·*Original Article*·

Immunohistopathology of the contralateral testis of rats undergoing experimental torsion of the spermatic cord

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Abstract

Aim: To evaluate the immunohistopathological changes in the contralateral testis of rats after an experimental spermatic cord torsion. **Methods:** Male Sprague–Dawley rats of 45-50 days old were subjected to a 720° unilateral spermatic cord torsion for 10, 30 and 80 days (experimental group, E), respectively or sham operation (control group, C). Histopathology of the contralateral testis as well as germ cell apoptosis were studied using the Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) technique. The number of testicular lymphocytes, mast cells and macrophages, and the expression of tumor necrosis factor-α (TNF-α) and its receptor (TNFR1) in testicular cells of the contralateral testis were quantified by histochemistry and immunohistochemistry. TNF-α concentration in testicular fluid was determined by ELISA. **Results:** In the contralateral testis of rats from the E group, the maximal degree of damage of the germinal epithelium was seen 30 days after torsion. At this time we observed in the E group *vs.* the C group increases: (i) the number of testicular T-lymphocytes; (ii) the number of testicular mast cells and macrophages; (iii) the percentage of macrophages expressing TNF-α; (iv) TNF-α concentration in testicular fluid; (v) the number of apoptotic germ cells; and (vi) the number of TNFR1$^+$ germ cells. **Conclusion:** Experimental spermatic cord torsion induces, in the contralateral testis, a focal damage of seminiferous tubules characterized by apoptosis and sloughing of germ cells. Results suggest humoral and cellular immune mediated testicular cell damage in which macrophages and mast cells seem to be involved in the induction of germ cell apoptosis through the TNF-α/TNFR1 system and in the modulation of the inflammatory process. *(Asian J Androl 2006 Sep; 8: 576–583)*

Keywords: testicular torsion; TNF-α/TNFR1; T-lymphocytes; macrophages; mast cells; germ cell apoptosis; spermatic cord

1 Introduction

Spermatic cord torsion is a medical emergency that mainly affects young men usually around puberty. Early

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surgical detorsion is the only effective treatment to improve testicular function. However, it is known that in some cases the treatment occurs too late because of delay by the patient in seeking medical consultation or because of misdiagnosis by the physician [1]. These patients and even those on whom detorsion was carried out might become infertile or subfertile in the future [2]. Most of the experimental models used to study spermatic cord torsion showed that testicular damage is induced in the contralateral testis [3, 4]. Some authors suggested that contrala-

teral testicular damage after spermatic cord torsion is a manifestation of preexisting testicular pathology [5]. However, the etiology of contralateral testicular damage after torsion is still unknown. Karaguzel *et al.* [6] suggested that contralateral testicular damage might occur through a reflex activating the sympathetic system. However, Sade *et al.* [7] and York and Drago [3] suggested that contralateral testicular damage is the result of an immunological mechanism. Based on the observation that testicular damage was found in animals undergoing spermatic cord torsion but not after spermatic vessel ligation, Cerasaro *et al.* [8] suggested that a minimum of blood circulation is necessary to trigger a systemic reaction being able to induce damage in the contralateral testis. Although initial studies reported damage to the contralateral testis in experimental models of spermatic cord torsion [4, 7], only recently was the phenomenom of germ cell apoptosis studied [9, 10]. Moreover, little attention has been focused on testicular immune cells and their secretion products in this model.

The aim of the present work was to study the effects of a prolonged spermatic cord torsion in the contralateral testis on the testicular histopathology, germ cell apoptosis, tumor necrosis factor-α (TNF-α)/TNF-α receptor (TNFR1) cell expression and the involvement of cellular and humoral immune response in rats.

2 Materials and methods

2.1 Animals

Male Sprague–Dawley rats aged 45–50 days were kept at 22ºC with a 14-h light, 10-h dark schedule and were fed standard food pellets and water *ad libitum*. Rats were killed according to protocols for animal use, in agreement with the National Institute of Health guidelines for the care and use of experimental animals.

2.2 Induction of spermatic cord torsion

Rats were randomly separated with 12 rats/group. Under ether anesthesia, a scrototomy on the left side and a 720° unilateral spermatic cord torsion were performed in rats from the experimental (E) group. The testis was fixed into the scrotum using a 4-0 silk suture. In rats from the control (C) group, a scrototomy was performed on the left side, and the testis was exposed and then replaced into the scrotum without torsion. Rats were killed 10, 30 and 80 days after surgery. After each rat was killed, the contralateral testis and epididymis were

removed, weighed, fixed in Bouin's solution, dehydrated and embedded in paraffin or quickly frozen to obtain cryostat sections for histopathology or immunohistochemistry. Blood was collected and sera was obtained for the detection of antispermatic antibodies. In some experiments, testicular fluid (TF) was collected as previously described [11]. Briefly, TF drained after an incision in the testicular capsule was collected for 16 h at 4ºC in the presence of proteases inhibitors.

2.3 Histopathology and morphometric analyses

Paraffin-embedded testis sections (6 μm) were stained with hematoxylin and eosin. The Crossmon's trichromic technique was used to detect collagen fibers. Tubular damage was quantified using a modified Johnsen score that analyzes the degree of damage of the seminiferous tubule germ cells with a score from 1 to 10 [12]. The mean tubular score was obtained by evaluating 50 seminiferous tubules/testes/rats. Tubular diameter was calculated in transversal testis sections with the aid of an ocular micrometer.

To identify mast cells, testis sections were stained with toluidine blue or alcian blue. Mast cells were counted using a 25 × objective and the ocular (12.5 ×) was fitted with a quadratic grid with a total area of 96 100 μm².

2.4 TUNEL

DNA fragmentation of cells was visualized by indirect immunoenzyme detection of digoxigenin-labeled genomic DNA. To facilitate antigen retrieval, deparaffinized and hydrated testis sections (< 5 μm) were microwaved at 400 W for 5 min in 10 mmol/L sodium citrate buffer (pH 6.0) and quickly cooled in phosphate buffered saline. Then, testis sections were processed as previously described [13]. For a negative control, TdT was replaced with the same volume of distilled water. Light counterstain was performed with eosin. TUNEL was performed in two non-consecutive testis sections of 5–8 rats/group and positive cells were counted in 100 seminiferous tubules/section using a 25 × objective.

2.5 Detection of antibodies to spermatic antigens

To detect antispermatic antibodies in the sera of rats, an indirect immunofluorescence technique was performed. Smears of spermatozoa obtained from cauda epididymis of normal rats were fixed in methanol for 10 min at 4ºC, incubated with sera of rats from E ($n = 12$) and C ($n = 12$) groups and then with a goat fluorescein isothiocyanate

conjugated anti-rat IgG (1:20) (Cappel Lab, Cochranville, PA, USA). As a technical control, primary antibody was omitted. Spermatozoa smears were observed under a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with epi-illumination.

2.6 Immunohistochemistry

An amplified immunoperoxidase technique was used to identify testicular T-lymphocytes, macrophage subsets, mast cell proteases, TNF-α and TNFR1 expression. To detect T-lymphocytes, frozen testis sections were incubated overnight with mouse monoclonal antibodies anti-rat Pan-T cells (1:50) (CBL 1503; Cymbus Biotechnology, Eastleigh, UK), that reacts with all T-lymphocytes. To identify macrophage subpopulations, an ED1 mouse monoclonal antibody, a marker of monocytes/macrophages recently arrived to the testis from circulation (10 μg/mL) (554954; BD Pharmingen, San Diego, CA, USA) or ED2 mouse monoclonal antibody, a marker of resident macrophages (5 μg/mL) (550573; BD Pharmingen, San Diego, CA, USA) was used (60 min at room temperature). A biotinylated horse anti-mouse rat adsorbed IgG (2 μg/mL) (Vector Lab, Burlingame, CA, USA) was used as a secondary antibody. The reaction was amplified with a Vectastin ABC Elite kit (Vector Lab) and the reaction product was visualized by adding diaminobenzidine substrate (Vector Lab). For a negative control, first antibodies were omitted. Sections were counterstained with hematoxylin and immunostained cells were counted. The number of macrophages and T cells in testis sections was obtained with the aid of a quadratic grid and the number per testis was calculated as previously reported [14].

To detect tryptase, rat mast cell protease 1 (RMCP1), TNF-α and TNFR1, testis sections were deparaffinized and microwaved at 400 W for 5 min in 10 mmol/L sodium citrate buffer (pH 6.0). Then testis sections were incubated overnight with a mouse monoclonal antibody anti-human mast cell tryptase (1:20) (M 7052; DAKO, Denmark A/S; a generous gift of Dr A. Mayerhofer), a goat polyclonal antibody anti-RMCP1 (1:200) (sc-17041; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a rabbit polyclonal antibody anti-mouse TNF-α that cross-reacts with rat TNF-α (1:100) (P-350; Endogen, Woburn, MA, USA) and a goat polyclonal antibody anti-mouse TNFR1 that cross-reacts with rat TNFR1 (1:50) (sc-1069; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A horse anti-mouse rat absorbed IgG (4 μg/mL) (Vector Lab), a goat anti-rabbit IgG and a rabbit anti-goat IgG (4 μg/mL) (Vector Lab) were used as secondary biotinylated antibodies.

For negative controls the first antibodies were omitted. The reaction was amplified as described above and sections were counterstained with hematoxylin or alcian blue to identify mast cells.

2.7 ELISA

TNF-α content in TF was measured using a solid phase sandwich ELISA (KRC 3011; Biosource, Camarillo, CA, USA). All procedures followed the manufacturer's instructions. Samples were processed in the same assay and each sample was measured by triplicate. The minimum detectable concentration of rat TNF-α is less than 4 pg/mL.

2.8 Statistical analysis

The unpaired t-test was used to analyze morphometry, Johnsen score, ELISA data and the number of TNFR1$^+$ cells. The non-parametric Mann-Whitney rank test was used to analyze the number of testicular lymphocytes, mast cells, ED1$^+$ (cells react positively with ED1 antibody) and ED2$^+$ (cells react positively with ED2 antibody) cells and germ cell apoptosis. $P < 0.05$ was considered significant.

3 Results

The torsioned left testis from the E group rats showed a fibrotic and necrotic scar, at 10, 30 and 80 days after surgery. Contralateral testis sections from the E group rats killed at 10 days after surgery showed a normal histology similar to the C group rats (Figure 1A). Focal damage of the contralateral testis was observed at 30 (Figure 1B) and 80 days after spermatic cord torsion. After comparing these three study periods, we found that the incidence of testicular damage was maximal in rats killed 30 days after surgery (10 days: 0%, 30 days: 44.8%, 80 days: 25%). Testicular lesions were focal and charac-terized by mild signs of inflammation, interstitial edema, sloughing of the germinal epithelium and seminiferous tubule atrophy (Figure 1B) with few signs of fibrosis of tubular walls. At 30 days after surgery we observed decreases of seminiferous tubule diameter (Figure 2) and Johnsen score (Figure 3) in the E group rats compared to the C group rats. Moreover, we observed a high correlation between these two parameters at this time (Figure 4).

TUNEL technique, identification of testicular mast cells and macrophages, immunodetection of TNF-α,
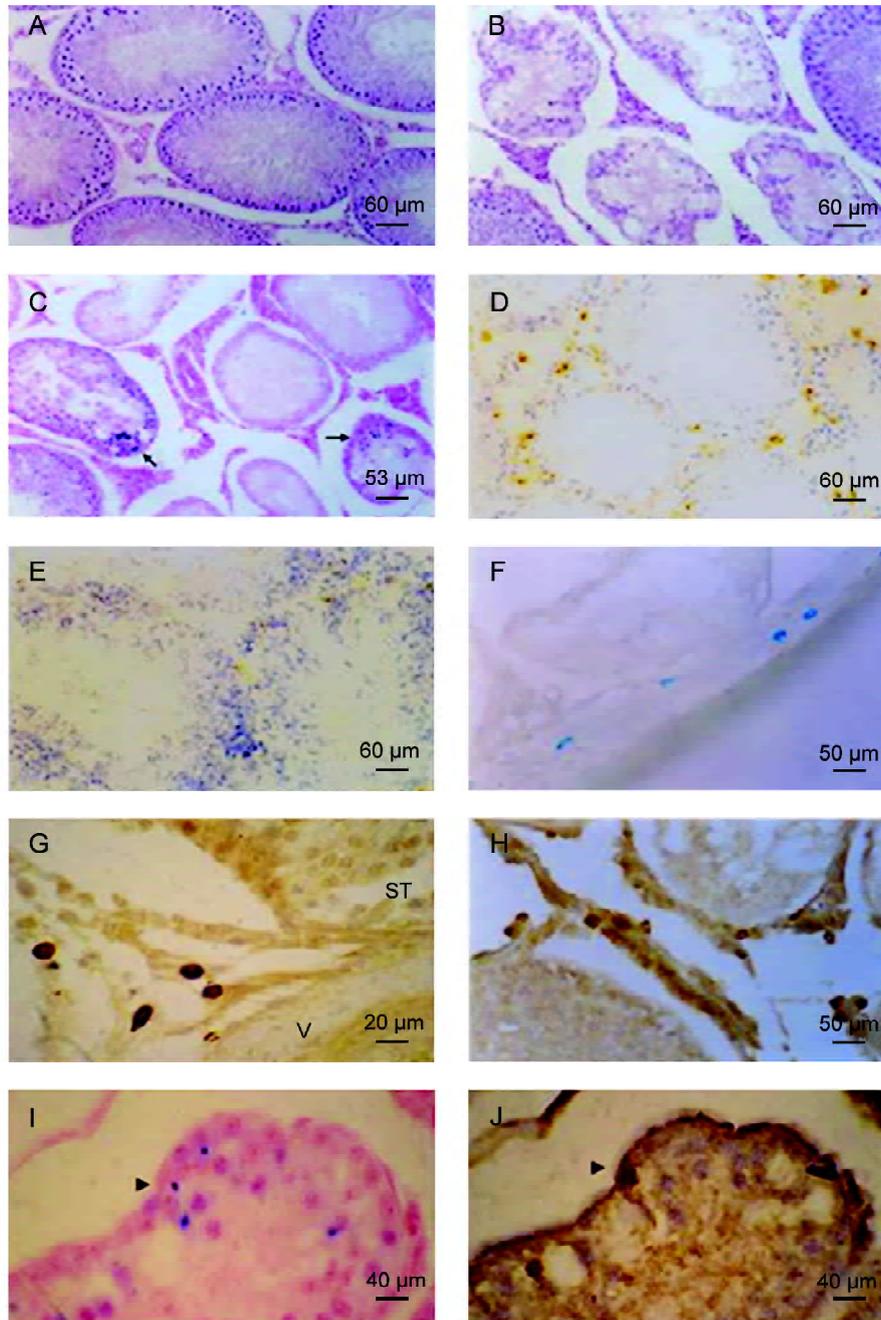
Figure 1. (A): Testis section from a control (C group) rat killed at 30 days after surgery. Normal seminiferous tubules are seen. Scale bar represents 60 μm. (B): Testis section from an experimental (E group) rat killed at 30 days after torsion. Several damaged seminiferous tubules with vacuolization and severe cell sloughing are observed. Scale bar represents 60 μm. (C): TUNEL technique: apoptotic germ cells are seen in a testis section from an E group rat killed at 30 days after surgery. TUNEL$^+$ nuclei are seen predominantly in pachytene spermatocytes (arrows). Scale bar represents 53 μm. (D), (E): Immunoperoxidase (IP) technique. ED1$^+$+ED2$^+$ macrophages in testis sections from a 30-day E rat (D) and C rat (E). Scale bar represents 60 μm. (F): Testis section from a 30-day E rat: subalbugineal mast cells are seen near a damaged seminiferous tubule. Alcian blue staining. Scale bar represents 50 μm. (G): IP: RMCP1$^+$ perivascular mast cells in a testis section from a 30-day E rat. ST, seminiferous tubule; V, subalbugineal vessel. Scale bar represents 20 μm. (H): Immunodetection of TNF-α in testicular macrophages in testis section from a 30-day E rat. Scale bar represents 50 μm. (I), (J): Co-localization of TNFR1 (J) in TUNEL$^+$ cells (I) in serial testis sections from a 30-day E rat. One TUNEL$^+$ germ cell shows TNFR1 immunoreactivity (arrowheads). Scale bar represents 40 μm.
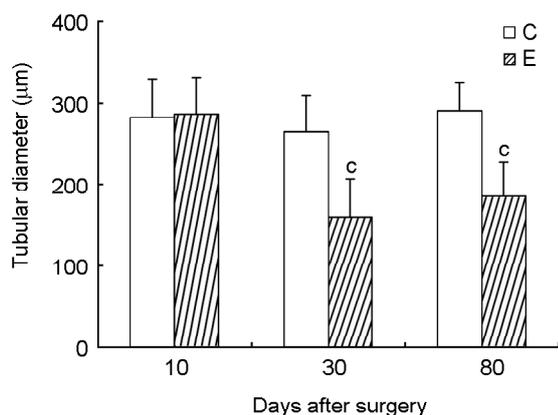
Figure 2. Quantification of seminiferous tubule diameter of testes from rats of control (C) and experimental (E) groups. Data represent mean ± SD of 50 seminiferous tubules randomly selected/rat testis; n = 6–9 rats/group. [c]P < 0.001, compared with respective control groups.
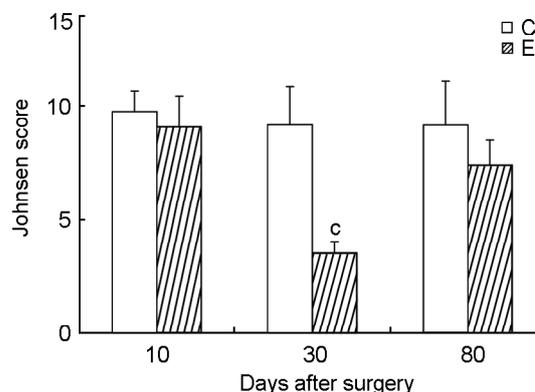
Figure 3. Johnsen score obtained from testis sections of rats of control (C) and experimental (E) groups. Data represent mean ± SD of 50 seminiferous tubules randomly selected/rat testis; n = 7–12 rats/group. [c]P < 0.001, compared with the control group.
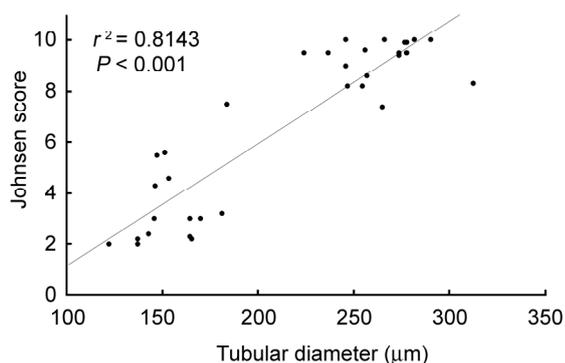
Figure 4. Lineal regression analysis showing the correlation between seminiferous tubule diameter and Johnsen score of rats from experimental (E) group killed 30 days after surgery. Data represent 1–3 testis sections/rat; n = 12 rats.
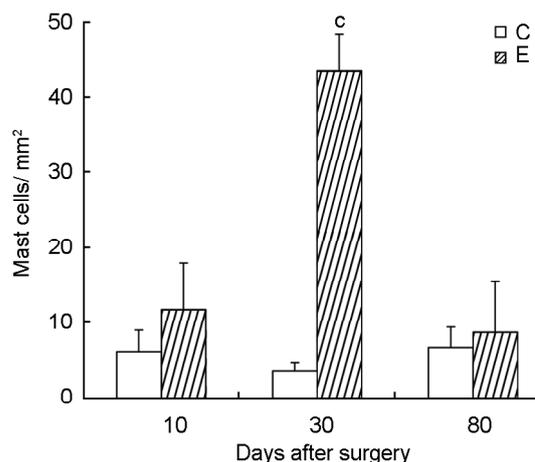
Figure 5. Quantification of mast cells in testis sections of rats from control (C) and experimental (E) groups. Data represent mean ± SD of 3 testis sections/rat; n = 6–8 rats/group. [c]P < 0.001, compared with the control group.
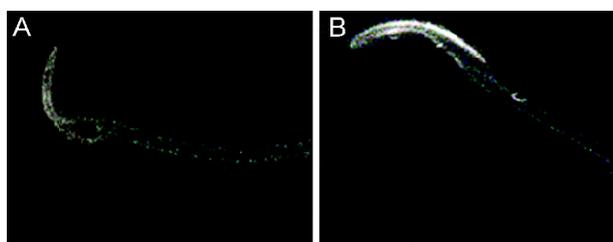
Figure 6. Indirect immunofluorescence (IF) to detect antisperm antibodies in the sera of rats. Negative (A) and positive (B) IF immunoreactivity in the sperm head was observed with sera obtained from C group and E group rats, respectively.

Table 1. Number of macrophages ($\times 10^6$/testis) in the contralateral testis of rats killed 30 days after surgery. Immunoperoxidase technique. The number of macrophages subpopulations was estimated by incubation with ED1 or ED2 and the total number of macrophages by incubation with ED1 + ED2 antibodies (Ab). Values are mean ± SD of 3 testis sections/rat; n = 7–10 rats/group; [c]P < 0.001, compared with respective control groups.

Groups	Number of macrophages		
	(ED1 + ED2) Ab	ED1 Ab	ED2 Ab
Control	2.95 ± 1.39	1.28 ± 0.42	1.87 ± 0.47
Experimental	11.00 ± 4.46[c]	4.98 ± 0.86[c]	6.35 ± 0.94[c]

TNFR1 and mast cell specific proteases were performed in rats killed 30 days after surgery because the incidence and degree of testicular damage was maximal at that time.

Using the TUNEL technique, we showed that germ cell death occurs by apoptosis, with spermatocytes and spermatids being the predominant cell types affected (Figure 1C). The number of apoptotic germ cells was significantly higher in the E group compared to that in the C group (E: 68.80 ± 28.80 cells/100 seminiferous tubules *vs*. C: 4.80 ± 3.30 cells/100 seminiferous tubules, mean $\pm$ SD of 2 testis sections/rat; $n = 5$–8 rats/group; $P < 0.05$).

T-lymphocytes were localized in the testicular interstitium and their number was increased in the testis of the E group rats at 30 days after surgery compared to that in the C group (E: $1.16 \pm 0.26 \times 10^6$ cells/testis *vs*. C: 0.63 ± 0.14 cells/testis, mean $\pm$ SD; $n = 4$ rats/group; $P < 0.05$).

Macrophages were localized in the testicular interstitium (Figure 1D) and many of them were close to the walls of seminiferous tubules. The total number of testicular macrophages also increased after surgery in rats from the E group compared to the C group (Table 1, Figure 1D, E). Both subpopulations of macrophages (ED1$^+$ and ED2$^+$) showed a similar increase in the testis of rats from the E group (3.9- and 3.4-fold increase, respectively).

In both the C and the E groups, mast cells were only localized near the tunica albuginea as cell clusters close to small blood vessels (Figure 1F, G). The number of mast cells significantly increased in the contralateral testis of rats from the E group compared to the C group (Figure 5). Mast cell tryptase and RMCP1 were detected by immunohistochemistry in the cell granules in both groups (Figure 1G).

ELISA data showed that TNF-α content in TF of the E group rats at 30 days was significantly higher compared to that in the C group rats (E: 58.31 ± 25.22 pg/mL *vs*. C: 30.24 ± 8.90 pg/mL, mean $\pm$ SD of triplicate samples/rat; $n = 4$ rats/group; $P < 0.05$).

We detected the expression of TNF-α in macrophages (Figure 1H), Leydig cells and also in mast cells in both groups. We observed a higher percentage of TNF-α^+ macrophages in the E group *vs*. the C group rats (E: $53.80 \pm 4.12\%$ *vs*. C: $13.60 \pm 2.10\%$, mean $\pm$ SD of 3 testis sections/rat; $n = 6$–10 rats/group; $P < 0.001$). For the quantification of TNF-α^+ macrophages, Leydig cells were excluded by the identification of the highly char-

acteristic nuclear morphology.

In contrast, we found no significant differences in the percentage of TNF-α^+ mast cells (E: $18.80 \pm 5.40\%$ *vs*. C: $18.70 \pm 9.60\%$, mean $\pm$ SD of 3 testis sections/rat; $n = 4$–9 rats/group).

By immunoperoxidase, we observed the expression of TNFR1 in interstitial cells (macrophages and Leydig cells) and in spermatocytes and spermatids. The number of TNFR1$^+$ germ cells significantly increased in the contralateral testis of the E group *vs*. the C group rats (E: 48.00 ± 32.05 cells/100 seminiferous tubules *vs*. C: 10.60 ± 6.02 cells/100 seminiferous tubules, mean $\pm$ SD of 4 testis sections/rat; $n = 6$–11 rats/group; $P < 0.05$). We also detected an increased percentage of TNFR1$^+$ germ cells sloughed in the lumen of epidimal tubules in rats from the E group *vs*. the C group rats (E: $59.90 \pm 7.70\%$ *vs*. C: $16.00 \pm 10.50\%$, mean $\pm$ SD of 2 testis sections/rat; $n = 5$–7 rats/group; $P < 0.001$). By combining immunohistochemistry and TUNEL in serial testis sections, we detected TNFR1 expression in $38.60 \pm 11.60\%$ ($n = 5$ rats) of TUNEL$^+$ cells (Figure 1I, J).

By indirect immunofluorescence we detected antisperm antibodies in the sera of all rats from the E group in contrast to the C group. Positive immunofluorescence was mainly observed in the sperm head (Figure 6).

4 Discussion

Our results demostrate that, in our experimental conditions, spermatic cord torsion induced maximal damage of the contralateral testis in 44.8% of rats at 30 days after surgery. We observed no testicular lesions in rats of the control group and in the experimental group before this time. By 80 days damage of the contralateral testis was observed in 25% of rats, suggesting the possible reversibility of the lesion.

Testicular damage was characterized by interstitial edema, apoptosis and sloughing of germ cells, mainly spermatocytes and spermatids and tubular atrophy. The histopathology had similar characteristics to that described in other torsion models [4, 8].

The presence of antisperm antibodies and the increase in the number of T-lymphocytes in the interstitium of the contralateral testis were observed in all rats of the experimental group. Therefore, our findings show that a humoral and cellular immune response occurs simultaneously to the histopathological damage of the contralateral testis after prolonged spermatic cord torsion. Testicular macrophages represent a heteroge-

neous population composed of ED1$^+$ (monocytes recently arrived to the testis from circulation) and ED2$^+$ cells (resident macrophages). Results of the present work showed a similar increase of ED1$^+$ and ED2$^+$ macrophages in the contralateral testis of rats after torsion. Both macrophage subsets were also increased in an experimental model of immune mediated testicular damage [14]. It is known that these cells are capable of producing proinflammatory cytokines, such as IL-1, IL-6 or TNF-α [15, 16]. In the contralateral testis of rats under experimental cord torsion the percentage of TNF-α macrophages and the concentration of TNF-α in testicular fluid increased simultaneously with the number of apoptotic and TNFR1$^+$ germ cells. Moreover, the observation that approximately 40% of apoptotic germ cells expressed TNFR1 suggests that the TNF-α/TNFR1 system could be involved in the apoptotic process. It is possible that TNF-α and other factors secreted by macrophages mainly localized in the peritubular area close to seminiferous tubules might induce apoptosis of germ cells. The role of mast cells in normal and pathological testis is not fully understood; however, the number of these cells increases in experimental autoimmune orchitis [17] and in testis biopsies of patients with infertility [18], suggesting that mast cells might be involved in the testicular damage. It has been demonstrated that mast cell tryptase can increase microvascular permeability, stimulate inflammatory cell migration and cytokine release [19]. Therefore, it is possible that, in our experimental torsion model, testicular mast cells could play a proinflammatory role indirectly triggering germ cell damage and also being involved in the induction of fibrosis of the walls of seminiferous tubules, as it has been suggested for other testicular pathologies [18]. In fact, in this model we observed signs of seminiferous tubular wall fibrosis at 30 days after torsion.

We conclude that spermatic cord torsion in rats induces focal damage of the contralateral testis with a maximal degree of lesion 30 days after surgery. The fact that histopathologic testicular damage and humoral and cellular immune responses occur simultaneously strongly suggests the involvement of an immunological mechanism in the induction of the lesion in the contralateral testis after spermatic cord torsion. Results suggest that lymphocytes, macrophages and mast cells modulate the inflammatory response and that the TNF-α/TNFR1 system is involved in the induction of germ cell apoptosis

observed in this pathology.

An early diagnosis and treatment of spermatic cord torsion is very important not only to preserve the normal function of the torsioned testis but also to avoid an immunological response to testicular antigens released by the ischaemic gonad.

Acknowledgment

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Edited by Prof. Susan Benoff

Erratum

Development of neonatal mouse and fetal human testicular tissue as ectopic grafts in immunodeficient mice.
Jie Yu, Zhi-Ming Cai, Hui-Juan Wan, Fang-Ting Zhang, Jing Ye, Jia-Zhi Fang, Yao-Ting Gui, Jiong-Xian Ye
Asian J Androl 2006 Jul; 8(4): 393-403.

The editorial office wishes to apologise for an error in Figure 3 in the above paper. The correct Figure is as follows:
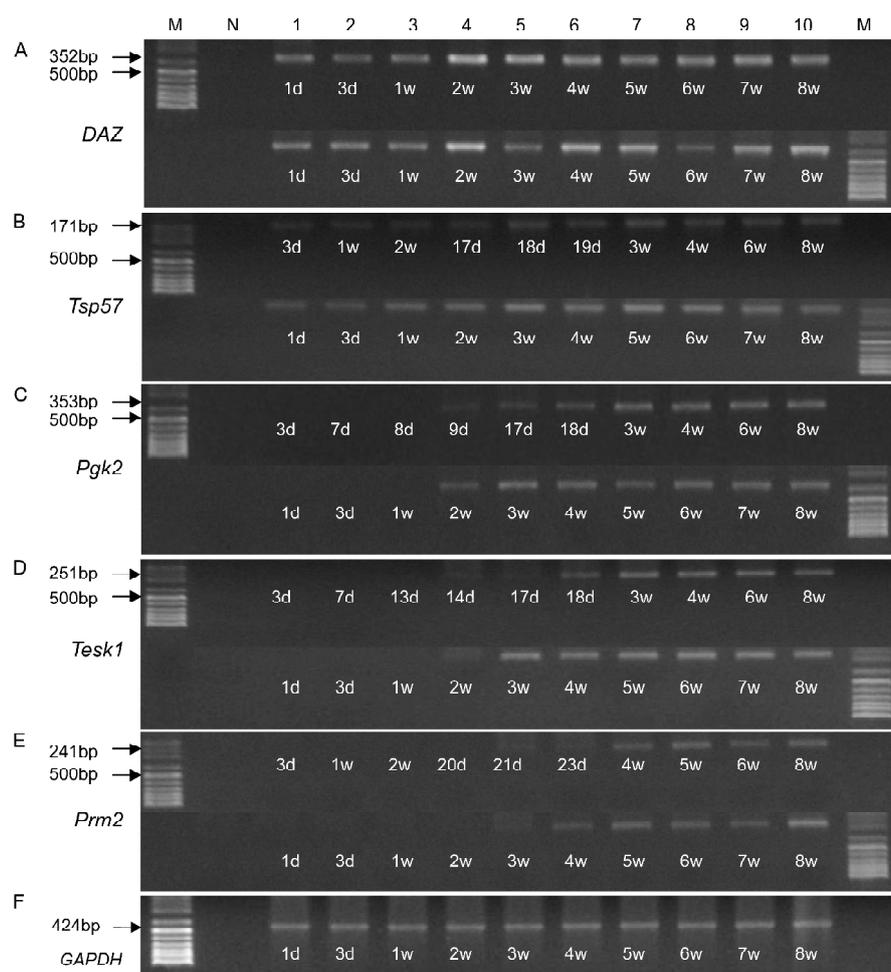
Figure 3. Expression of *DAZL*, *Tsp57*, *Pgk2*, *TESK1* and *Prm2* mRNA in grafts and normal donor testes. (A)–(E): Presenting *DAZL*, *Tsp57*, *Pgk2*, *TESK1* and *Prm2* reverse transcriptase-polymerase chain reaction (RT-PCR) products, respectively. (F): *GAPDH* RT-PCR products of grafts as RNA loading control. Lanes 1–10 (top): Testicular tissues from normal donor mice. Lanes 1–10 (bottom): Grafts obtained at different time intervals. Lanes M: DNA ladder (bands from bottom to top: 1 000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100 and 50 bp). Lane N: negative control.