
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$.

Effects of 2,4-D on AR expression

In the 2,4-D-treated rats, VP AR protein abundance decreased significantly at PND 45 (22%). However, we observed an increase in AR (37.7%) with respect to controls (Figure 3) at PND 90.

Effects of 2,4-D on T, DHT, GH and IGF-I circulating levels

T and DHT serum concentrations at PND 45 and 60 were significantly lower than controls (97% and 96%; 88% and

Table 3. Effects of 2,4-D (70 mg/kg/day) on the epithelial thickness and cell numbers per selected field.

Age	Epithelial thickness (μm)	Cell number
45 PND		
Control	12.9 ± 0.8	52.6 ± 1.2
2,4-D	8.2 ± 0.4*	48.5 ± 1.5
60 PND		
Control	12.2 ± 1.2	51.7 ± 2.5
2,4-D	10.3 ± 1.5	58.1 ± 2.4
90 PND		
Control	16.8 ± 0.9	55.4 ± 2.1
2,4-D	9.8 ± 1.1*	39.1 ± 1.5*

Data are expressed as mean ± SE for at least eight rats for each experimental group.
*$p < 0.01$ with reference to control values.

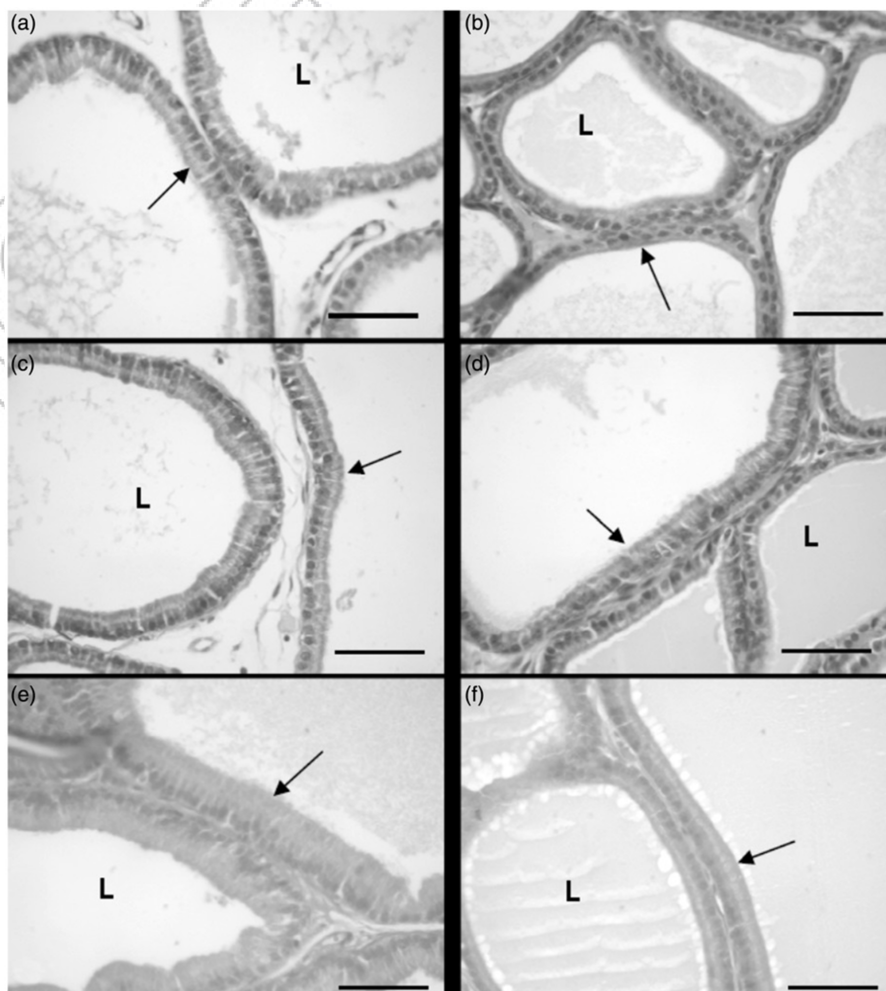

Figure 2. Prostate for males at 3 ages studied. Sections stained with H-E. The bar represents 50 μm (A) and (B) rats of 45 days old, control and treated respectively. (C) and (D) rat 60-day-old control and treated. (E) and (F) rat 90-day-old control and treated. Lumen (L) of prostatic alveoli and prostatic epithelium (arrow).

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76%, respectively) (Figure 4A and B). However in adult treated animals (PND 90), the levels of both androgens were similar to control values.

IGF-1 serum levels were significantly reduced by 2,4-D at all ages compared with the respective controls (70%, 55% and 71% at PND 45, 60 and 90, respectively) (Figure 4C).

Conversely, 2,4-D did not affect serum GH levels at any age studied (Figure 4D).

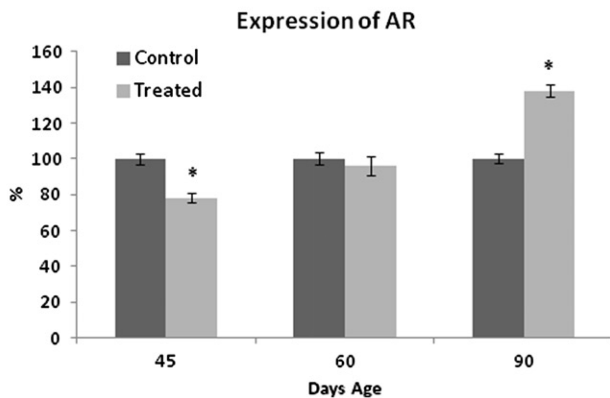

Figure 3. Effects of 2,4-D on the expression of AR in the ventral prostate gland. Rats were treated as described in Figure 1. Densitometric scanning of the AR bands after being normalized to the levels of β-actin. Data are expressed as the mean of 8 samples ± SEM. Control, its value was considered as 100% of the intensity of the band.

Effect of 2,4-D on AR, IGF-1 and IGF-1R mRNA from VP

To assess the effect of 2,4-D on VP at 45, 60, and 90 days of age, AR, IGF-1 and IGF-1R mRNA levels of in VP were determined by real time quantitative PCR. As indicated in Figure 5(A), IGF-1 mRNA levels decreased significantly in VP from treated groups with compared controls at PND 45 and 60 (31% and 32%, respectively). On the other hand, IGF-1R mRNA levels increased significantly 20% and 42% with respect to controls at PND 45 and PND 60, respectively (Figure 5B). However, 2,4-D treatments did not affect AR mRNA levels at any age studied (data not shown).

Discussion

Many studies have focused on chemicals that modify the function of the endocrine system. Depending on the beginning and length of exposure, such chemicals alter growth and development of hormone-sensitive organs such as the prostate gland.

The present study shows that rats exposed through the mother during pregnancy and postnatal life until weaning and treated later during development with 70 mg/kg/day of 2,4-D through diet, did not show external signs of toxicity, such as changes in body weight of pups at birth or fetal toxicity. However, a slight decrease in body weight (between 8 and 12%) at sacrifice (45, 60 and 90 days of age) was detected. In no case such decrease surpassed 15% and therefore, according to previous data from our laboratory, it was not considered to be toxicologically relevant since it did not critically affect the overall development of the animal (Bortolozzi et al., 1999).

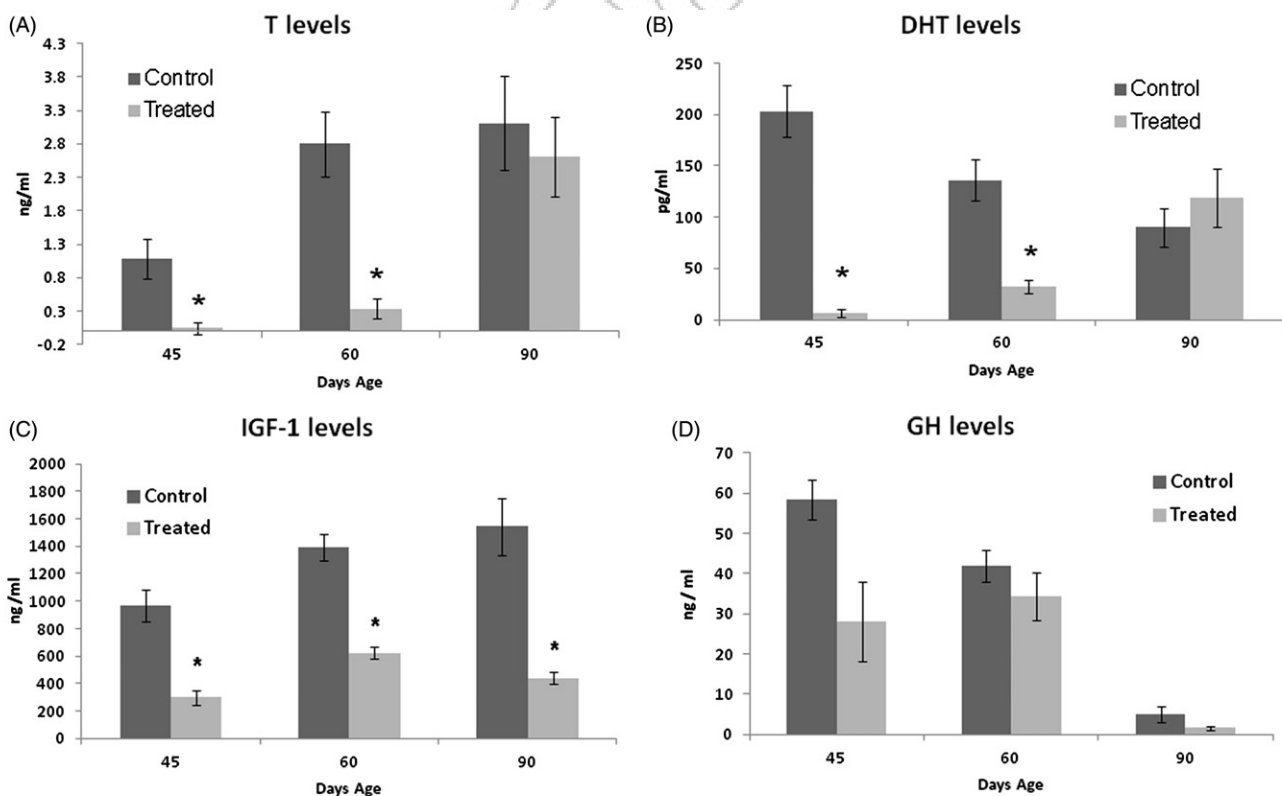

Figure 4. Serum T (A), DHT (B), IGF-I (C) and GH levels (D) expressed as ng/ml in male rats treated with 2,4-D at all ages studied. Control (black bar) and treated group (70 mg/kg/day, gray bar). Data are expressed as mean ± SE for at least eight rats for each experimental group. (*) Significance levels observed are $p < 0.05$ in comparison with control group.

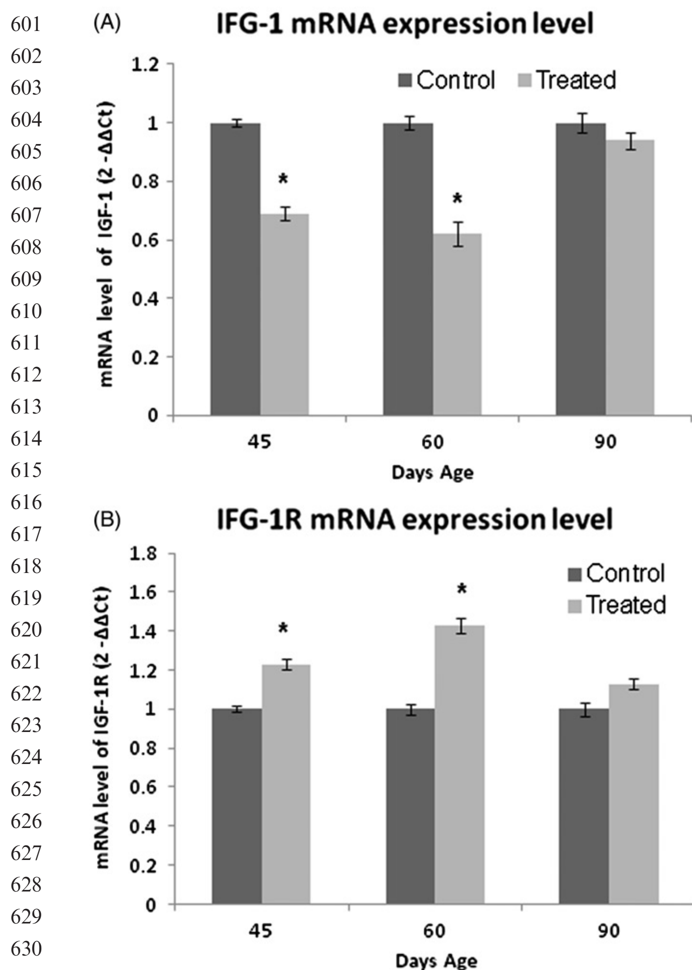

Figure 5. Effect of 2,4-D on IGF-1 (A) and IGF-1R (B) mRNA expression level in rat ventral prostate at the ages of 45, 60 and 90 days by real time quantitative PCR. The housekeeping gene GADPH was used as an internal positive control standard for quantitative analysis. The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$. At each age controls were assigned the reference value 1 and all data were expressed as mean ± SEM of at least 4 independent determinations. (*) Significance levels observed are $p < 0.05$ in comparison with control group.

The neonatal development period is considered the most vulnerable to the action of xenobiotics (Dencker & Eriksson, 1998). Pharmacological doses of diethylstilbestrol (DES) (Singh & Handelsman, 1999) and vinclozolin (Yu et al., 2004) in rats exposed during development produced a reduction in the growth and size of the prostate gland. Moreover, *in utero* exposure to these chemicals induced a higher incidence of prostate lesions in old age, including atrophy and prostatitis (Cowin et al., 2008).

In this work, we show that 2,4-D diminished VP weight as well as height of epithelial the cell layer of the alveoli in animals of 45 and 60 days of age. These data correlate with previous results from our laboratory showing delayed puberty in male rats treated with the herbicide, evidenced as a decrease in the number of sperm cells with normal morphology (Madariaga, 2007). This observation is supported by the markedly low T levels observed in peri and pubertal (45 and 60 days) animals reported in this study. At 60 days of age, the low T level was accompanied by similarly low levels of DHT, the androgen responsible for stimulating growth and function of the prostate gland, thus explaining the delayed organ development.

Since androgens exert their action on the prostate gland through AR, AR levels were also determined. It has been reported that certain chemicals, including bisphenol A, nonylphenol and fenthionare capable of interacting directly with the AR, activating transcription of AR-dependent genes in mammalian cells (Kitamura et al., 2003; Lee et al., 2003). It has also been shown that estrogenization during development reduces AR protein levels, which also decreases the response capacity to DHT and T, without modification of AR mRNA expression (Prins, 1997). The decrease in receptor protein levels was due to increased proteolytic degradation (Woodham et al., 2003). In the present work we found that AR levels, were decreased in treated animals compared to controls at 45 days of age, without changes in mRNA expression, indicating that the herbicide, may be increasing the degradation or decreasing AR protein synthesis in peripubertal rats. On the other hand, since AR expression in epithelial prostatic tissue increases with age (Prins & Birch, 1995), decreased AR protein expression may be a consequence of delayed puberty and maturation of reproductive organs. Q1

Even though the prostate is sensitive to T and DHT during development, ductal branching morphogenesis occurs before puberty, when androgen levels are low (Donjacour & Cunha, 1988). At this early stage, circulating IGF-1 and GH are elevated and therefore play a critical role in prostate development (Sandhu et al., 2006). For this reason, serum levels of GH were evaluated showing the typical pattern observed during growth, with high levels that decline with age in the young control animals, and no significant effect of 2,4-D treatment. Since GH levels are typically pulsatile, and measurement at only one point in time may not reflect possible effects of the herbicide upon GH pulsatility (amplitude or frequency), we decided to determine circulating IGF-1 that, as previously mentioned, displays more stable circulating levels. This approach, reflects more accurately the state of GH secretion, since systemic and local IGF-I synthesis depends largely on GH (Rajaram et al., 1997). IGF-1 levels decreased in 45 and 60 day old animals treated with 2,4-D. IGF-1 −/− mice show overall reduction in prostate size and specific structural deficit, including the number of ductal terminals. Administration of IGF-1 reverses these effects (Ruan et al., 1999), demonstrating the role of circulating IGF-1 in prostate development. Therefore we can conclude that at least some of the effects of 2,4-D in the development of the gland in prepuberal and puberal animals may be mediated through the decrease in circulating IGF-1 produced by the herbicide.

In addition to hormonal influences and circulating IGF-1, local factors may play a critical role in prostate normal growth. IGF-1 is produced by stromal cells. acting as a paracrine factor on epithelial cells through IGF-1R (Lipschutz et al., 1999). We found diminished IGF-1 mRNA level at 45 and 60 days of age in treated animals, accompanied by increased expression levels of its receptor. IGF-1 mRNA expression at the tissue level is also regulated by circulating GH and thus the herbicide could affect IGF-1 transcription on tissues directly, or through undetected effects on GH

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pulsatility, as mentioned above. On the other hand, the increase in IGF-1R expression levels at the local level could be compensating for the decreased local or circulating IGF-1.

Kim et al. (2005) reported that 2,4-D (50 mg/kg/day) caused an increase in the weight of androgen-dependent tissues. When administered simultaneously with T to castrated adult animals, an increase in the VP weight was observed. In this work similar results were found in 90 day old animals, in the presence of normal T and DHT levels. Thus, it can be concluded that the increase in gland weight at this age may be produced by 2,4-D acting synergistically with the normal circulating androgens.

It is worth mentioning that, some pesticides such as atrazine (nonsteroidal compound) produce different changes in the prostate. Also, a brief exposure to this compound through breastfeeding on postnatal days 1–9, suppressed suckling induced PRL release in the mothers and resulted in an adverse effect such as inflammation of the prostate in adult male offspring (Stoker et al., 1999). Also in a previous work from our laboratory, exposure to 2,4-D during lactation (postnatal days 1–16) resulted in a partial blockade in suckling induced oxytocin and PRL release in treated mothers (Stürtz et al., 2010). Therefore, the observed increase in prostate gland weight may not only be associated with hormonal induction, but also with the effects that the herbicide may have caused during lactation. This is due to the fact that, in our experimental scheme, animals are exposed from gestation to adulthood.

It has been reported that some phytochemicals, depending on their concentration, may exhibit agonist/antagonist activity on the steroid hormone system. For example, *in vitro* studies have shown that quercetin at low concentrations acts as agonist (Taepongsorat et al., 2008). The *in vivo* effect of this compound is not well known, but it has been observed that treatment with quercetin caused a dramatic expansion of the prostatic lumen, which was filled with secretion, indicating that quercetin may have increased the secretory activity of the epithelial cells. Likewise, the increase in luminal volume produces a decrease in the number of cells per unit area compared to the control (Ma et al., 2004). In the histological and morphometric observation of prostates of 90 day old male rats treated with 2,4-D, we found changes similar to those found in the previously mentioned paper, suggesting that the herbicide may act similarly. Additionally, although androgens and IGF-1 levels were restored in the adult glands of treated animals, epithelial tissue morphology remained altered, as it is expressed in the decrease in height of its cells.

Administration of low doses of estrogen to the mother during gestation increases the size of the prostate as well as AR expression in the adult offspring (Nagel et al., 1997). Studies performed on cell line cultures of human prostate cancer indicate that 2,4-D and 2,4-dichlorophenol (DCP) in combination with DHT have androgenic activity in cell proliferation and induce transactivation by androgen, possibly through increased translocation to the nucleus without alteration in AR expression levels (Kim et al., 2005). On the contrary, in this work we found a 40% increase in AR protein levels for the treated group at 90 days of age. However, the aforementioned results were observed in isolated tumoral epithelial cells, and thus cannot always be interpreted as a reflection of what would take place in normal tissue *in vivo*, as the prostatic epithelial tissue depends on stromal factors for its correct development and maintenance. Since AR mRNA levels in adult animals were not modified, the action of 2,4-D could be exerted on AR protein synthesis or degradation rates, increasing its abundance. It is interesting to point out that AR synthesis is induced by DHT, thus the herbicide would enhance this effect in animals of 90 days of age, since in the absence of androgen, 2,4-D alone did not increase AR expression in mature animals.

Conclusion

2,4-D belongs to the group of non-steroidal environmental substances with the ability to act as endocrine disruptors (EDs) (Diamanti-Kandarakis et al., 2009). The National Institutes of Health (USA) considers 2,4-D as a potential ED (Anon, 2004). Numerous papers indicate that IGF-1 and RIGF-1 expression are influenced by steroid and peptide hormones (Yu & Rohan, 2000). Therefore, variations detected in the members of the IGF family could be partially due to a modification of gonadal steroid concentrations and their receptors since the herbicide produces:

(1) Decrease of serum androgen and AR levels in the prostate of male pups at their youngest age. This effect is similar to the one observed for environmental substances with estrogenic effect (Singh & Handelsman, 1999). The opposite effect was observed in adult age, where T levels were normal and AR protein expression was induced in the prostate was induced.
(2) Serum IGF-1 levels were diminished in pups at the three ages studied.
(3) VP from prepub and puber males showed a decrease in IGF-1 mRNA levels along with an increase in its receptor expression.

These results indicate that 2,4-D could behave as an ED, affecting prostate development. Future research should focus on the nature of the major deleterious effects produced by the herbicide on VP, establishing whether if such changes are permanent or reversible and able to if they affect male fertility and/or prostate function.

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Declaration of interest

The authors report no declarations of interest.

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