Protective Effects of the Nuclear Factor Kappa B Inhibitor Pyrrolidine Dithiocarbamate on Experimental Testicular Torsion and Detorsion Injury

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INTRODUCTION

Testicular torsion is an urologic emergency that affects newborns, children and adolescents. This condition also requires immediate surgical intervention to prevent testicular damage [1]. Testicular torsion leads to altered hormone production, and subfertility and infertility [2]. Ischemia and reperfusion (I/R) injury leads to the production of excessive reactive oxygen species (ROS), which can initiate lipid peroxidation, and oxidise proteins to inactive states and cause reactive oxygen species (ROS), which can initiate lipid peroxidation, and oxidise proteins to inactive states and cause DNA strand breaks [3,4]. It has been known that during ischemia, cells and tissues undergo rapid changes which lead to perturbations in signaling pathways and surface molecule expression. These events are thought to contribute to the tissue damage during I/R in various organs. The main cause of testicular damage after torsion is ischemia followed by reperfusion, which are known to have deleterious consequences of I/R [5]. The ischemic tissue leads to a complex cascade of events that includes the activation of nuclear factor kappa B (NF-κB), which controls cytokine, chemokines and adhesion molecules [6]. Previously, many preventative pretreatment agents like antioxidants and ROS scavengers were shown in testicular torsion-detorsion (T/D) experimental models. However, the molecular mechanism of antioxidants which control testicular torsion-induced male fertility has not yet been clearly identified [7]. Pyrroldidine dithiocarbamate (PDTC) is a low-molecular weight thiol compound. PDTC has different properties such as, redox state alternation, heavy metal chelation, and enzyme inhibition [8,9]. Studies have suggested that potent inhibitor of NF-κB which used as an antioxidant compound to counteract the toxic effects of free radicals and to interfere with the generation of proinflammatory cytokines [10,11]. Although studies showed that the protective effects of PDTC might inhibit NF-κB activation, PDTC has a potential protective agent for preventing the biochemical and histological changes related to oxidative stress in testicular injury caused by testis torsion.

Key Words: Antioxidant enzyme, Ischemia-reperfusion, Pyrrolidine dithiocarbamate, Testicular torsion

PDTC, pyrrolidine dithiocarbamate; NF-κB, nuclear factor kappa B; T/D, testicular torsion-detorsion; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; ROS, reactive oxygen species; HO-1, heme oxygenase-1.

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tial to activate gene expression of endogenous antioxidants and independent of any effects on NF-κB [12,13]. In addition, some studies suggested that the protective effects of PDTC might inhibit NF-κB activation via stabilization of IκB-α and inhibit of the ubiquitin-proteasome pathway [14,15]. Also, it has been demonstrated that PDTC is one of the most effective inducers of heme oxygenase-1 (HO-1), which also provides cytoprotection against oxidative stress [13,16].

The aim of the study is to evaluate the effects of PDTC on testicular ischemia reperfusion injury, by determining biochemical parameters and evaluating histological examinations.

**METHODS**

**Animals**

The experimental protocol was approved by the institutional animal ethics committee. Forty adult male Sprague-Dawley rats weighting 220 to 250 g were obtained from Medical and Surgical Experimental Research Center (Eskisehir-Turkey). Rats were housed in polycarbonate cages in a room with controlled temperature (22±2°C), humidity (50±5%), and a 12 h. cycle of light and dark and were fed laboratory pellet chows and water was given water ad libitum. The experiment was performed after a stabilization period in the laboratory for five days.

**Experimental protocol**

The surgical procedures were done under general anesthesia induced by intraperitoneal injection of ketamine HCl (50 mg/kg) and chlorpromazine (25 mg/kg). The skin of the scrotal area was shaved and prepared with 10% povidone iodine solution. A mid-scrotal vertical incision was performed for access to both testes. Torsion was created by twisting the right testis 720° in a counterclockwise direction and maintained by fixing the testis to the scrotum with a 5-0 nylon suture passing through the tunica albuginea and dartos. After 2 hour of ischemia, the suture was removed, and the right testis was untwisted and replaced in the scrotum for 4 hours of reperfusion. During the sham operation, the right testis was brought through the incision and then replaced without twisting, and a nylon suture was placed through the tunica albuginea. After each surgical intervention, the incision was closed. The animals were decapitated at the end of the experiment. Bilateral orchiectomies were performed for histologic examination and measurement of tissue malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) levels.

**Groups**

The rats were randomly separated into four equal groups (10 animals each). Group I (sham-operated control group) underwent a sham operation to determine basal values for biochemical and histopathological evaluation. The testes were brought through the incision and then replaced with a fixation to the scrotum with no additional intervention. Group II (torsion) was designed to study the effects of testicular torsion. In this group, torsion is performed for 2 hours by 720 degree extravaginally testis. Group III (torsion/detorsion) was designed to study the effect of detorsion after testicular torsion. In this group, 4 h reperfusion of the testis was performed after 2 h of testicular torsion. Group IV (torsion—PDTC treatment—detorsion) was designed to determine the effect of PDTC after torsion. After performing the same surgical procedures as in group III, PDTC (100 mg/kg, intravenous’s) (Dithiocarbamate; Sigma-Aldrich, Steinheim, Germany) was administered before 30 min of detorsion.

**Histopathological evaluation**

At the end of the surgical procedure, testicular specimens were individually immersed in Bouin's fixative (7.5 ml of saturated picric acid, 2.65 ml of glacial acetic acid and 2.5 ml of 7% formaldehyde) and they were dehydrated in alcohol and embedded in paraffin. Five micrometer sections were obtained, deparaffinized and stained with haematoxylin and eosin (H&E). The testicular tissue was evaluated in random order with standard light microscopy (NIKON, Japan) by an observer unaware of which group the rat had belonged. Three slides prepared from the upper, lower and midportions of the testes were evaluated completely for each testis.

**Biochemical analysis**

1. **Homogenate preparation**

After sacrificing the animals, testes were quickly removed and perfused immediately with ice-cold normal saline, and homogenized in chilled potassium chloride (1.17%). The homogenate was centrifuged at 800g for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500g for 20 min at 4°C to get the homogenate which was used to assay MDA, CAT, and SOD activity.

2. **Determination of lipid peroxidation**

MDA production was an end product of lipid peroxidation reacts with thiobarbituric acid to form a red colored complex. 0.1 ml of homogenate, 3 ml of 1% phosphoric acid, 0.5 ml of distilled water and 1.0 ml of 0.0% 2-thiobarbituric acid were added. The mixture was boiled in water bath for 45 min, followed by cooling in an ice, and addition of 4.0 ml of n-butanol to extract the cold thiobarbituric acid reactants. The optical density of the n-butanol layer was determined at 532 nm after centrifugation at 1,000 g for five minutes and expressed as nmol MDA/g of wet tissue [17].

3. **Assay of SOD activity**

SOD activity was spectrophotometrically assayed with commercial kits (Fluka SOD kit USA). It is an indirect assay method based on xanthine oxidase and a novel color reagent. SOD activity in the homogenate was determined by inhibition of Formosan dye (450 nm) employing the xanthin-xanthin oxidase enzymatic method to generate superoxide radicals and calculated the active SOD concentration according to inhibition curve graphic expressed as U/g of wet tissue.

4. **Assay of CAT activity**

One unit (1U) of CAT equals the enzyme activity that recognized 1 μmol of hydrogen peroxide in 60 s at 37°C. CAT activity was measured with determination of absorbance of three blank samples at 405 nm. CAT activity (kU/L)
Table 1. Antioxidant enzyme levels in testes

| Group                      | Group number | MDA (nmol/mg Hb) | SOD (Inh %) | CAT (kU/L) |
|----------------------------|--------------|------------------|-------------|------------|
| Group I (Sham)             | GI           | 4.24±2.69        | 68.90±6.90  | 7.03±3.93  |
| Group II (Torsion)         | GII          | 8.30±3.39        | 58.20±3.39  | 2.93±1.27  |
| Group III (Torsion/Detorsion) | GIII       | 9.60±3.32        | 56.30±6.39  | 3.00±1.54  |
| Group IV (PDTC)            | GIV          | 3.82±1.35        | 65.70±4.02  | 6.46±3.44  |

p values and Multiple comparison of the groups
GI-GII .013 .000 .012
GI-GIII .001 .000 .014
GI-GIV .987 .552 .968
GII-GIII .734 .859 1.000
GII-GIV .006 .018 .038
GIII-GIV .000 .002 .043

Fig. 1. Mean MDA, SOD, CAT activities of all groups.

was calculated as=[(Absblank1−Absblanksample)/Absblank2−Absblank3]×271 [18].

Statistical analysis

All statistical analysis was performed with the computer program “SPSS for Windows” (SPSS Inc; Release 11.5; Sep 6, 2002). All of the data were expressed as means±SD. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. The significance was tested as n.s p > 0.05, p < 0.05, p < 0.01 and p < 0.001.

RESULTS

The values of MDA, SOD and CAT measurements for the different groups are shown in Table 1 and Fig. 1. The MDA levels of group II and III in testes tissue were significantly higher than group I and group IV. PDTC administration had significantly decreased MDA levels in group IV when compared with group II and III (p < 0.05). But, there was no significant difference between the group II and group III (p > 0.05). The SOD and CAT levels were significantly decreased in group II and III when compared with group I and IV, but after PDTC administration SOD and CAT levels increased and there weren’t any difference between group I and IV (p > 0.05). Also these results suggested that PDTC may be effective in preventing oxidative injury. The findings of the histopathological evaluation for each group were shown in Fig. 2. The testes of rats in sham-operated control group showed the presence of normal testicular architecture and regular seminiferous tubular morphology with normal spermatogenesis (Fig. 2A). In Group II (ischemia) severe tubular degeneration was observed, many tubule of spermatogenic cell lines completely disappeared, also thickening of the basal laminae of tubules, interstitial edema and vascular congestions in the area were seen, and some of the tubules necrotic (pyknotic nuclei and eosinophilic cytoplasm) cells spermatocytes, which was more pronounced with detorsion (Fig. 2B). In addition, in the T/D group we observed closed tubule lumen with many cellular desquamation, degeneration of germ cells, severe tubular degeneration necrotic cells and eosinophilic accumulation of fluid in the interstitial space and vascular congestions (Fig. 2C). The PDTC-treated group showed maturation up to the level of spermatozoa, with preservation of tubular morphology. Administration of PDTC caused significant rescue of testicular function by preserving the intact seminiferous tubular morphology.

DISCUSSION

Testicular torsion and detorsion leads to biochemical and morphologic changes caused by ischemia-reperfusion (IR) injury in the testicular tissue [19]. Various pathogenic mechanisms have been reported to explain the tissue damage that occurs during testicular torsion and detorsion such
Fig. 2. (A) Sham group shows normal testicular architecture and regular seminiferous tubular morphology with normal spermatogenesis (star) (H&E, 50×). (B) Torsion group shows severe tubular degeneration, many of the tubules of spermatogenic cell lines completely disappeared (black arrows), thickening of the basal laminae of tubules (yellow arrows), Interstitial edema (stars) and vascular congestions, hemorrhage (black arrowhead) in the area, some of the tubules has necrotic (pyknotic nuclei and eosinophilic cytoplasm) cells spermatocytes (white arrows) (H&E, 20×, 50×). (C) Torsion/Detorsion group shows tubule lumen be filled with many cellular desquamation, degeneration of germ cells (stars), severe tubular degeneration (black arrowhead), necrotic cells (white arrowhead) and eosinophilic accumulation of fluid in the interstitial space and vascular congestions (black arrows) (H&E, 20×, 100×). (D) The PDTC-treated group shows maturation up to the level of spermatozoa, with preservation of tubular morphology (H&E, 20×).
as testicular lipid peroxidation, vascular leukocyte margina-
lation and apoptosis, which in turn leads to membrane dam-
age [4]. The detorsion involves the production of toxic re-
active oxygen species (ROS) with the return of blood flow
following ischemia [5]. Testicular torsion and detorsion in-
jury decrease also frees radical scavengers (SOD and CAT),
as shown in our study. Thus, defining the activities of free
radical scavenger enzymes in testicular tissue provides
some important clues about free radical formation. Exces-
sive amounts of oxygen free radicals cause membrane lipid
peroxidation which can be measured by tissue MDA levels
and it is a well-known parameter for determining the raised
free radical formation in reperfused tissue [17]. In our
study, the level of MDA significantly increased in the T/D
group when compared to the sham group. Treatment with
PDTC significantly decreased MDA levels. Histologically,
the group when compared to the sham group. Treatment with
PDTC significantly increased MDA levels. Histologically,
the level of MDA significantly increased in the T/D
perfusion injury [6,20,21]. Previous studies have demon-
strated the role of NF-kB in injuries of organs such as the
kidney, liver, pancreas and nerve injury [21-25]. PDTC is
shown to potent protective effect against cisplatin-resis-
tant neuroblastoma cells [37]. Recently, it has been demonstrated in an animal model that
PDTC markedly ameliorated renal, brain, intestinal, mes-
enteric, colonic, liver, lung and gastric I-R injury [38-45].
PDTC has been shown to potent protective effect against
the testicular damage of cisplatin-induced testicular tox-
icity and demonstrate that blockade of NF-κB activation
by an antioxidant could be an effective strategy for prophy-
laxis of cisplatin-induced testicular damage [46].

In conclusion, the present study demonstrates that PDTC
prevents testicular T/D injury induced biochemical and his-
tologic changes testicular tissues in the rat. The clinical im-
plications of these results merits further experimental and
clinical studies to be performed.

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