Selective Nuclear Factor Kappa b (NFκB) Inhibitor, Pyrrolidium Dithiocarbamate Prevents, Long-Term Histologic Damage in Ischemia-Reperfusion Injuries after Delayed Testicular Torsion

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Purpose: Nuclear factor kapa b (NFKB) is a transcription factor that is required for cytokine-mediated induction of the human inducible nitric oxide synthase (iNOS) gene. Recent studies have shown that in the pathophysiology of ischemia-reperfusion (IR) injuries NFKB is involved. In our study we aimed to determine the efficacy of the selective NFKB inhibitor, pyrrolidium dithiocarbamate (PDTC), on long-term histological damage in testicular IR injuries.

Materials and Methods: Twenty-one adult male Wistar albino rats were divided into 3 equal groups. In groups 1-2, the left testes in rats underwent 4 hours of 720° experimental torsion. In group 2, PDTC (100 mg/kg) was administered intraperitoneally in the last 1 hour before detorsion; and group 3 underwent a sham operation. All rats underwent bilateral orchiectomy 45 days after the experiment. The testes weights were measured and compared to the other groups and their contralateral values. Testes samples were fixed with Bouin solution for histological (Johnsen score) and immunohistochemical examination. Immunohistochemically iNOS and an active subunit of NFKB, p65 were evaluated using mouse primary monoclonal antibodies and were evaluated semi quantitatively.

Results: Testicular weights and Johnsen scores in ipsilateral testes were 0.67 ± 0.85, 1.54 ± 0.11, 1.84 ± 0.64 and 1.63 (1-4), 6.94 (4-10), 5.29 (1-9) in the torsion, sham and PDTC groups, respectively. In contralateral testes the same values were 1.74 ± 0.84, 1.59 ± 0.13, 1.50 ± 0.54 and 5.38 (2-8), 7.17 (5-10), 6.30 (4-9). Testicular weights and Johnsen scores were significantly different in the ipsilateral torsion group ($P < .05$). In the PDTC group testicular weights and Johnsen scores were similar with the control group ($P > .05$). Immunohistochemically there was marked staining in the iNOS and p65 expressions in the torsion group compared with group 2 and 3. In rats administered PDTC, iNOS and p65 expressions were significantly reduced compared with the torsion group. There were no significant differences between the histological and immunohistochemical results of groups 2 and 3.

Conclusion: This data suggests that IR induces iNOS expressions through the activation of NFKB, p65. The NFKB pathway plays major role in testicular reperfusion injuries. It is possible to prevent reperfusion injuries using selective the NFKB inhibitor.

Keywords: torsion abnormality; reperfusion ınjury/pathology; rats, wistar; antioxidants/therapeutic use; testis/blood supply; testis/metabolism.

INTRODUCTION

Testicular torsion (TT) is common urological emergency in infants and adolescents that can lead to testicular necrosis. The incidence of TT has been estimated to be 1 in 158 males by the age of 25 years or approximately 1 in 4,000 per annum in males.[1] To avoid testicular loss and eventual impaired fertility, rapid diagnosis and immediate surgical intervention are the most important issues for the treatment of these patients. Testicular salvage rates with surgery have been reported to range from 42% to 88%.[2,3] Surgical intervention within 6, 12 and 24 hours of beginning results in a salvage rate of 90%, 50% and less than 10%, respectively.[4] Several experimental studies suggest the role of ischemia and reperfusion injuries in the pathophysiology of TT and the salvage of testicles from ischemia reperfusion induced testicular injuries, antioxidants and reactive oxygen species (ROS) scavengers have been used widely in literature.[5-9]

Previous studies have demonstrated that nuclear factor kappa b (NFKB) is involved and is an important transcription factor during the inflammatory process and ischemia reperfusion. Activation of NFKB by ROS are responsible for several proinflammatory molecules including intercellular adhesion molecular-1, inducible nitric oxide synthase (iNOS), cyclooxygenase-2, interleukin-1β, interleukin-6, tumor necrosis factor-α, etc.[10-12]

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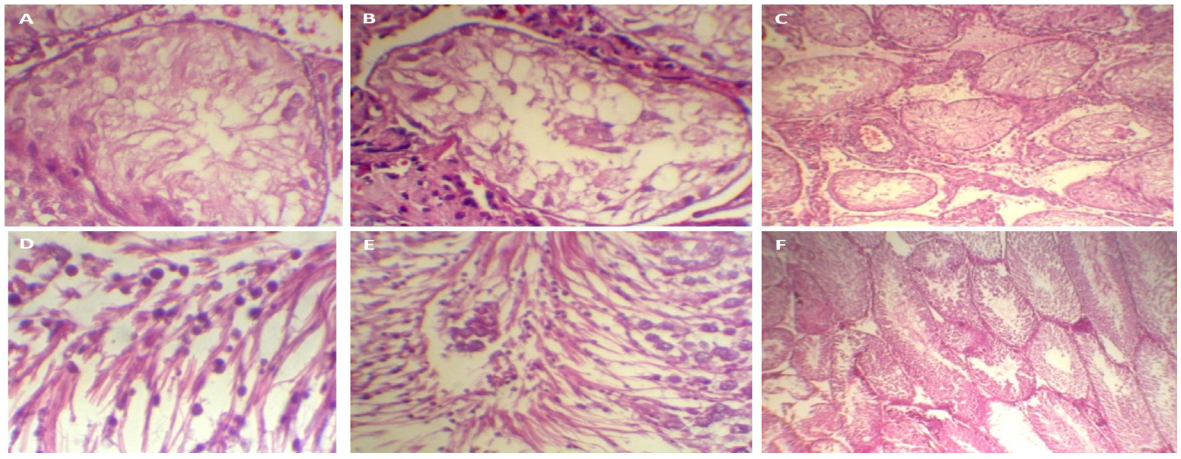

Figure 1. Haematoxylene eosine staining.
A: G-I, there is only Sertoli cells in tubular lumens (×400).
B: G-I, seminifer tubules containing only Sertoli cells (×100).
C: G-I, there is moderate narrowing in seminifere tubules (×400).
D-E: G-II, active spermatagenesis, spermatids in seminifer tubules and spermatozoas (×400).
F: G-II, partially protected seminifer tubules (×400).

In this study we tested the preventive role of selective the NFKB inhibitor, pyrrolidium dithiocarbamate (PDTC) in testicular ischemia reperfusion ischemia induced testis damage in rats in the long term.

MATERIALS AND METHODS

Experimental Procedure

Adult, male, Wistar albino rats (230-250 g) were used. The animals were kept under standard laboratory conditions (12 h light/dark cycle, 26-28°C) for at least 1 week before the experiment and those conditions were preserved until the end of the experiment. Animal cages were kept clean, and food and water were given regularly every day. All experiments in this study were performed in accordance with the guidelines for animal research issued by the National Institutes of Health and were approved by the Local Committee on Animal Research.

The rats were divided into 3 groups containing 7 rats each, Group 1: Sham control; Group 2: ischemia-reperfusion; Group 3: ischemia-reperfusion + PDTC. All anesthesia was performed with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). After anesthesia, the rats were kept in a supine position and underwent antisepsis of the scrotal region with 2% iodine alcohol. Surgery was performed through a left scrotal incision. In group 2 and 3, unilateral testicular torsion was created by rotating the left testis 720° in a clockwise direction and fixed within hemiscrotum with a 3/0 silk suture and the incision was then closed using 2/0 silk suture.[8] Torsion was maintained for 4 hours. At the end of 4 hours, anesthesia was repeated. Then detorsion of the testis was performed in group 2 and a testis was placed into the scrotum and wound closed again. In group 3, PDTC (Sigma-Aldrich Chemical Corp, MO, USA; 100 mg/kg) was administered intraperitoneally in the last 1 hour before detorsion and then the testis was detorsioned and placed into scrotum and wound closed. The doses of PDTC were selected based on the results of recent studies in which the antioxidant and anti-inflammatory action of this agent were apparent.[13,14] PDTC was dissolved in 0.9% saline. All rats underwent bilateral orchiectomy 45 days after the experiment. Contralateral testes were used as an internal control. Testes weights were measured and compared to other groups and contralateral values. Testes samples were fixed with Bouin solution for histological (Johnsen score) and immunohistochemical examinations.

Immunohistochemistry and Johnsen Scoring

For the immunohistochemical evaluations, the specimens were processed for light microscopy and sections incubated at 60°C overnight and then de-waxed in xylene for 30 min. After rehydrating them in a decreasing series of ethanol, sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin in a 50 mM Tris buffer (pH 7.5) at 37°C for 15 min, and then washed with PBS. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. Then, sections were incubated with NF-κB/P65 (Rel A) Ab-1 (R-B-1638-R7, Neomarkers, Labvision, Fremont, CA, USA) and iNOS Ab-1 (R-B-1605-R7, Neomarkers, Labvision, Fremont, CA, USA)

Table. Testicular weights and Johnsen score among the groups.

Parameters	Sham Group	Torsion Group	PDTC + Torsion Group
Testicular weight/i (g)	1.54 ± 0.11	0.67 ± 0.85a	1.84 ± 0.64b
Testicular weight/c (g)	1.59 ± 0.13	1.74 ± 0.84	1.51 ± 0.54
Johnsen score/i	6.94 ± 2.13	1.63 ± 1.12a	5.29 ± 2.92b
Johnsen score/c	7.17 ± 2.72	5.38 ± 1.86	6.3 ± 2.76

Abbreviations: PDTC, pyrrolidium dithiocarbamate; i, ipsilateral; c, contralateral.
Values are expressed as mean ± SD for seven rats in each group.
a Significantly different from sham.
b Significantly different from torsion group ($P < .05$).

antibodies. The Ultra-vision (Labvision, Fremont, CA, USA) horseradish peroxidase/3-amino-9- ethylcarbazole staining protocol was used at this stage.

Sections prepared for each case were examined by light microscopy. Sections of rat lung were used as the control for immunohistochemical staining specificity, in accordance with data provided by the antibody manufacturer. The sections were evaluated for diffuseness and staining. Testicular changes were evaluated according to the diffuseness and intensity of staining. For staining diffuseness, sections were graded as: 0, no staining; 1, staining < 25%; 2, staining 25-50%; 3, staining 50-75%; 4, staining > 75%. For staining intensity, sections were graded as: 0, no staining; 1, weak but detectable above control; 2, distinct; 3, intense.[14] Immunohistochemical values were obtained by adding the diffuseness and intensity scores, and the results were compared using chi-square test.

Johnsen Tubular Testicular Biopsy Scores

The Johnsen score was used to assess testicular morphological damage as described above.[15] Briefly, paraffin sections (4 mm thick) were cut and mounted on glass slides and then sections were defaraffinized and hydrated by processing them with xylene and a series of graded alcohols. The sections were stained by hematoxylin eosin were observed under a light microscope, and then seminiferous tubular sperm formation disorders were evaluated in each group following the Johnsen score. To evaluate spermatogenesis, at least 40 seminiferous tubules were examined per slide, and each slide was scored using the Johnson score. Seminiferous tubules were scored on a scale of 1 to 10, with complete inactivity of tubules scored as 1 and those with maximum activity (≥ 5 spermatozoa in the lumen) scored as 10.

Statistical Analysis

Testicular weights and Johnsen biopsy scores in the

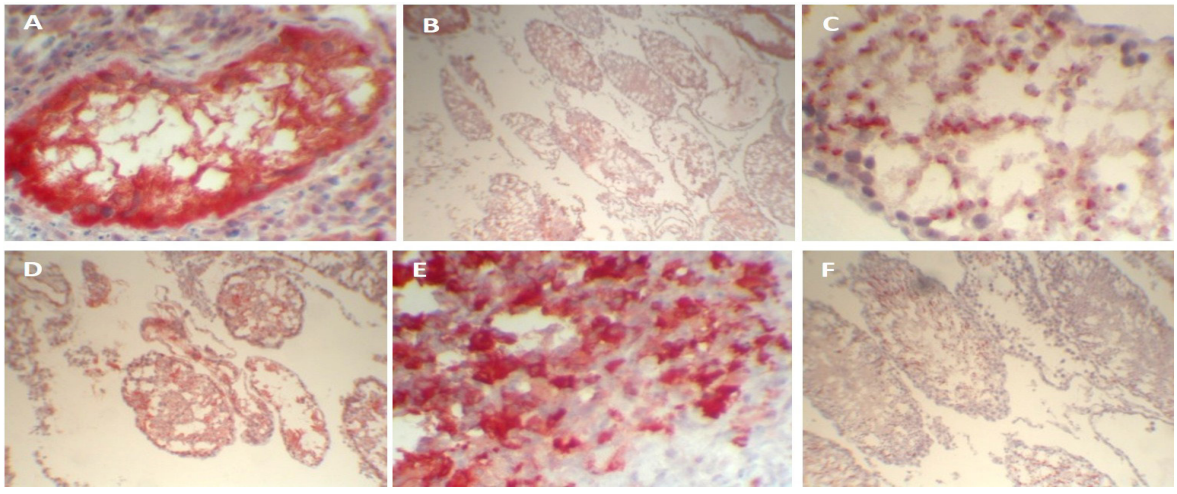

Figure 2. Immunohistochemical staining.
A: G-I, strongly positive staining p65 in seminiferous tubules (×400).
B: G-III, weak staining of p65 (×400).
C: G-II, weak iNOS staining in seminiferous tubules (×400).
D: G-II, moderate staining of p65 in seminiferous tubules (×100).
E: G-I, strong iNOS staining in interstitial space (×400).
F: G-III, weak iNOS staining (×100).

groups are expressed as the mean ± SD. Analysis of variance (ANOVA) was used for statistical analysis of the data among the groups. The significance between two groups was determined by the Tukey's multiple comparison test and $P < .05$ was accepted as statistically significant value.

RESULTS

Testicular weights and Johnsen scores in the ipsilateral testes were 0.67 ± 0.85, 1.54 ± 0.11, 1.84 ± 0.64 and 1.63 (1-4), 6.94 (4-10), 5.29 (1-9) in torsion, sham and PDTC group, respectively. In contralateral testes the same values were 1.74 ± 0.84, 1.59 ± 0.13, 1.50 ± 0.54 and 5.38 (2-8), 7.17 (5-10), 6.30 (4-9). Testicular weights and Johnsen scores were significantly different in the ipsilateral torsion group ($P < .05$). In PDTC group testicular weights and Johnsen scores were similar with the control group ($P > .05$) (**Table**). Immunohistochemically there was marked staining in iNOS and a p65 expression in the torsion group compared with group 2 and 3. In rats administered PDTC, iNOS and p65 expression were significantly reduced compared with torsion group (**Figures 1 and 2**). There were no significant differences between the histological and immunohistochemical results of groups 2 and 3.

DISCUSSION

The severity of testicular damage is related to the duration and degree of torsion and reperfusion time.[16] Increased reactive oxygen species after detorsion are the most important mediators in testicular damage. To prevent or minimize the ischemia reperfusion injuries, antioxidants are the most commonly used agents.[7,8] One of them NFKB, is a transcriptional factor and plays a significant role in the pathophysiology of ischemia reperfusion induced tissue damage.

To prevent testicular damage after ischemia reperfusion injury several agents have been tested. Ekici and colleagues have showed the protective effect of ozone and melatonin in TT.[8] In another study Altunoluk and colleagues evaluated the protective effects of zofenopril on TT. According to their study treatment with zofenopril decreased damage in ipsilateral testis caused by ischemia/reperfusion. They also conducted clinical application of zofenopril which might be a new approach for the treatment of TT in addition to conventional detorsions.[17] In recently published study, the involvement of NFκB has been reported. According to this study the authors demonstrates that PDTC prevents testicular torsion-detorsion injury induced biochemical and histologic changes testicular tissues in the rat.[18] The protective role of NFκB, other antioxidants and ROS scavengers has been used in TT for short time periods. But in this study we evaluated the protective role of selective NFκB inhibitors, PDTC, after a long time period.

Animal studies have known significant decreases in testicular weights and Johnsen scores in ipsilateral testes, but there are some controversies about the possible deleterious effect of torsion on contralateral testis.[19-21] The present study showed that unilateral TT-detorsions caused significant loss in ipsilateral testicular weight in torsion group.

Ischemia-reperfusion of the testes results in increased testicular oxidative stress and germ cell apoptosis. In this study we evaluated spermatogenesis by Johnsen scores. We showed that unilateral TT-detorsions caused significant loss in ipsilateral testicular Johnsen score in the torsion group.

One of the mechanisms involved in ischemia reperfusion injuries is the increased intratesticular nitric oxide levels the through activation of iNOS.[22] Another of the most accepted possible mechanisms of nitric oxide is through increased peroxynitrite production.[23] Excess amounts of nitric oxide production by iNOS react rapidly with the superoxide radicals that are produced during reperfusion injuries to form peroxynitrite which induces protein damage by forming nitrotyrosine.[24,25] Wang and colleagues showed that natural phenolic antioxidant compound tyrosol administration attenuated ischemia-reperfusion-induced NFκB activations, iNOS expression and improved kidney functions. They proposed that that tyrosol may have a protective effect against acute kidney injury through inhibition of iNOS-mediated oxidative stress.[24] In immunohistochemical examination there was severe staining in iNOS and the active subunit of NFκB, p65, in the torsion group, however, there was weak staining in sham and PDTC treated group in this study. We propose that activation of NFκB by IR, activates iNOS expressions and this results in increased intratesticular nitric oxide and testicular injury.

The limitation of this study is the biochemical parameters of IR injuries which were not analyzed and the lack of a vehicle group.

CONCLUSIONS

This data suggest that IR induces iNOS expression through activation of the NFkB pathway which plays a major role in testicular reperfusion injuries. It is possible to prevent reperfusion injuries using the selective NFkB inhibitor. Our study needs to be supported by further experimental and clinical studies.

CONFLICT OF INTEREST

None declared.

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