

## ORIGINAL ARTICLE

# Protective effect of $\alpha$ -tocopherol on damage to rat testes by experimental cryptorchidism

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## INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

doi: 10.1111/j.1365-2613.2010.00757.x

Received for publication:  
28 July 2010

Accepted for publication:  
1 December 2010

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## Summary

It is thought that the degeneration of germ cells associated with an increase in the temperature due to cryptorchidism involves oxidative stress.  $\alpha$ -Tocopherol is a powerful antioxidant that prevents oxidation of polyunsaturated fats found in membranes and stabilizes peroxy radicals. For this reason we were interested in determining the role of  $\alpha$ -Tocopherol using experimental cryptorchidism, followed by orchidopexia in neonatal rats. Eighty-four, 10-day-postpartum (dpp) male rats (Wistar strain) were used and divided into 7 groups: healthy control, sham with  $\alpha$ -Tocopherol treated with 30 or 100 mg/kg doses, sham vehicle, cryptorchidism treated with  $\alpha$ -Tocopherol at 30 or 100 mg/kg doses and cryptorchidism vehicle. Cryptorchidism was surgically induced at 10 dpp. At 25 dpp the animals were treated with  $\alpha$ -Tocopherol and the vitamin vehicle. Lipoperoxidation and testicular morphology was determined in half of the animals at 40 dpp (short term). The remaining animals underwent orchidopexia and fertility was determined at 90 dpp. Testicular morphology was determined at 120 dpp (long term) in these animals. A significant reduction of lipoperoxidation was observed in the cryptorchid group treated with  $\alpha$ -Tocopherol compared to the untreated cryptorchid group, in addition to short-term histological alterations. At long term, we observed an increase in the area and maturation of the seminiferous epithelium, a decrease in apoptosis and histological alterations and an increase in fertility from  $\alpha$ -Tocopherol treatment.  $\alpha$ -Tocopherol treatment decreased lipoperoxidation, possibly stabilizing free radicals produced during cryptorchidism, reducing morphological testicular alterations and favoring fertility.

## Keywords

cryptorchidism, fertility, male, oxidative stress and testicle

Cryptorchidism is present in more than 5% of newborn males. The index increases in premature and low-birth-weight infants. At 3 months of age, this index spontaneously decreases and is maintained at 1.6%. Subfertility is a recognized effect of cryptorchidism, even after testicular position has been corrected, owing to alterations in the maturation of germ cells (Cooper & Little 1985; Elder 1988; Lee & Coughlin 2001; Hutson *et al.* 2010), possibly caused by inguinal heat stress (DeFoor *et al.* 2004). Heat stress induces the generation of reactive oxygen species (ROS) in the testis (Ahotupa & Huhtaniemi 1992; Ikeda *et al.* 1999; DeFoor *et al.* 2004; Viguera *et al.* 2009) and

the reduction in endogenous antioxidant enzymes such as superoxide dismutase and catalase (Ahotupa & Huhtaniemi 1992). The superoxide anion, hydroxyl radical, nitric oxide and hydrogen peroxide are among the ROS generated (Zini & Schlegel 1997; Kumagai *et al.* 2002; Ishii *et al.* 2005). These ROS are transitory molecules with a high degree of chemical reactivity that could stimulate lipoperoxidation. They cause a deleterious change in cell membrane lipoprotein complexes in addition to testicular damage (Peltola *et al.* 1995), affecting spermatogonia for life and reducing sperm production and male fertility (El-Missiry 1999; Ghosh *et al.* 2002).

$\alpha$ -Tocopherol ( $\alpha$ T) is an important antioxidant that localizes to cell membranes. It interrupts the lipoperoxidation chain reaction and traps the ROS created during univalent reduction in molecular oxygen (Traber & Kayden 1987). Therefore, it is important to determine whether  $\alpha$ T prevents or reduces the damage caused by heat stress because of the testis' inguinal position.

## Material and methods

We used 84, 10-day-postpartum (dpp), male rats (Wistar strain) that stayed with their mothers until they weaned. Food and water were given on demand. All animals were handled according to the ethical principles and regulations specified by the Official Mexican Norm (NOM-062-200-1999). The protocol was approved by the Institutional Committee for the care and use of laboratory animals.

A total of 48 animals were used in both control and sham groups. The only intervention handling of animals from the sham group was surgical manipulation of the inguinal area without testicular fixation. The other 36 rats underwent surgical unilateral left cryptorchidism, and 12 animals were assigned randomly to each group which were defined in the following manner:

*Group 1:* Healthy control without surgical or pharmacological intervention (Control).

*Group 2:* Animals with simulated operation (sham) and daily administration of propylene glycol for 15 days, beginning at 25 dpp (Sham-Vehicle).

*Group 3:* Animals with sham operation and daily administration of 30 mg/kg i.p. of  $\alpha$ T (Sigma-Aldrich, St Louis, MO, USA) for 15 days, beginning at 25 dpp (Sham- $\alpha$ T-30).

*Group 4:* Animals with sham operation and daily administration of 100 mg/kg i.p. of  $\alpha$ T (Sigma-Aldrich) for 15 days, beginning at 25 dpp (Sham- $\alpha$ T-100).

*Group 5:* Animals with unilateral surgical cryptorchidism and daily administration of propylene glycol i.p. for 15 days in the 25 dpp (Cryptorchid-vehicle).

*Group 6:* Animals with unilateral surgical cryptorchidism and daily administration of 30 mg/kg i.p. of  $\alpha$ T for 15 days, beginning at 25 dpp (Cryptorchid- $\alpha$ T-30).

*Group 7:* Animals with unilateral surgical cryptorchidism and daily administration of 100 mg/kg i.p. of  $\alpha$ T for 15 days, beginning at 25 dpp (Cryptorchid- $\alpha$ T-100).

### Surgical procedures

*Cryptorchidism.* Surgical cryptorchidism was performed as described by Shono et al. (1996): extra-abdominal exposition of the gubernaculum by means of a transversal inguinal incision, fixing the inguinal fascia with a 6-0 nylon suture, which impedes the testicle's descent.

Xylazine hydrochloride (10 mg/kg i.p.) and ketamine (80 mg/kg i.p.) were administered as anaesthetics. Dipyrone (5 mg/kg i.p.) was administered as an analgesic.

At 40 days of age (short term), the lipoperoxidation and testicular morphology were determined in six animals from

each group. Orchidopexy and orchidectomy of the contralateral testis, with the aim of preventing the participation of the healthy testicle in fertility test, were performed and were euthanized at 120 dpp (long term).

*Orchidopexy.* After anaesthetizing the abdomen, an approximately 3-cm transverse inguinal incision was made in the skin and subcutaneous tissue. The cryptorchid testis was identified in the inguinal region and dissected from its attachments. If descended, it was fixed to the scrotal bag with a 6-0 prolene suture on the inferior pole of the vaginal tunic, intertegumentary subcutaneous tissue and fascia of the scrotal sac on the corresponding side. Once the testis' permanence in the scrotal sac was verified, the incision was sutured on one plane with a 6-0 prolene suture.

*Orchidectomy.* A transversal inguinal incision was made through the different subcutaneous layers until the testicle was localized. Taking care not to cut the albugineous tunic, the testicle and epididymis were freed from adherences, identifying the sperm duct and tying it off with a double suture of absorbable 2-0 material and its elements separately (vessels, cremaster, etc.), removing the testicle and epididymis. The stoppage of bleeding was checked, and the inguinal wall sutured in layers with 6-0 prolene.

### Fertility evaluation

Fertility was evaluated at 90 dpp by placing individual males from all groups with two fertile females for two oestrous cycles; vaginal swabs were performed in the morning to determine the presence of sperm. Once the presence of sperm was confirmed, females were separated to verify pregnancy, and if positive, until delivery to confirm the birth of healthy offspring.

### Processing of biological material

All of the animals were sacrificed with an overdose of sodium pentobarbital (100 mg/kg) between 12:00 and 13:00 h to prevent circadian fluctuations and were examined *post-mortem*. Testicles were dissected, weighed and washed with saline solution (0.9%). For animals sacrificed at short term, half of each testicle was used to determine lipoperoxidation and the other half was distributed for inclusion in EPON 812 (Ted Pella, Inc., Redding, CA, USA) and paraffin. Tissue for lipoperoxidation was placed on ice. A sample of the left testicle of animals evaluated at long term was processed for inclusion in EPON and the rest for inclusion in paraffin. Tissue included in EPON was processed to determine the histopathological index (HI) of maturation or the Johnsen index (JI) and the epithelial area (EA), and material included in paraffin was used to determine the apoptosis index (AI) and cellular proliferation index (PI), as described below.

*Lipoperoxidation through thiobarbituric acid-reactive substances.* Production of thiobarbituric acid-reactive sub-

stances (TBARS) was measured according to the modified technique described for *in vitro* studies (Ríos & Santamaría 1991). A 1 ml aliquot containing the homogenized testis was added to 2 ml of the thiobarbituric acid (TBA) reagent (0.375 g of TBA, 15 g of trichloroacetic acid and 2.5 ml of concentrated HCl in 100 ml of distilled water), and the final solution (3 ml total volume) was heated in a boiling water bath for 30 min. Samples were cooled on ice and centrifuged at 3000 g for 15 min. The absorbance was measured in supernatants by spectrophotometry at 532 nm. Concentrations of TBARS were calculated by the interpolation of a periodic oxidation of malondialdehyde standard curve. The final result was expressed as nmol of TBARS per mg of protein.

Protein content in testis tissue samples was measured with the Folin phenol reagent (Lowry *et al.* 1951). The results of lipid peroxidation were normalized to the protein content in each sample.

### *Morphological evaluation*

Tissue specimens were fixed overnight with Karnovsky's aldehyde solution (without  $\text{Ca}^{2+}$ , pH 7.4) (Karnovsky 1965); postfixed with 1%  $\text{OsO}_4$ , dehydrated in a graded series of ethanol and embedded in EPON 812 (Ted Pella). One-micron-thick sections were cut from EPON blocks using an Ultracut UCT microtome (Leica, Vienna, Austria) and stained with 0.5% toluidine blue.

Histological analysis of seminiferous tubes was performed using a BX 51 Olympus light microscope with fluorescence (Tokyo, Japan). Twenty to 30 transversal sections of seminiferous tubes per animal were analysed. Seminiferous epithelium area was determined by subtracting the internal area from the external area using an image analysing system (Image-Pro Plus 5.1, Media Cybernetics, INC. MD, USA) and was only assessed in transverse sections. Seminiferous epithelium degree of maturity was determined by means of the JI (Johnsen 1970). A score was assigned to each seminiferous tubule according to the main cell-type present, ranging from one (no cells) to ten (complete spermatogenesis with many late spermatids). The HI was determined by Viguera *et al.* (2009).

*Cellular apoptosis determined by TUNEL.* Cellular apoptosis was determined using TUNEL (*In situ* Cell Death Detection Kit, Roche Diagnostic Corporation, IN, USA). Testes samples were fixed in 4% paraformaldehyde for 18 h and processed for embedding in paraffin. Transverse 4- $\mu\text{m}$ -thick sections were cut with a microtome (Leica RM 2155; Microsystems, Nussloch GmbH, Germany) and mounted on poly-L-lysine-coated slides (Sigma-Aldrich). The sections were deparaffinized and hydrated through a graded ethanol series. Sections were delineated by Dako pen (Dako, Carpinteria, CA, USA). To identify cells, slides were first contrasted with 4',6-diamidino-2-phenylindole (DAPI) as follows: they were washed with Hanks' balanced solution (Gibco, Grand Island, NY, USA) and incubated with DAPI (Sigma-Aldrich) at a concentration of 1  $\mu\text{g}/\text{ml}$ , after which they were incu-

bated with proteinase K (20 mg/ml) (Stratagene, La Jolla, CA, USA) for 30 min and placed in 0.3%  $\text{H}_2\text{O}_2$  in methanol for 30 min. They were subsequently permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) for 2 min and incubated in TUNEL solution (50  $\mu\text{l}$  terminal deoxynucleotidyl transferase and 450  $\mu\text{l}$  nucleotide mixture) for 1 h at 37 °C. As a control for staining specificity, some sections were processed throughout the incubation steps and treated with DNase (Stratagene) at a concentration of 1  $\mu\text{g}/\text{ml}$  for 10 min at 37 °C, before incubation in TUNEL solution to induce DNA strand breaks.

Sections were mounted in Entellan (Merck, Darmstadt, Germany) and observed with a fluorescent microscope (Olympus DX51). The number of apoptotic cells per transversally cut seminiferous cord was calculated. Twenty to 30 transversal sections of seminiferous tubes per animal were analysed. All dilutions and washes between stages were performed using phosphate-buffered saline (PBS) unless otherwise specified.

*Determination of cellular proliferation.* Paraffin sections, prepared as above, were mounted on poly-L-lysine-coated slides (Sigma-Aldrich). Six sections from each testis were prepared. Slides were deparaffinized in xylene for 30 min. After rehydration through a graded ethanol series, sections were delineated by Dako pen (Dako). Endogenous peroxidase activity was blocked with 1%  $\text{H}_2\text{O}_2$  (Merck) in methanol for 30 min, after which sections were washed with distilled water. Slides were incubated in 1% Triton X-100 solution (Sigma-Aldrich) for 10 min, blocked with 1% bovine serum albumin (Amersham Biosciences, Buckinghamshire, UK) for 2 h and incubated with a rabbit polyclonal antibody against histone-H3 (1:500 dilution) (Upstate Biotechnology, Lake Placid, NY, USA) for 18 h at 4 °C. Slides were then incubated with biotinylated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA USA) at a 1:100 dilution for 1 h and then with streptavidin-peroxidase conjugate (Rabbit Immunocruz staining system; Santa Cruz Biotechnology) for 30 min in accordance with the manufacturer's instructions. Sections were incubated in a peroxidase substrate solution [1.6 ml distilled  $\text{H}_2\text{O}$ , 20  $\mu\text{l}$  10 $\times$  substrate buffer and 40  $\mu\text{l}$  50 $\times$  diaminobenzidine chromogen (Santa Cruz Biotechnology)] for 6 min, counterstained with haematoxylin, dehydrated through a graded series of ethanol and cleared with xylene. PBS was used for all dilutions and washes between stages unless otherwise specified.

Control sections were processed in an identical manner with the exception that the primary antibody incubation step was omitted. Sections were mounted with Entellan mounting medium (Merck) and were examined using a light microscope. All tissue sections from control and experimental animals were processed at the same time to minimize any potential variance in the labelling procedure. Slides from both groups were randomized and coded in such a way that all subsequent analyses were conducted in a blinded manner. All histological examinations were performed by a single observer.

The PI was evaluated in transversally cut sections, and the number of cells stained with the anti-histone-H3 antibody was determined in relation to the total number of cells in the transversally cut tube. A total of 50 seminiferous tubules were evaluated per animal.

**Stereology.** The rest of the tissue included in paraffin was sectioned in a serial manner and cut at 15- $\mu$ m-thick with a 150  $\mu$ m separation. It was stained with haematoxylin-eosin-periodic acid-Schiff.

A systematic random procedure termed the optical fractionator (West *et al.* 1991) was used to count the number of spermatocytes and spermatids because of the fact that they are the germ cells most vulnerable to damage caused by ROS (Bauché *et al.* 1994). The size of the counting frame was 35  $\times$  35  $\mu$ m, the sampling grid was 2500  $\times$  2500  $\mu$ m and the periodicity of the section was 20.

The height of the counting frame was 8  $\mu$ m, and the guard zones were defined at 1  $\mu$ m from the upper and lower borders of the counting frame. Nuclei cells that intersected with the upper guard zone and with the lateral exclusion boundaries of the counting frame were excluded from the count. The estimated total number of germ cells was calculated by using the Stereo investigator 9 program in a semi-automatic stereological system (MicroBrightField Inc., Williston, VT, USA). The program estimates the total number of cells considering the total area of the region of interest and the average volume of the tissue sampled in various sites randomly taken through the sampled section. The counting was carried out at 100 $\times$ , and the coefficient of error (Gundersen,  $m = 1$ ) was <0.07.

### Statistical analysis

All parametric results were analysed using one-way analysis of variance test (ANOVA) followed by the Tukey test. Non-parametric results were analysed using the 'Kruskal-Wallis test'. Fertility was evaluated using a chi square test. A  $P < 0.05$  was considered significant.

## Results

### Short term (40 dpp)

The testes of all animals in the control, sham and sham- $\alpha$ T groups were localized in the scrotum. In animals with unilateral cryptorchidism, the left testis was localized in an inguinal position and the contralateral testis in the scrotum.

The testicular weight of the groups with cryptorchid-vehicle and cryptorchid- $\alpha$ T (30 and 100 mg/kg) showed a decrease when compared to control, sham-vehicle and sham- $\alpha$ T groups (Table 1).

Lipoperoxidation in different groups was determined using the TBARS technique, which was present in low concentrations in the testes of the control, sham-vehicle and sham- $\alpha$ T (30 and 100 mg/kg) ( $P > 0.05$ ) (Table 1). The values increased significantly (+42%,  $P < 0.05$ ) in testes from the cryptorchid-vehicle group compared to the control group. In cryptorchidism, animals that had been administered  $\alpha$ T (30 and 100 mg/kg), lipoperoxidation was reduced by -25% ( $P < 0.05$ ) and -15%, respectively (Table 1), in comparison with the cryptorchid-vehicle group. The cryptorchid- $\alpha$ T with 30 mg/kg showed no significant differences compared to the control group; however, the cryptorchid- $\alpha$ T-100 group was significantly different from the control group.

Histologically, the testicles of the control, sham-vehicle and sham- $\alpha$ T groups showed normal cytoarchitecture, characterized by seminiferous tubules in different stages of the seminiferous epithelial cycle and by germ cells in different stages of development from spermatogonia to round spermatids. Some tubules showed elongated spermatids (Fig. 1a,b,c). Testicles of animals in sham-vehicle and sham- $\alpha$ T groups (30 and 100 mg/kg) showed no significant differences, compared to the control group, in most of the histological parameters evaluated (HI, EA, AI and PI) (Fig. 2a,d; Table 1).

The seminiferous tubules of animals from cryptorchid-vehicle and cryptorchid- $\alpha$ T groups (30 and 100 mg/kg)

**Table 1** Effects of  $\alpha$ -tocopherol on the lipoperoxidation and morphological parameters of cryptorchidism testes in 40-day-old rats

	Controls groups			Cryptorchid groups			
	Control	Sham-Vehicle	Sham $\alpha$ T-30	Sham $\alpha$ T-100	Vehicle	$\alpha$ T-30	$\alpha$ T-100
Testis weight (g)	0.88 $\pm$ 0.032 <sup>†</sup>	0.80 $\pm$ 0.031 <sup>†</sup>	0.79 $\pm$ 0.012 <sup>†</sup>	0.87 $\pm$ 0.043 <sup>†</sup>	0.62 $\pm$ 0.018*	0.68 $\pm$ 0.016*	0.54 $\pm$ 0.034* <sup>‡</sup>
TBARS (nmoles of TBARS per mg of protein)	0.14 $\pm$ 0.007 <sup>†</sup>	0.14 $\pm$ 0.002 <sup>†</sup>	0.12 $\pm$ 0.001 <sup>†</sup>	0.13 $\pm$ 0.001 <sup>†</sup>	0.20 $\pm$ 0.002*	0.15 $\pm$ 0.001 <sup>†</sup>	0.17 $\pm$ 0.001*
HI	0.80 $\pm$ 0.05 <sup>†</sup>	0.94 $\pm$ 0.02 <sup>†</sup>	0.56 $\pm$ 0.02 <sup>†</sup>	0.50 $\pm$ 0.02 <sup>b</sup>	8.31 $\pm$ 0.16*	6.81 $\pm$ 0.18* <sup>†</sup>	7.00 $\pm$ 0.08* <sup>†</sup>
EA ( $\mu$ m <sup>2</sup> )	42.57 $\pm$ 0.82 <sup>†</sup>	38.79 $\pm$ 0.52* <sup>†</sup>	41.63 $\pm$ 2.05 <sup>†</sup>	47.04 $\pm$ 1.08* <sup>†</sup>	34.99 $\pm$ 0.88*	32.17 $\pm$ 0.78*	30.48 $\pm$ 0.68* <sup>†</sup>
AI	1.37 $\pm$ 0.10 <sup>†</sup>	1.10 $\pm$ 0.07 <sup>†</sup>	1.24 $\pm$ 0.08 <sup>†</sup>	0.99 $\pm$ 0.13 <sup>†</sup>	10.93 $\pm$ 1.08*	4.81 $\pm$ 0.49* <sup>†</sup>	6.28 $\pm$ 0.32* <sup>†</sup>
PI	7.76 $\pm$ 1.12	11.43 $\pm$ 1.46	9.92 $\pm$ 1.61	12.27 $\pm$ 1.49*	5.56 $\pm$ 0.50	8.36 $\pm$ 0.84	9.41 $\pm$ 0.85

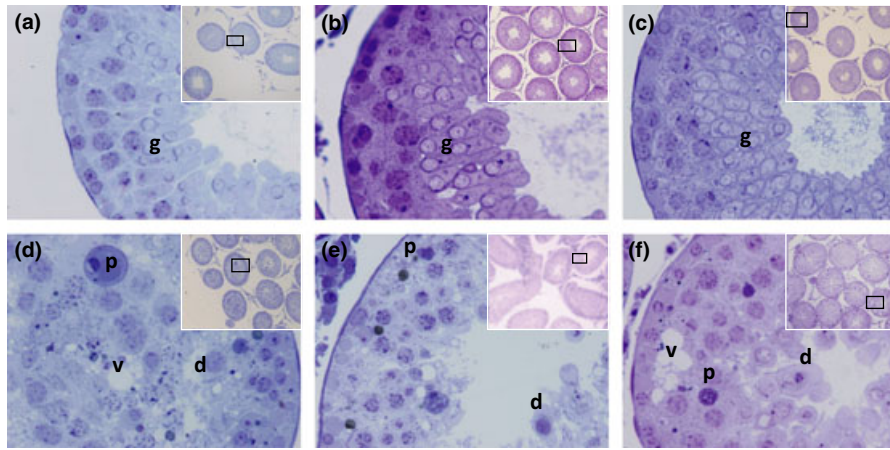
Data were collected from six animals per group and are expressed as the mean  $\pm$  SEM.

$\alpha$ T,  $\alpha$ -Tocopherol; TBARS, thiobarbituric acid-reactive substances; HI, histopathological index; EA, epithelial area; AI, apoptosis index; PI, proliferation index.

\* $P < 0.05$  (*vs.* control).

<sup>†</sup> $P < 0.001$  (*vs.* cryptorchid-vehicle).

<sup>‡</sup> $P < 0.001$  (cryptorchid- $\alpha$ T-30 *vs.* cryptorchid- $\alpha$ T-100).



**Figure 1** Histological images of seminiferous tubules from 40 dpp animals in different experimental conditions. (a–c) Healthy control, sham-vehicle and sham- $\alpha$ T-100, respectively, in which normal cytoarchitecture is observed with abundant germ cells (g). (d) Cryptorchid-vehicle showing cellular desquamation (d), pyknosis (p) and vacuolization (v). (e) Cryptorchid- $\alpha$ T-30 showing cellular desquamation (d), pyknosis (p) and spermatogenesis detention. (f) Cryptorchid- $\alpha$ T-100 showing cellular desquamation (d), pyknosis (p) and vacuolization (v) (60 $\times$ ). The inset shows a panoramic view of seminiferous tubules (10 $\times$ ), toluidine blue.

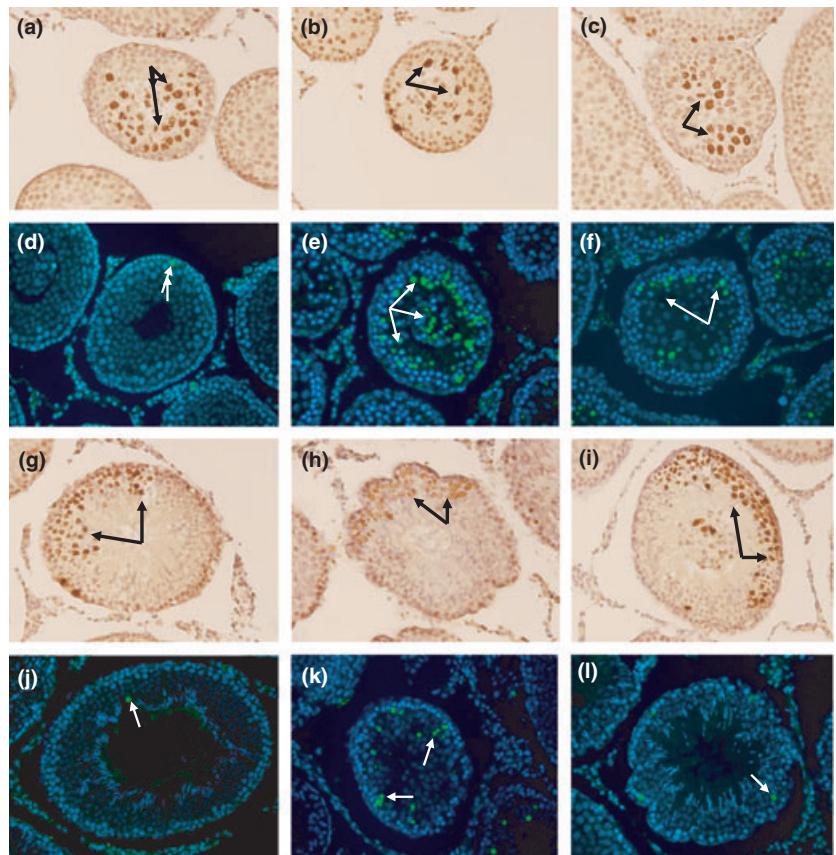
showed cell desquamation, folding of the basal lamina, vacuolization of germ and Sertoli cells, pyknosis, giant cells and hypoplasia of germ cells (Fig. 1d,e,f). In these three groups, the HI, EA and AI were significantly affected with respect to the control group (Table 1). The cryptorchidism-vehicle group had a significant increase in HI and AI with respect to cryptorchidism- $\alpha$ T-30 and 100 mg/kg groups

(Fig. 2b,c,e,f). The most advanced germ cells were the round spermatids (Table 1 and Fig. 1d–f).

*Long term (120 dpp)*

The testicles of all the animals were localized in the scrotum. The testicular weight of the animals in the chryptorchid-

**Figure 2** (a–f) Seminiferous tubules from 40 dpp animals with different experimental conditions. (a–c) Control, cryptorchid-vehicle and cryptorchid- $\alpha$ T-30, respectively, proliferating cells (arrow). (d) Apoptotic control cells (arrow). (e) Cryptorchid-vehicle showing a large number of apoptotic cells (arrow), both desquamated cells and spermatocytes. (f) Cryptorchid- $\alpha$ T-30 showing some spermatocytes and spermatids in apoptosis (arrow). (g–l) Seminiferous tubules from 120 dpp animals under different experimental conditions. (g–i) Control, cryptorchid-vehicle and cryptorchid- $\alpha$ T-30, respectively, showing a large amount of proliferative cells (arrow), even in the cryptorchid-vehicle group. (j) Seminiferous tubules with an apoptotic cell (arrow) from the control group. (k) Spermatocytes undergoing apoptosis (arrow) from cryptorchid-vehicle group. (l) Cryptorchid- $\alpha$ T-30 with a smaller number of apoptotic cells (arrow) compared to the cryptorchid-vehicle group. Immunocytochemistry (20 $\times$ ) using the H3S anti-histone antibody and TUNEL technique to detect apoptosis.



**Table 2** Effects of  $\alpha$ -tocopherol on the lipoperoxidation and morphologic parameters of cryptorchidism testes in rats at 120 days of age

	Controls groups				Cryptorchid groups		
	Control	Sham-Vehicle	Sham $\alpha$ T-30	Sham $\alpha$ T-100	Vehicle	$\alpha$ T-30	$\alpha$ T-100
Testis weight (g)	1.84 $\pm$ 0.063 <sup>†</sup>	1.78 $\pm$ 0.056 <sup>†</sup>	1.80 $\pm$ 0.017 <sup>†</sup>	1.90 $\pm$ 0.035 <sup>†</sup>	1.28 $\pm$ 0.096*	1.61 $\pm$ 0.028	1.49 $\pm$ 0.142* <sup>‡</sup>
HI	0.25 $\pm$ 0.04 <sup>†</sup>	0.33 $\pm$ 0.05 <sup>†</sup>	0.31 $\pm$ 0.04 <sup>†</sup>	0.27 $\pm$ 0.05 <sup>†</sup>	3.08 $\pm$ 0.21*	0.92 $\pm$ 0.08* <sup>†</sup>	1.49 $\pm$ 0.11* <sup>‡</sup>
EA ( $\mu$ m <sup>2</sup> ) $\times$ 10 <sup>3</sup>	79.91 $\pm$ 1.10 <sup>†</sup>	74.75 $\pm$ 8.94 <sup>†</sup>	74.73 $\pm$ 8.41 <sup>†</sup>	74.51 $\pm$ 8.24 <sup>†</sup>	45.71 $\pm$ 2.76*	75.22 $\pm$ 1.57 <sup>†</sup>	68.93 $\pm$ 0.82* <sup>‡</sup>
AI	1.00 $\pm$ 0.13 <sup>†</sup>	1.19 $\pm$ 0.12 <sup>†</sup>	1.08 $\pm$ 0.11 <sup>†</sup>	0.53 $\pm$ 0.04 <sup>†</sup>	3.24 $\pm$ 0.26*	1.07 $\pm$ 0.11 <sup>†</sup>	1.48 $\pm$ 0.16 <sup>†</sup>
PI	12.21 $\pm$ 1.64	11.68 $\pm$ 1.78 <sup>†</sup>	12.65 $\pm$ 1.21 <sup>†</sup>	15.71 $\pm$ 1.14	10.0 $\pm$ 1.21	12.67 $\pm$ 1.33	11.68 $\pm$ 1.78
JI	10.0 $\pm$ 0 <sup>†</sup>	9.74 $\pm$ 0.09 <sup>†</sup>	9.93 $\pm$ 0.25 <sup>†</sup>	10 $\pm$ 0.01 <sup>†</sup>	8.43 $\pm$ 0.38*	9.83 $\pm$ 0.07 <sup>†</sup>	9.74 $\pm$ 0.07 <sup>†</sup>
Spermatocytes and spermatids number ( $\times$ 10 <sup>6</sup> )	82.64 $\pm$ 5.52 <sup>†</sup>	78.37 $\pm$ 14.44 <sup>†</sup>	71.20 $\pm$ 1.48 <sup>†</sup>	78.41 $\pm$ 2.91 <sup>†</sup>	34.30 $\pm$ 3.67*	60.51 $\pm$ 3.98	51.52 $\pm$ 2.44*
Pregnant female/total evaluated female	12/12 <sup>†</sup>	8/12 <sup>†</sup>	10/12 <sup>†</sup>	12/12 <sup>†</sup>	2/12*	6/12	5/12*

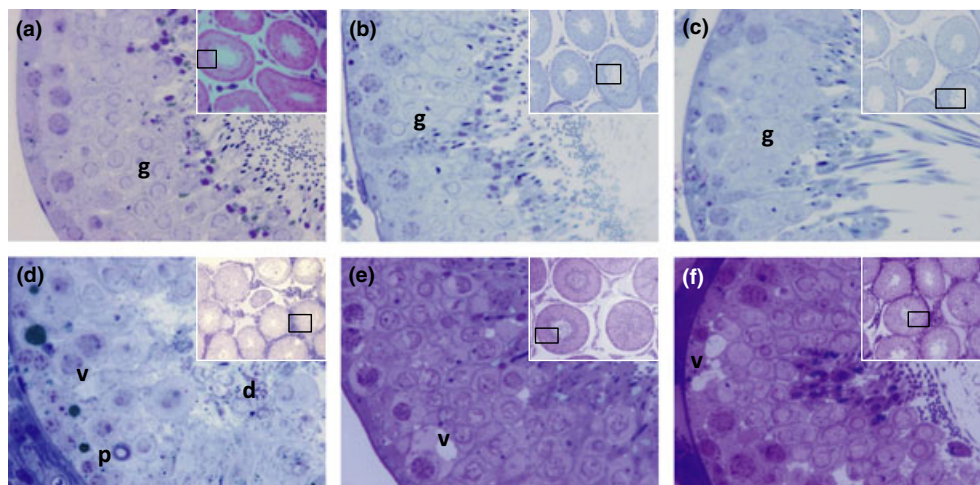
The data were collected from six animals by group and are expressed as the mean  $\pm$  SEM.

$\alpha$ T,  $\alpha$ -tocopherol; TBARS, thiobarbituric acid-reactive substances; HI, histopathological index; EA, epithelial area; AI, apoptosis index; PI, proliferation index; JI, Johnsen index.

\* $P$  < 0.05 (*vs.* control).

<sup>†</sup> $P$  < 0.001 (*vs.* cryptorchid-vehicle).

<sup>‡</sup> $P$  < 0.001 (cryptorchid- $\alpha$ T-30 *vs.* cryptorchid- $\alpha$ T-100).



**Figure 3** Histological tubules from 120 dpp animals with different experimental conditions. (a–c) Control, sham-vehicle and sham- $\alpha$ T-100, respectively, in which complete spermatogenesis is appreciated, as well as abundant germ cells (g) with normal cytoarchitecture. (d) Cryptorchid-vehicle showing obvious histological alterations such as cellular desquamation (d), epithelial vacuolization (v), pyknosis (p) with spermatogenesis detention in spermatide. (e) Cryptorchid- $\alpha$ T-30 with an obvious reduction in histological alterations compared to the cryptorchid-vehicle group, although some epithelial vacuoles still persist (v), complete spermatogenesis is appreciated. (f) Cryptorchid- $\alpha$ T-100 showing a clear reduction in histological alterations, although vacuoles (v) persist (60 $\times$ ). The inset shows a panoramic view of seminiferous tubules (10 $\times$ ), toluidine blue.

vehicle group was significantly reduced when compared with control (–30%), sham-vehicle (–28%), sham- $\alpha$ T-30 (–28%) and sham- $\alpha$ T-100 (–32%) groups. However, in the cryptorchid- $\alpha$ T-30 group, the reduction in the testicular weight was approximately of –12% without a significant difference when compared to control, sham-vehicle and sham- $\alpha$ T groups. Although it also did not show any difference with the cryptorchid-vehicle group, the testicular weight in the cryptorchid- $\alpha$ T-100 group showed significant difference with

the control group and no difference with the cryptorchid-vehicle group (Table 2).

At this age, the seminiferous tubules of control and sham animals (vehicle,  $\alpha$ T-30 and 100 mg/kg) had normal cytoarchitecture with abundant germ cells in different stages of the seminiferous epithelium cycle and spermatids in different phases of development: from round to elongated (Fig. 3a–c). The HI, EA, AI and PI of sham groups showed no differences in comparison with the control group (Table 2, Fig. 2g,j).

The seminiferous tubules of the animals from the cryptorchid-vehicle group showed histological alterations such as pyknosis, cellular desquamation, vacuolization of Sertoli cells and creasing of the basal lamina, until seminiferous tubules with only Sertoli cells and spermatogonia (Fig. 3d). The JI and EA of this group were significantly reduced, and the HI and AI increased in comparison with the rest of the groups (Table 2).

Application of  $\alpha$ T in animals with cryptorchidism led to a reduction in HI and AI and an increase in JI and EA, as well as the presence of elongated spermatids, compared to the cryptorchid-vehicle group (Table 2, Fig. 3d-f). The HI in cryptorchid  $\alpha$ T groups was significantly reduced by -70% and -51% with 30 and 100 mg/kg of  $\alpha$ T, respectively, compared to the cryptorchid-vehicle group. The AI was also greatly reduced by -67% and -54% with 30 and 100 mg/kg of  $\alpha$ T respectively. The JI increased by +16% and +15% with 30 and 100 mg/kg of  $\alpha$ T, respectively, and the EA significantly increased by +64% and +50% with 30 and 100 mg/kg of  $\alpha$ T respectively. Cellular proliferation did not seem to be modified in any of the groups. Favourable  $\alpha$ T outcome was more evident with a 30 mg/kg dose, but only in some parameters (like AI and JI) were the values of the control group reached (Table 2, Fig. 2h,i,k,l).

The sterologic study showed that the germinal cell population was reduced significantly in the cryptorchid-vehicle compared with control (-58%), sham-vehicle (-56%), sham- $\alpha$ T-30 (-51%) and sham- $\alpha$ T-100 (-56%) groups. This reduction in the germinal cell population was less compared with control in the groups with cryptorchidism and administration of vitamin (cryptorchid- $\alpha$ T-30 of -26% and cryptorchid- $\alpha$ T-100 of -37%), and only the administration of 30 mg/kg was the one that reached the level of the control group, without showing a significant difference ( $P > 0.05$ ). It is important to point out that the cryptorchid- $\alpha$ T-30 and  $\alpha$ T-100 groups did not show a difference with the cryptorchid-vehicle group (Table 2).

All females in the control group were pregnant. There were fewer pregnant females in the sham-vehicle and sham- $\alpha$ T (30 and 100 mg/kg) groups. The number of pregnant females was also reduced in the cryptorchid-vehicle group, but the administration of  $\alpha$ T led to an increase in pregnant females (at least 50%) in the two doses used (Table 2).

The fertility of the cryptorchid-vehicle group was significantly reduced compared with control, sham-vehicle and sham- $\alpha$ T groups. The administration of the vitamin in the cryptorchid- $\alpha$ T-30 and  $\alpha$ T-100 groups increased the fertility to more than +50% without difference in the cryptorchid-vehicle group although and without achieving the fertility level of the control group (Table 2).

## Discussion

It has been suggested that cryptorchidism is associated with a decrease in antioxidant enzyme activity (Zini & Schlegel 1997) or an increase in the production of ROS, such as superoxide anion, hydroxyl radical, nitric oxide and

hydrogen peroxide (Zini & Schlegel 1997; Kumagai *et al.* 2002; Ishii *et al.* 2005), which stimulates lipoperoxidation (Janero 1990). Antioxidant treatments could increase a cell's endogenous antioxidant defense system, inhibiting ROS production or working to trap free radicals and impede lipoperoxidation (Tilly & Tilly 1995). Attempts have been made to decrease the damage caused by free radicals in cryptorchidism (Kumagai *et al.* 2002; DeFoor *et al.* 2004), but there are no reports on the use of  $\alpha$ T as a powerful antioxidant in this pathology.

$\alpha$ T is an important antioxidant, found mainly in cell membranes, that acts by interrupting the chain reaction involved in lipid peroxidation and traps ROS generated during the univalent reduction in molecular oxygen (Traber & Kayden 1987; Sen Gupta *et al.* 2004; Shirpoor *et al.* 2007; Aybek *et al.* 2008).  $\alpha$ T has been shown to decrease lipoperoxidation of different organs undergoing oxidative stress (Nemmiche *et al.* 2007).

The results of this study confirm that the generation of lipoperoxidation plays an important role in the cryptorchid testicular damage mechanism. The administration of  $\alpha$ T significantly reduces lipoperoxidation because of the  $\alpha$ T action mechanism. This mechanism has been suggested to decrease lipoperoxidation by ceding hydrogen from the hydroxyl group in position 6 of its ring, in addition to its capacity to stabilize radicals produced during this process (Traber & Kayden 1987), reducing the ROS and the histological damage caused by them, leading to the conservation of spermatogenesis and increasing fertility. It has also been reported that  $\alpha$ T reduces damage caused by ROS generated by exposure to metals and toxic substances inside the testicle (Lucesoli & Fraga 1999; Jedlinska-Krakowska *et al.* 2006; Acharya *et al.* 2008).

Short-term observation of animals with cryptorchidism without the administration of  $\alpha$ T had a number of alterations similar to those described previously in testicles subjected to elevated temperature (Huff *et al.* 1989; Brehm & Steger 2005), such as the reduction in the EA, maturation index and testicular weight. These alterations are possibly owing to the fact that spermatocytes and spermatids are the germ cells most vulnerable to damage caused by ROS because, even though they are capable of converting superoxide anion to hydrogen peroxide, they have difficulty metabolizing peroxide, which saturates its protective system against peroxide that becomes a highly toxic hydroxyl radical (Bauché *et al.* 1994).

In our study,  $\alpha$ T was shown to partially protect rat testicles exposed to cryptorchidism, which was evaluated at short term because of the extrascrotal position of the testicle at this age. Protection by  $\alpha$ T was more obvious at 120 dpp (long term), because of the testicle's position in the scrotum and at the 30 mg/kg/day dose, wherein there was an increase in JI, EA, testis weight, number of spermatocytes and spermatids and a decrease in HI and AI.

In the cryptorchid-vehicle group, even with orchidopexia, histological alterations were similar to those mentioned previously. Desquamation of their germ cells was probably

due to the destabilization of Sertoli cell membranes caused by lipoperoxidation, which altered the cell union with germ cells. By administering  $\alpha$ T, lipoperoxidation of the membrane was impeded, conserving cell union and increasing the amount of germ cells in the seminiferous epithelium, resulting in a greater EA and JI.

Cryptorchidism is associated with high levels of apoptosis in germ cells (Shikone *et al.* 1994). In this work, we showed that  $\alpha$ T reduced apoptosis in germ cells. The mechanism through which this is believed to occur is through  $\alpha$ T inhibition of the production of lipoxygenases, enzymes that initiate membrane lipoperoxidation and activate apoptosis (Maccarrone *et al.* 2001), promoting cell survival and reducing histological alterations to the seminiferous epithelium.

At long term, it is not enough to have performed an effective orchidopexia in addition to  $\alpha$ T treatment because altered seminiferous tubules are seen in the testicles of these rats, besides a partial recovery of testicular weight and the number of germinal cells that although they did not show a significant difference with the control group, a significant increase was not seen when compared to the cryptorchid-vehicle group. It is possible that there is a high degree of damage at the spermatogonia level that is probably permanent.

There is the possibility that the ROS or another damage way generates irreversible alterations in Sertoli cells, which inhibit the production of factors (including epidermal growth factors) that maintain the survival of germ cells. This factor has been used successfully for fertility in animal models with cryptorchidism and orchidopexy (Kurokawa *et al.* 2005).

The partial fertility recovery of the groups with cryptorchid and  $\alpha$ T makes us think on an alternate damaging mechanism to the epididymis level. De Miguel *et al.* (2001) using immunohistochemical and morphometric techniques showed a reduced development of the human cryptorchid epididymis that could explain that surgical descent would probably not be able to completely reverse the fertility alterations.

It attracted our attention that by administering a 100 mg/kg/day dose of  $\alpha$ T in the cryptorchidism group, the lipoperoxidation was not as reduced as in the 30 mg/kg/day group. This could be attributed to the saturation of the lipoperoxidation regeneration system.  $\alpha$ T, by ceding hydrogen, oxidizes and gives rise to a new stable free radical (Burton & Traber 1990). However,  $\alpha$ T reduction, or regeneration, is caused by ascorbic acid and glutathione peroxidase (Pita 1997). It is possible that the reduction in glutathione peroxidase seen in cryptorchid (Bauché *et al.* 1994), and an elevated dose of  $\alpha$ T (100 mg/kg/day), caused an accumulation of the  $\alpha$ T radical (as there are less enzymes to reduce it) leading to an increase in the amount of free radicals.

Lastly, our results have been pointed out by different authors (Hadziselimovic 2008). Orchidopexia alone is not enough to completely restore spermatogenesis, justifying the search for new germinal epithelium protective substances in the cryptorchid testicle at a stage before orchidopexia.

## Conclusion

Treatment with  $\alpha$ T prior to orchidopexia, particularly at 30 mg/kg, partially protects the undescended testicle from damage caused by ROS (demonstrated by the significant improvement in seminiferous epithelium damage), restored spermatogenesis and increased fertility.

## Acknowledgements

We are grateful to Pedro Medina Granados and Edgar Daniel Cervantes Arias for technical assistance. We thank Julieta Mendoza Torreblanca and Leticia Granados Rojas for their technical support from the Instituto Nacional de Pediatría, Stereological Unit. This work was supported by 12/2006 INP grants.

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