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Original Article

Testicular alterations in cryptorchid/orchiopexic rats chronically exposed to acrylamide or di-butyl-phthalate

Thania R. R. Lima[1], Nathália P. Souza[1,2], Ana P. Ferragut Cardoso[1,3], Lígia M. M. Gomide[1,4], Merielen G. Nascimento e Pontes[1,5], Hélio A. Miot[6], Lora L. Arnold[7], Samuel M. Cohen[7], and João Lauro V. de Camargo[1]

[1] Center for Evaluation of Environmental Impact on Human Health (TOXICAM), Department of Pathology, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, 18618-687, São Paulo, Brazil
[2] Present address: Allergisa Pesquisa Dermato Cosmética, Campinas, 13083-897, São Paulo, Brazil
[3] Present address: Department of Pharmacology and Toxicology, University of Louisville, Louisville, 40202, KY, USA
[4] Present address: Centro Universitário Sudoeste Paulista (UniFSP), Itapetininga, 18213-145, São Paulo, Brazil
[5] Present address: Syngenta Crop Protection, São Paulo, 04730-000, São Paulo, Brazil
[6] Department of Dermatology and Radiotherapy, Botucatu Medical School, São Paulo State University (UNESP), Botucatu, 18618-687, São Paulo, Brazil
[7] Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, 68105, NE, USA

Abstract: Exposure of Sprague-Dawley (SD) rats to acrylamide (AA) or di-butyl-phthalate (DBP) from the 12th gestational day to the 16th postnatal week (PNW) has been shown to reduce the effectiveness of orchiopexy in recovering the testicular alterations associated with experimental cryptorchidism established at weaning. Herein, we provide information about the long-term effects of AA or DBP on the testes of cryptorchid/orchiopexic rats. Male offspring exposed *in utero* to 10 mg/kg/day AA or 500 mg/kg/day DBP underwent bilateral surgical cryptorchidism at the 3rd PNW and orchiopexy at the 6th week, with continuous exposure to the chemicals through diet until the 58th week. Regardless of the test chemical, there were severe qualitative/quantitative alterations in the seminiferous tubules and increased numbers of Leydig cells. There was an increase and decrease in the number of tubules with c-Kit- and placental alkaline phosphatase-labeled germ cells, respectively, as compared to those in the control group, suggesting an imbalance between apoptosis and cell proliferation processes. The histological scores of the testicular lesions at the end of this one-year study were higher than those in the previous 16-week study, indicating that exposure of rats to the toxicants AA or DBP enhanced the testicular alterations induced by the chemicals beginning at the intra-uterine life, and impaired the effectiveness of orchiopexy in restoring the testes to normal morphology. Although the present experimental protocol does not completely replicate the natural human undescended testes, our findings may contribute to understanding the alterations occurring in cryptorchid/orchiopexic testes potentially exposed to exogenous chemicals for extended periods. (DOI: 10.1293/tox.2021-0045; J Toxicol Pathol 2022; 35: 159–170)

Key words: rat surgical cryptorchidism, orchiopexy, chronic toxicity, testicular germ cells, di-butyl-phthalate, acrylamide

Introduction

The incidence of male reproductive tract disorders, including cryptorchidism (CPT), hypospadias, infertility, and testicular germ cell tumors, has been reported to be increasing worldwide. These disorders seem to have a multifactorial origin and, although the role played by genetic factors in them remains unclear, geographical and temporal variations in their prevalence suggest that environmental factors, such as exposure to chemical contaminants, contribute to their development. Among these disorders, undescended testes or CPT occurs in 2–9% of full-term and more than 30% of pre-term males[1–3]. In CPT, the gonadal microenvironment is modified, and there may be an increase in the susceptibility of the testes to xenobiotics[4]. Although most cryptorchid testes later spontaneously descend to the scrotum, a considerable number continue into puberty and adulthood, and are variably associated with increased risks of infertility and malignant germ cell tumors[2, 5, 6]. To recover testicular function and minimize any future risk, surgical correction of CPT, *i.e.*, orchiopexy, should be performed early in the pre-pubertal period[5, 7].

Various rodent models of CPT have been established using surgical intervention, hormonal manipulation, or

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*Corresponding author: TRR Lima
(e-mail: thania.lima49@gmail.com)

transgenic techniques, all of which present advantages and limitations with respect to similarity to the human condition[8, 9]. These different rat models of CPT show atrophy of seminiferous tubules, a germinative epithelium presenting germ and Sertoli cell maturation arrest and degeneration[8–10], a scenario that resembles the human undescended testes. As in humans, it has been suggested that experimentally cryptorchid rats should receive orchiopexy as early as possible to re-establish normal spermatogenesis[8–13].

Experimental exposure to environmental chemicals, such as acrylamide (AA) or phthalates, has been associated with testicular disorders in rats[14–16]. Humans are also potentially exposed to low levels of these chemicals because of their common use in industrial processes and occurrence in household items. As a chemical product, AA has a wide range of technical applications, including as a flocculant in wastewater treatment, and in gel electrophoresis, paper processing, and permanent press fabrics. AA may also occur as a by-product when starchy foods are cooked at high temperatures, thus resulting in an estimated potential average intake of about 0.3 to 0.8 μg/kg/day, as reported by studies conducted in the United States of America and Switzerland[17, 18]. AA is a well-established human and rodent neurotoxin that has an estimated no-observed adverse effect level of 0.5 mg/kg/day and lowest observed adverse effect level of 2 mg/kg/day for male rats, with these data being slightly variable among experimental studies[17, 19].

Likewise, phthalates belong to a very common chemical group; they confer malleability, transparency, and durability to an enormous variety of plastic products such as household objects, toys, medical equipment, and drugs, among other applications[20], and are ubiquitous environmental contaminants. Human exposure to di-butyl-phthalate (DBP) occurs mainly through oral, inhalation, and dermal routes, with food being the major source of exposure, at an estimated range of 7–10 μg/kg/day[21, 22]. While the detailed mechanisms of phthalate action are still under investigation, reduction of circulating androgen levels seems to be the most sensitive outcome[23]. Accordingly, DBP has been included in the endocrine disruptor group of chemicals, based on animal and *in vitro* studies[24], although evidence for this is lacking in humans. In addition to its anti-androgenic effects, induction of oxidative stress has also been considered among the modes of action of phthalate[25]. *In utero* exposure to DPB has been proposed as a possible model of human testicular dysgenesis syndrome[14].

Experimental and epidemiological studies indicate that both AA and phthalates are found in breast milk and can cross the placental barrier, leading to exposure to the offspring[26–28]. In a previous study, we reported the effects of AA or DBP on the testes of cryptorchid/orchiopexic Sprague-Dawley (SD) rats continuously exposed to each of those chemicals, *in utero* and postnatally[13]. The animals were made cryptorchid at weaning and underwent orchiopexy three weeks later. At the end of the study, *i.e.*, the 16th postnatal week (PNW), CPT was found to be associated with testicular atrophy and severe histological alterations. Orchiopexy was effective in promoting the structural recovery of the non-chemically exposed cryptorchid testes, which became morphologically similar to the controls when evaluated within 10 weeks after relocation of the testes to the scrotum. Animals exposed continuously to 10 mg/kg/day AA or 500 mg/kg/day DBP, but not subjected to CPT, did not develop histological lesions, which implies that the applied doses did not damage the gonads within the period studied, at least morphologically. However, when the cryptorchid animals received AA or DBP, the testicular alterations could not be entirely reversed by means of orchiopexy, *i.e.*, upon exposure to these testicular toxicants, the recovery of the cryptorchid testes was impaired[13].

Since continuous exposure to AA or DBP impaired the restoration of the rat cryptorchid testes to normal in a short-term 16-week long study, questions were raised about the eventual progression of the CPT-induced lesions, after an extended period of AA or DBP exposure, such as 52 weeks (one year) after performing the orchiopexy. This later assessment could be particularly interesting, because there is an increased risk of development of infertility and malignant testicular germ cell tumors in humans with a history of current or previously corrected CPT. To address this question, two independent groups of cryptorchid/orchiopexic rats exposed to AA or DBP that were established during the Souza *et al.*[13] experiment were kept alive under the same experimental conditions until the 58th PNW. No other groups were established for comparisons, because the aim of evaluating these two groups was to register the type of alterations that would eventually occur after such an extended period of observation.

Materials and Methods

General

The present experiment is part of an already published study[13] and was approved by the Local Committee for Ethics in Animal Experimentation (protocol no. 1209/2017). Briefly, eight-week-old female and male SD rats were mated overnight at the proportion of two females to each male. Vaginal smears were collected daily, and the day of sperm detection was defined as gestational day 0 (GD0). Seventeen pregnant female SD rats (control, n=4; AA-exposed, n=5; DBP-exposed, n=8) were exposed daily by gavage to a dose of 10 mg/kg body weight/day of 99% pure AA (CAS 79-06-1; cat. A9099, Sigma-Aldrich, St. Louis, MO, USA) or 500 mg/kg body weight/day of 99% pure DBP (CAS 84-74-2; cat. 524980, Sigma-Aldrich), from the 12th GD to the 21st GD. These dose levels were selected because they were able to induce testicular alterations when exposed *in utero* and induced dysgenetic areas in postnatal testes of rat[14, 15, 29, 30]. Although these dose levels did not induce testicular lesions in rats not made cryptorchid in our previous short-term studies with SD rats[13, 31], the doses were kept at the same levels for the sake of comparison with other studies performed at the same laboratory. After birth, the pups were putatively exposed to AA or DBP through maternal milk until postna-

tal day (PND) 21, when up to three male pups from each litter were allocated to their respective test groups and underwent surgical procedures for CPT. The time-point adopted for surgical induction of CPT (weaning, PND 21, and 3rd PNW) corresponded to a period of considerable testicular activity. From this time-point to the end of the study (the original study at the 16th PNW[13] and the present study at the 58th PNW), the animals were fed dietary concentrations of either chemical, corresponding to the doses provided to the dams (AA, 120 ppm; DBP, 6,000 ppm). During the 6th week, the animals were submitted to orchiopexy (CPT-R) (Fig. 1). The interval between the two surgical procedures was defined as the period between the appearance of the first round spermatids in the rat and the complete maturation of these cells in mature elongated spermatids, which are expected to be observed around PND 44[32, 33]. Based on the xenobiotic exposure and surgical procedures, the groups were designated as AA/CPT-R or DBP/CPT-R (Fig. 1). Detailed descriptions of CPT and orchiopexy procedures and the establishment of controls can be found elsewhere[10, 13]. Proper care for asepsis and infection control was performed before and after surgery. At the end of the experiment, the animals were anesthetized between 08:00 a.m. and 10:00 a.m. and euthanized by exsanguination *via* heart puncture.

Immediately after euthanasia, the testes were removed, weighed, fixed in modified Davidson's fixative for 24 h[34], and processed for histological analyses. The gonadosomatic index (GSI, or testicular relative weight) was determined according to the formula: $GSI = \left(\frac{WG}{WT}\right) \times 100$, where WG is the testis weight and WT is the animal body weight[35].

Histological examination

Sections of 5 µm thickness were cut through the middle transverse plane of the paraffin-embedded testes and stained with hematoxylin and eosin (H&E). Histological analysis of the seminiferous tubules was performed blindly, without identification of the corresponding experimental chemical exposure. A two-step analysis of H&E-stained testicular sections was performed. First, the seminiferous tubules were analyzed qualitatively, according to the standardized nomenclature of rat testicular histological lesions[36]. Next, an average of 250 rounded sections of seminiferous tubules per animal were classified using a four-class ranking system, based on the most frequent histological tubular alterations, as described in the original study[13]: Class 1, normal tubules; Class 2, tubules with spermatid-like and sperm mature cells, but also showing alterations such as intra-epithelial vacuoles, apoptotic bodies, and/or multi-nucleated germ cells; Class 3, tubules with the same elements as Class 2, but presenting only spermatocytes and spermatogonia (germ cell maturation arrest/epithelial layer atrophy); and Class 4, increased number of Sertoli cell-only (SCO) tubules. The results obtained using this system were adjusted to an adapted H-score, which is applied for semi-quantitative evaluations in immunohistochemistry analyses[37, 38]. The incidence

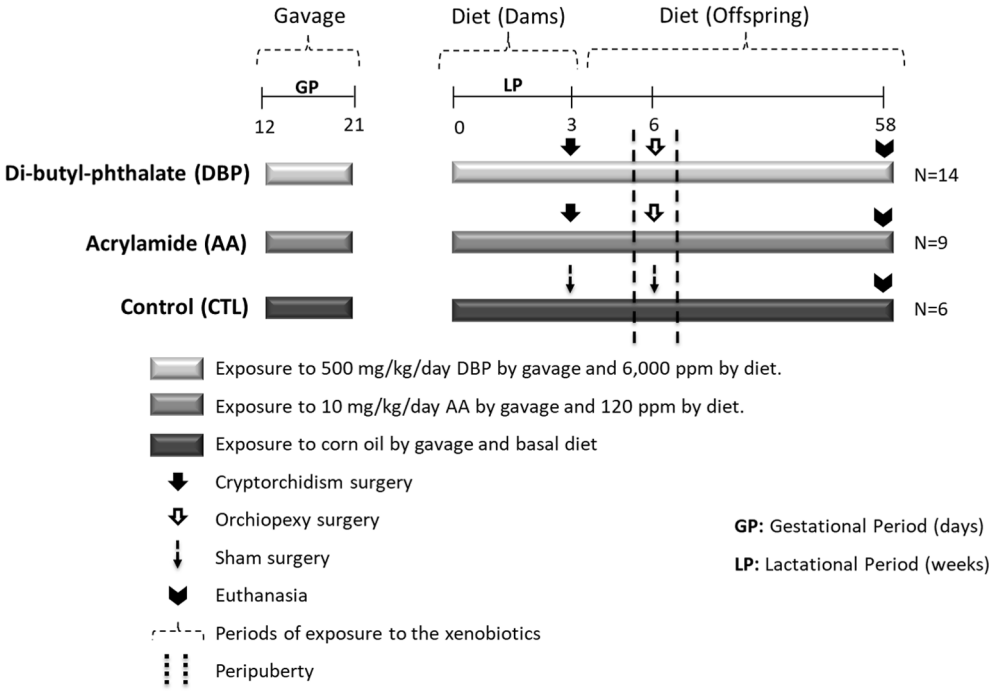

Fig. 1. Experimental design. Dams were exposed by gavage to a dose of 10 mg/kg/day AA or 500 mg/kg/day DBP during the gestational days 12 to 21. At the 3rd postnatal week (weaning), the male offspring underwent surgery for cryptorchidism, which was reversed by orchiopexy 3 weeks later. After weaning, the animals were exposed to the chemicals through diet. Euthanasia was performed when the rats were 58 weeks old. The number of animals in each group represents the number of animals that survived to the end of the study.

(%) of tubules in each class per animal was calculated by multiplying the number of tubules in that class by 100 and dividing by the total number of tubules counted in the testis section. The score for each group was generated by adding the number of tubules in class 1 (1×), class 2 (2×), class 3 (3×), and class 4 (4×), and then dividing by the total number of tubules counted in the respective tissue sections.

Morphometric analysis of seminiferous tubules and Leydig cells (LCs)

Images of the seminiferous tubules were acquired from the H&E-stained slides using an optical microscope coupled to a charge-coupled device camera (BX41/Q-capture Pro 5.1, Olympus, Tokyo, Japan) with a resolution of 1280 × 960 pixels in TIFF format. Using ImageJ software - version 1.49d (National Institutes of Health, Bethesda, MD, USA), the diameter and height of the seminiferous epithelium were measured in 200×-magnified images captured from 15 randomly selected round or rounded seminiferous tubules[35, 39]. The images of the selected fields were scaled from pixel to metric (micra) values.

To obtain the volumes of the germinative epithelium, as well as, of the epithelium plus lumen of the seminiferous tubules, fifteen 400×-magnified testicular histological fields were randomly analyzed under a 475-intersection point grid. The epithelial volume was estimated by counting the intersections of the germinative cells using the ImageJ "point or multi-point" tool; the total volume was obtained by adding the number of intersections on the lumen. The total number of points obtained in each analysis was divided by the total number of points generated by the reticulated grid (7125) and multiplied by the total testicular volume, which was assumed to be equal to the net weight, because the density of the testis was assumed to be 1.0. To obtain the total net testicular volume, the relative weight of albuginea (6.5%) was subtracted from the gross testicular weight[35, 39].

The length of the seminiferous tubules (TL) was estimated according to the formula $TL = \frac{TVS}{\pi r^2}$, where TVS is the total volume of the seminiferous tubule, πr^2 is the seminiferous tubule area, with π equal to 3.14 and r the tubular diameter divided by two[36]. All tubular morphometric analyses preferentially examined tubules in the stages VII–VIII of spermatogenesis, which represent the most active phases of the spermatogenic process[36].

Receptor tyrosine kinase (c-Kit)-immunostained slides (see below) were used to facilitate the morphometric data collection of LCs[40]. The slides were digitalized using Pannoramic MIDI hardware (3DHISTECH Ltd., Budapest, Hungary), with images captured using Pannoramic Viewer software (version 1.15.4), at 400× magnification. All morphometric analyses were performed using ImageJ software (version 1.48). A reticulated grid with 504 points of intersection was randomly allocated over 15 histological fields, to determine the volume occupied by LCs within the testes. The number of intersections on the LCs was counted using the ImageJ "point or multi-point" tool. The number of points was divided by the total number of points generated by each reticulated grid (7560) and multiplied by the total testicular volume (as indicated above)[39, 40].

The LC somatic index (LSI) was calculated according to the formula, $LSI = \left(\frac{TVOLC}{BW}\right) \times 100$, where TVOLC corresponds to the total volume occupied by LCs in the testis and BW is the body weight of the animal[41]. Using the number of LCs in 15 histological fields (NLC/μm²), the average number of these cells per ml was estimated according to the formula, $NLC/ml = \left(10^{10}\right) \times \left(Cells/\mu m^{2^{1.5}}\right)$. The result was multiplied by the testicular volume of each animal, to determine the number of LCs/testis. Further, the average area occupied by an individual LC was obtained by evaluating 30 cells/animal. Each LC was delimited with the aid of the ImageJ "polygon" tool, and the average area of these 30 cells[40] was considered the average area of the individual LC in each animal.

c-Kit and placental alkaline phosphatase (PLAP) immunohistochemistry

Testicular histological sections of 4 μm thickness were mounted on silanized slides (Knittel Glass, Braunschweig, Germany) and subjected to specific immunohistochemical staining protocol for detection of c-Kit or PLAP, marker antigens of human seminoma, to evaluate damaged rodent testes[42, 43]. Antigen retrieval was performed using Trilogy solution® (Cell Marque Corp., Rocklin, CA, USA) in a water bath, for approximately 30 min in case of c-Kit, or NaCitrate (Sigma-Aldrich) in a microwave for approximately 5 min at 95–99°C in case of PLAP. Endogenous peroxidase and proteins were blocked using Bloxall® (Vector Corp., Burlingame, CA, USA) and Sniper (Biocare Medical, LLC, Pike Lane Concord, CA, USA) for c-Kit, or using 3% H_2O_2 (Sigma-Aldrich) and horse serum (Vector Corp.) for PLAP. The sections were then incubated overnight at 4°C with each of the primary antibodies diluted to a ratio of 1:100 (c-Kit – Bioss Inc., Woburn, MA, USA, rabbit polyclonal antibody, bs-0672R; PLAP – LS BioSciences Inc., Seattle, WA, USA, rabbit monoclonal antibody, LS-C189575). c-Kit signal amplification was performed using a biotin-free polymer detection system with the MACH 4 Universal HRP-Polymer kit (Biocare Medical), according to the manufacturer's instructions, while for PLAP, the Avidin-Biotin Complex kit was used for signal detection (Vectastain® Elite® ABC Kit, Vector Laboratories). Lastly, 3,3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories) was used as the chromogenic substrate. The sections were counterstained with Harris hematoxylin. Histological samples from a human classical seminoma were used as positive controls for immunohistochemistry. For the negative control, the same material was processed without application of the respective primary antibodies.

Since depletion of germ cells occurred with variable intensity in many seminiferous tubules of the chemically exposed animals, the determination of the number of labeled cells for each marker could be misleading. To mini-

mize biased estimates, c-Kit- and PLAP-positive cells were assessed qualitatively within the seminiferous epithelium. A ratio was then generated between the number of tubules with labeled germ cells ("positive tubules", %) and the total number of seminiferous tubules in each testicle section analyzed.

Statistical analysis

The study experimental units were male rats, adjusted to their dams. As more than one pup from the same litter comprised each of the experimental groups, both the pups and dams were considered for the statistical analyses, in order to minimize the "litter effect". Variables were analyzed using a mixed-effects generalized linear model followed by a *post-hoc* Sidak correction[44] and presented as mean ± standard deviation or as the median (p25–p75), depending on their distribution pattern. Analyses were performed using Statistical Package for Social Science (SPSS) 25.0 Statistics software (SPSS Incorporation, IBM, Armonk, NY, USA). Differences were considered significant at $p<0.05$. Variations in the number of samples among parameters occurred either due to testicular atrophy and cell loss impairing the acquisition of data or because some data were excluded based on being statistically determined to be outliers.

Results

Chemical exposure and body/testicular weights

At the end of the study, the final mean daily intake of each chemical (495 mg/kg/day for DBP and 8.25 mg/kg/day for AA) was estimated from the mean dietary consumption during the postnatal period (data not shown). Compared to the control group, decreases in the body weights of the animals exposed to the chemicals occurred from the beginning of the study, with decreases more evident in the AA-exposed group. Decreases intensified from the 22nd week, although the difference was not statistically significant. Accordingly, there were no significant differences in body weight at the end of the study (58th week) (Fig. 2; Table 1). The absolute and relative (GSI) testicular weights and volumes of AA/CPT-R and DBP/CPT-R animals were significantly lower than those of the sham-operated controls ($p<0.05$) (Table 1), indicating that orchiopexy was not effective in allowing normal testicular growth.

Histology of the testes

Control animals presented morphologically normal seminiferous tubules, with active and complete spermatogenesis (Fig. 3A). Rats exposed to AA or DBP showed several similarly altered tubules among the majority of morphologically normal rats. Tubular alterations included germ cell exfoliation and maturation arrest (Fig. 3B–C), atrophy of the germinative epithelium, vacuoles within the seminiferous epithelium (vacuolated Sertoli cells), multi-nucleated giant cells in the lumen, and interstitial edema (Fig. 3B inset b, and 3C). Some tubules were variably atrophic, unevenly presenting a SCO pattern (Fig. 3D–F). Other tubules presented some isolated and enlarged germ cells, apparently spermatogonia, with hyperchromatic nuclei and pleomorphic cytoplasm (Fig. 3C inset c, and 4B). A few tubules presented intraluminal calcifications (Fig. 3D inset). Apparent LC hyperplasia was observed in some areas of the testes sections, where hyperplasia occurred not as nodules but as sheets of LCs intermingled with degenerated seminiferous tubules (Fig. 3E).

Histological examination was used to classify the alterations into four classes (Table 2) according to the scoring system described in the Materials and Methods section. Control animals had the lowest score (median=1.03, 1.03–1.06) (Table 2). The AA/CPT-R and DBP/CPT-R groups had significantly ($p<0.05$) higher scores, as compared to the controls, with the DBP/CPT-R group scoring (median=3.63; 2.31–3.90) slightly higher and with a wider range, as compared to the AA/CPT-R group (median=3.48; 3.11–3.60).

Morphometry of the seminiferous tubules and LCs

At the 58th week, the seminiferous tubules of the chemically exposed groups had significantly lower values for the following parameters, when compared to the controls (Table 3): diameter and area, length (only the AA/CPT-R

Table 1. Body Weights and Absolute and Relative Testicular Weights of Rats Exposed *In Utero* and Postnatally to Xenobiotics Acrylamide (AA) or Di-n Butyl-phthalate (DBP), Followed by Surgery for Induction of Cryptorchidism and Orchiopexy (CPT-R)

Groups	Body weight (g)[a]	Absolute testis weight (g)[b]	GSI (%)[a,c]	Testicular volume (mL)[b]
CTL	553.8 ± 48.6	1.7 (1.6–1.7)	0.3 ± 0.0	1.6 (1.5–1.6)
(n)	(6)	(6)	(6)	(6)
AA/CPT-R	501.0 ± 73.8	0.7 (0.7–0.8)*	0.1 ± 0.0 *	0.6 (0.6–0.7)*
(n)	(9)	(5)	(5)	(5)
DBP/CPT-R	541.4 ± 69.4	0.6 (0.5–1.3)*	0.1 ± 0.1*	0.5 (0.4–1.2)*
(n)	(14)	(7)	(7)	(7)

Values obtained at the end of the experiment (58th week).
Values have been expressed as [a]mean ± SD or [b]median (p25–p75). Generalized linear mixed-effects model with gamma distribution followed by *post-hoc* Sidak correction. [c]GSI: gonadosomatic index or testicular weight relative to body weight (%); n is the number of animals per group.
*Significantly different from the control group ($p<0.05$).

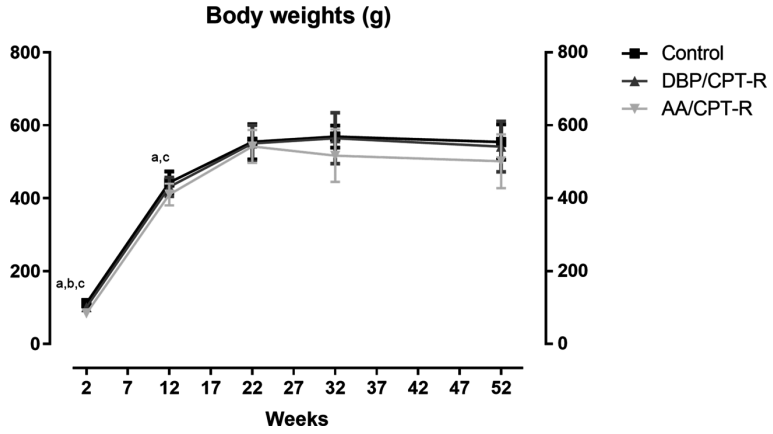

Fig. 2. Body weights of Sprague-Dawley rats. Generalized linear mixed-effects model with gamma distribution. [a]AA/CPT-R and [b]DBP/CPT-R significantly differed from control (week 2: AA/CPT-R p=0.000 and DBP/CPT-R p=0.008; week 12: AA/CPT-R p=0.002 and DBP/CPT-R p=0.394); [c]Significant differences between the chemically-exposed groups (week 2: p=0.000; week 12: p=0.002).

Table 2. Histological Ranking[a] of the Testes of Rats Exposed *In Utero* and Postnatally to Xenobiotics Acrylamide (AA) or Di-n Butyl-phthalate (DBP), Followed by Surgery for Induction of Cryptorchidism and then Orchiopexy (CPT-R)

Groups	Score[b]
CTL	1.03 (1.03–1.06)
(n)	(6)
AA/CPT-R	3.48 (3.11–3.60)*
(n)	(9)
DBP/CPT-R	3.63 (2.31–3.90)*
(n)	(14)

Values obtained upon examination at the end of the experiment (58th week).
[a]See *Materials & Methods* for classification of the testes according to histological alterations and calculation of scores. [b]Histological score values have been expressed as median (p25–p75); generalized linear mixed-effects model with gamma distribution followed by *post-hoc* Sidak correction. n: number of animals per group. *Significantly different from the control group ($p<0.05$).

group was significantly lower), total volume, and height and volume of the germinative epithelium, in line with the reduced weights of the respective gonads at that time-point. Additionally, these groups presented significantly ($p<0.05$) increased number of LCs/ml when compared to the control. Other LC parameters did not differ significantly between each chemically exposed group and the control group.

c-Kit and PLAP labeling

Weakly labeled c-Kit and PLAP cells occurred diffusely throughout the germinative epithelium in the testes of the AA/CPT-R and DBP/CPT-R animals. These cells were found less frequently in the control group, where they were scattered among basal spermatogonia up to the level of primary spermatocytes (Fig. 4A–F).

Although both chemically treated groups had higher proportions of tubules with positively labeled cells relative to the control, a significant predominance of c-Kit-positive tubules was observed only in the AA/CPT-R group (Table 4). In contrast, the proportion of PLAP-positive tubules was significantly lower in both the AA/CPT-R and DBP/CPT-R groups than in the control animals, with the difference being more pronounced in the AA/CPT-R group, corresponding to 78% of the control value (Table 4).

Discussion

The present observations indicate that one year after performing orchiopexy during the pre-pubertal period (6th PNW), the gonadal morphologic alterations in rats continuously exposed to AA or DBP since *in utero* life and treated with CPT/orchiopexy were not restored to normal. This finding should be considered from the perspective of previous short-term studies carried out by this laboratory that documented complete morphological restoration of cryptorchid testes upon orchiopexy in untreated animals[10], but not in rats simultaneously exposed to AA or DBP[13]. In the present study, animals that underwent only CPT/orchiopexy or only AA or DBP treatments were not evaluated, as we have already reported that orchiopexy was effective in restoring normal morphology within 10 weeks[10, 13]. In addition, the long-term testicular effects of individual exposure to each chemical have been reported at doses equal to or close to those used in this study: 500 mg/kg DBP after 90 days[29] and 2 mg/kg AA after 1 year[45]. By using the limited experimental groups of interest, this study also attended to the 3R principles of using a reduced number of test animals[46].

At the end of the study, the animals exposed to the chemicals had mean body weights that did not differ significantly from the control group (Table 1), indicating that a systemic adjustment of the animals to each of the chemical stressors occurred. However, while the gonads of non-exposed animals had no conspicuous alterations, the gonads of chemically treated animals showed severe and similarly altered changes, irrespective of the chemical. The testes were atrophic, with absolute and relative weights ranging from 30% to 50% of the control values (Table 1). In line with the atrophy, all tubular morphometric parameters were reduced, resulting in total volumes of the seminiferous tubules in the AA/CPT-R and DBP/CPT-R animals being 33%–34% of the control (Table 3). When the histological scoring system developed by our laboratory[13] was applied, alterations of the germinative epithelium scored 3.48 and 3.63 in AA/CPT-R and DBP/CPT-R animals, respectively, both contrasting significantly with the control group, which scored 1.03 (Table 2). As expected, due to the extended duration of this experiment, these lesions were more severe than those observed in the previous 16-week long study that registered histological scores of 2.9 and 2.5 in rats treated

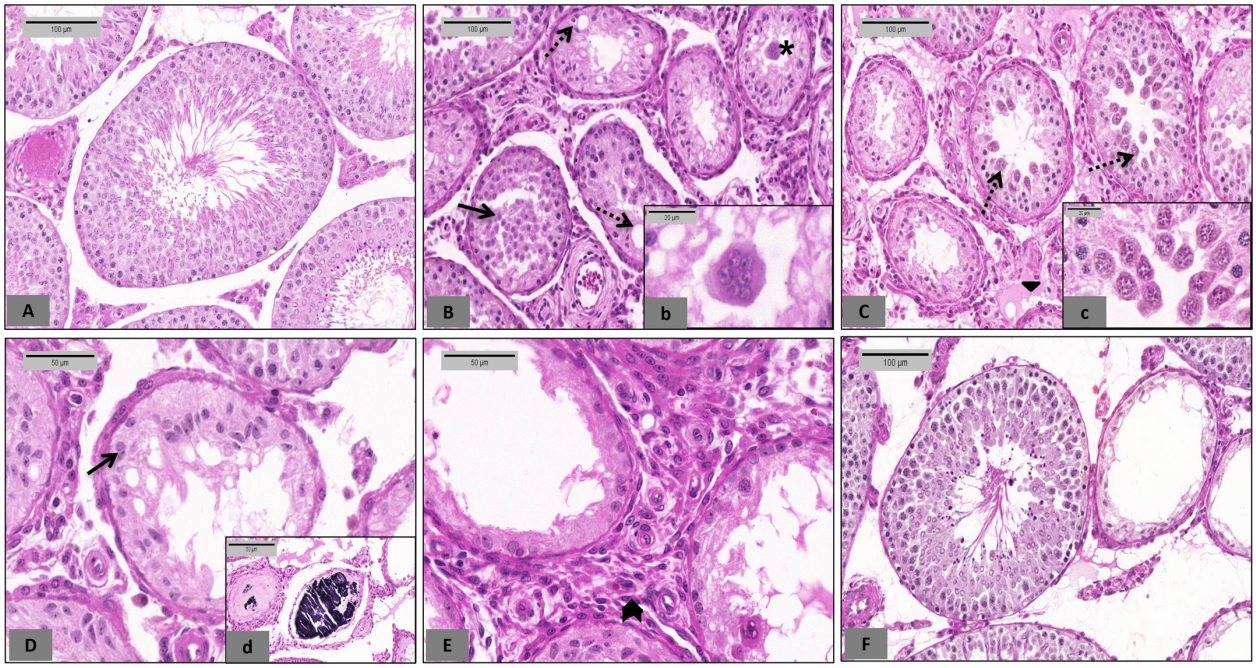

Fig. 3. Testes of Sprague-Dawley rats (H&E-stained) at 58 weeks of age. (A, 100 µm) Control - normal seminiferous tubules with complete spermatogenesis. Treated groups with several histopathological changes: (B, 100 µm) vacuolization (dotted arrow), multi-nucleated germ cells (asterisk and b, inset - 20 µm), germ cells exfoliation (black arrow); (C, 100 µm) hyperchromatic nuclei and pleomorphic cytoplasm (dotted arrow and c, inset - 20 µm), and interstitial edema (triangle); (D, E, 50 µm) tubules with "Sertoli cells only" (black arrow), "apparent" Leydig cell hyperplasia (black arrowhead); (d, inset - 50 µm) intratubular calcification of debris within an atrophic tubule; (F, 100 µm) tubular atrophy with spermatogenesis arrest occurring among apparently normal tubules.

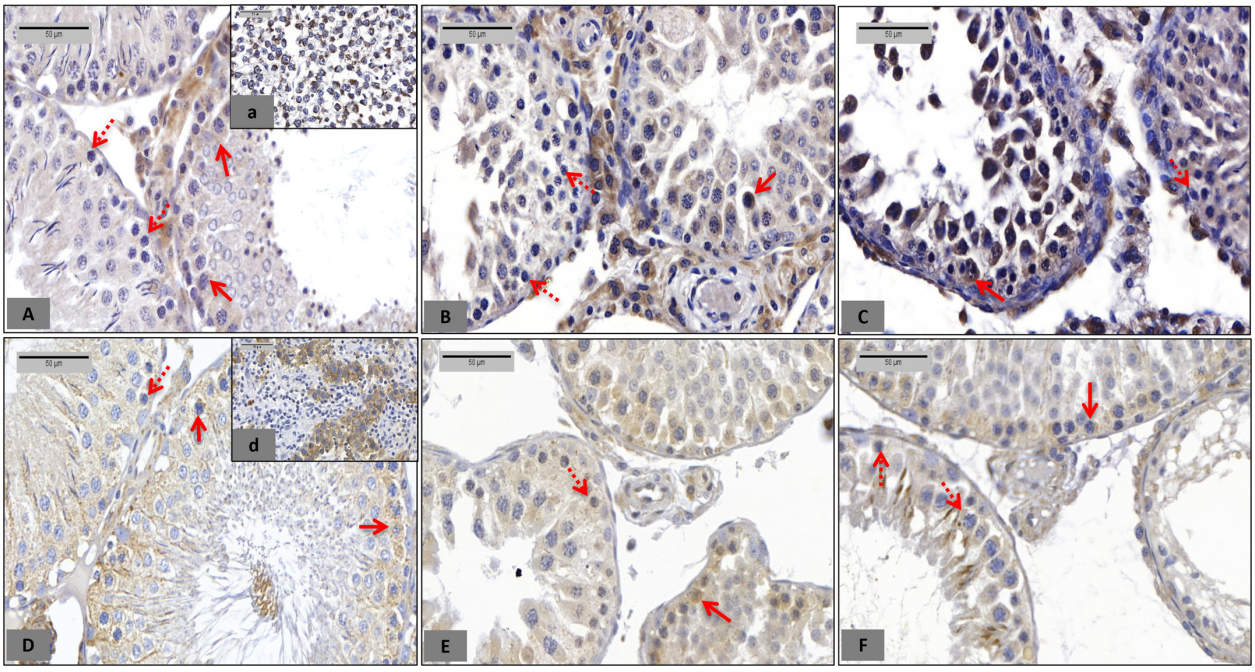

Fig. 4. Testes of Sprague-Dawley rats at 58 weeks of age. Immunohistochemistry staining for c-Kit and PLAP (50 µm, solid red arrows). (a and d, inset) - Human classical seminoma, positive control for immunohistochemistry; c-Kit: (A) Control – Positive staining scattered among basal spermatogonia up to the level of primary spermatocytes, (B) AA/CPT-R and (C) DBP/CPT-R - Spermatogonia occurred diffusely in the germinative epithelium, with weak positive cytoplasmic staining. PLAP: (D) Control – Positive staining scattered among basal spermatogonia up to the level of primary spermatocytes, (E) AA/CPT-R and (F) DBP/CPT-R - Weak positive cytoplasmic staining in germ cells, with a decrease in the number of labeled tubules, especially in the AA/CPT-R group. Non-stained cells in the dotted red arrows.

Table 3. Morphometric Parameters of the Seminiferous Tubules and Leydig Cells of Rats Exposed *In Utero* and Postnatally to Acrylamide (AA) or Di-n Butyl-phthalate (DBP), Followed by Surgery for Induction of Cryptorchidism and then Orchiopexy (CPT-R)

Parameters	CTL (n)	AA/CPT-R (n)	DBP/CPT-R (n)
Seminiferous tubules diameter (μm)[a]	275.6 ± 13.2 (6)	193.0 ± 51.3* (7)	193.1 ± 66.2* (14)
Seminiferous tubules area (mm^2)[a]	59.8 ± 57.9 (6)	31.0 ± 17.1* (7)	32.5 ± 21.2* (14)
Seminiferous tubules length (m)[a]	18.8 ± 1.9 (6)	12.9 ± 4.9* (5)	15.1 ± 7.7 (7)
Seminiferous tubules total volume (μL)[a]	1112.7 ± 87.7 (6)	362.5 ± 137.7* (5)	384.0 ± 273.9* (7)
Germinative epithelium height (μm)[a]	75.0 ± 5.2 (6)	54.5 ± 15.8* (7)	49.7 ± 15.8* (14)
Germinative epithelium volume (μL)[a]	959.1 ± 77.4 (6)	301.7 ± 109.8* (5)	307.6 ± 224.3* (7)
Leydig cells total number/ml ($\times 10^5$)[b]	2.1 (1.8–2.5) (6)	9.4 (5.3–17.7)* (6)	5.5 (3.1–13.6)* (6)
Leydig cells total number/testis ($\times 10^5$)[b]	3.3 (2.8–3.5) (6)	5.7 (2.2–6.8) (5)	3.6 (3.2–7.4) (5)
Leydig cells total volume (μL)[b]	120.4 (111.6–130.8) (6)	130.0 (73.9–157.2) (5)	116.0 (102.6–149.5) (5)
Leydig cell individual area (μm^2)[a]	72.7 ± 7.7 (5)	65.2 ± 9.9 (6)	61.3 ± 5.6 (6)
Leydig cell somatic index (LSI; %)[b]	21.9 (20.4–23.5) (6)	22.4 (15.2–31.2) (5)	19.5 (18.8–21.6) (5)

Values obtained upon examination at the end of the experiment (58th week).
Values have been expressed as [a]mean ± SD or [b]median (p25–p75); generalized linear mixed-effects model with gamma distribution followed by *post-hoc* Sidak correction. n: number of animals per group; LSI: volume occupied by Leydig cells in the testes relative to body mass. *Significantly different from the control group ($p<0.05$).

Table 4. Percentage of Seminiferous Tubules with Cells Immunohistochemically Stained with c-Kit or PLAP in the Testes of Rats Exposed *In Utero* and Postnatally to Acrylamide (AA) or to Di-n-butyl-phthalate (DBP), Followed by Surgery for Induction of Cryptorchidism and then Orchiopexy (CPT-R)

	Positive seminiferous tubules (%)[a]	
Groups	c-Kit	PLAP
CTL (n)	58.5 ± 22.9 (6)	99.3 ± 1.1 (4)
AA/CPT-R n)	90.6 ± 6.7*[b] (6)	75.5 ± 22.4*[c] (6)
DBP/CPT-R (n)	75.0 ± 9.4 (5)	93.2 ± 4.8*[d] (8)

Values obtained upon examination at the end of the experiment (58th week).
[a]Values have been expressed as the mean percentage of tubules with positive cells/total seminiferous tubules ± SD; generalized linear mixed-effects model with gamma distribution followed by *post-hoc* Sidak correction. n: number of animals per group. *Significantly different from the control group ($p<0.05$). [b]$p=0.009$; [c]$p=0.012$; [d]$p=0.001$.

under the same conditions and chemicals but for a shorter period[13]. These values underscore the adequacy of our system in stratifying the experimental groups according to the histological alterations (Fig. 3). It should be noted that similar histological alterations [interruption of spermatogenesis, germ cell multi-nucleation and exfoliation, intra-epithelial vacuolated Sertoli cells, and tubules almost completely devoid of germ cells (SCO pattern)] have been described not only in cryptorchid rats[9, 11, 13, 47], but also in the testes of rats given short- or long-term exposure to 500 mg/kg DBP or 2–10 mg/kg AA[14, 15, 30, 46]. However, we observed no alterations[31] or only mild alterations[13] in the otherwise normal non-cryptorchid testes of SD rats exposed for 45 days or 16 weeks, respectively to AA and DBP, at the same dose levels used in the present experiment. Therefore, it is likely that the testicular lesions observed in the current study resulted from the cryptorchid condition, combined with the continuous damage induced by the testicular toxicants, with each of the chemicals contributing to the failure of orchiopexy in reversing the changes through their respective known adverse modes of action. DBP may exert an anti-androgenic influence[30, 48, 49], and AA possibly operates by mechanisms such as overproduction of reactive oxygen species and cytoskeletal dysfunction, which may have induced altered metabolism and cell degeneration[50].

The testicular dysplastic condition with CPT is possibly due to non-mutually exclusive causes, such as a common primary testicular defect during embryo fetal development[2, 51] and the high intra-abdominal temperature that leads to changes in those testes, which, for whatever reason, fail to descend[52–54]. Since the cryptorchid model discussed here differs from the human congenital undescended testes, as it was surgically established early in the postnatal life of otherwise normal animals, it is possible that the intra-abdominal temperature was a contributing factor for the

morphological alterations observed.

The damage to seminiferous tubules and germ cell depletion observed after AA or DBP exposure, as reported in this study and by other research groups[14, 15, 29, 45] can be linked to alterations in germ cell proliferation and/or apoptosis. Critical genes involved in pluripotency, proliferation, and apoptosis of spermatogonia were investigated by Souza *et al.* [31], who observed reduced Pou5f1, Mki67, and Spry4 expression on PND 24 and PND 45 after *in utero* and postnatal exposure to AA or DBP. The authors suggested that the chemicals reduced the potential for proliferation of spermatogonia, possibly by means of impairment of cellular differentiation[31]. The present observations support this hypothesis. Both chemically exposed groups showed higher or lower proportions of tubules with c-Kit- or PLAP-positive spermatogonia, as compared to the control group. The immunohistochemical findings were expressed more in the AA/CPT-R group, possibly associated with the putatively more complex mode of toxicological damage by this substance[55]. c-Kit is an anti-apoptotic factor, and its expression during the fetal period could lead to the survival of primordial germ cells and trigger cell proliferation[56, 57]. However, progressive cellular differentiation results in loss of its expression[58] and a hypothesis could be suggested that increased expression of this protein is associated with inhibition of cell death, favoring the persistence of undifferentiated germ cells in the testes exposed to the chemicals. Additionally, PLAP has been postulated to regulate the cell division cycle in organs such as the placenta[59]. Assuming that the activity of PLAP is the same in testicular cells, its decreased expression in chemically exposed animals may be related to the observed alterations in germ cell proliferation and differentiation. These alterations could at least partially be involved in the persistence of undifferentiated spermatogonia and explain the occurrence of isolated and enlarged germ cells, apparently undifferentiated type A spermatogonia (Fig. 3C, inset c and 4B)[60]. Additional longer experiments could shed light on the very nature of these seemingly less differentiated germ cells that are associated with chronic exposure to AA or DBP.

In addition to the severely damaged seminiferous tubules, interstitial LCs were altered in the groups exposed to each of the chemicals. The hyperplasia observed morphologically was expressed quantitatively by a significant increase in the mean number of LC/ml, particularly in the group exposed to AA (Table 3, Fig. 3E). The other four quantitative LC parameters (total number/testis, total volume, individual cell area, and somatic index) showed largely variable values and did not differ significantly from the control values (Table 3). It has been reported that adult male rats whose dams had been exposed to DBP (at doses of 100 mg/kg body weight and higher) during gestation present LC hyperplasia associated with dysgenetic seminiferous tubules in atrophic testes[61–63]. These alterations have been attributed to DBP interference in fetal LC steroidogenesis and impairment of testicular testosterone synthesis[61, 64, 65]. However, the mechanisms by which AA is associated with LC hyperplasia are less clear, but it has been suggested that AA directly targets LCs[19, 55], establishing a defective response to luteinizing hormone (LH) stimulation and reduced levels of circulating testosterone[15, 55, 66]. Regardless of exposure to DBP or AA, LC hyperplasia in the adult rat seems to be associated with a compensatory adaptation of the hypothalamic-pituitary-gonadal axis and increased levels of LH[55, 62]. In addition to systemic influences, local modulation may also play a role in the emergence and maintenance of proliferation and hyperplasia of adult LCs[67, 68]. Among these local influences are the soluble modulating factors secreted by other interstitial cells such as macrophages, fibroblast-like cells, smooth muscle cells, pericytes, and peritubular cells, which are eventually recruited to trans-differentiate into LCs, and also by the surviving Sertoli cells within degenerated atrophic tubules[67, 68]. The present study does not provide a complete understanding of the mechanisms that result in LC hyperplasia, because functional assessments were not performed herein.

It should be taken into consideration that the experimental protocol adopted in the present study differs from those reported in the literature in that a non-exposure interval (days or weeks) was not established, and the animals were continuously held under the influence of DBP or AA beginning at intra-uterine life. Accordingly, it may be assumed that whatever the mechanisms were operative on the testes, the effects of each chemical were cumulative.

In summary, this study indicated that sustained exposure of SD rats to the testicular toxicants AA or DBP since the intra-uterine life aggravated the testicular alterations induced by the chemicals and impaired the effectiveness of orchiopexy in restoring the testis to normal morphology after CPT. Although the present experimental protocol does not replicate the conditions that occur in human males, it is a potentially useful model to inform the functional and morphological lesions occurring in cryptorchid humans chronically exposed to exogenous chemicals.

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