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Mechanism mediating the protective effect of diacerein in ischemia-reperfusion-induced testicular injury in rats

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ABSTRACT

Aims: Torsion of the spermatic cord is a common urologic emergency and can lead to testicular necrosis and infertility. Diacerein (DIA) is interleukin-1b (IL-1b) blocker which has a protective role against myocardial ischemia-reperfusion, however, to-date this role has not been investigated in testicular ischemia- reperfusion (TIR). We aimed to investigate the role and mechanism of action of DIA in induced TIR.
Main methods: DIA (50 mg/kg) was administered i.m (intramuscular) to rats in the presence or absence of TIR. Testicular weight changes and serum testosterone and total cholesterol levels were evaluated. In addition; the level of testicular tissue reduced glutathione (GSH), malondialdehyde (MDA), total nitrites (NOx) and the activity of superoxide dismutase (SOD) were measured. Histopathology and interleukin1b (IL-1b) immunoexpression were evaluated.
Key findings: TIR manifested by significant decrease in testicular weight, serum testosterone and testicular tissue GSH levels and SOD activity as well as increase in serum total cholesterol, testicular MDA and NOx levels. TIR showed the histopathological changes of marked testicular damage with increase in IL-1b immunoexpression. DIA was able to normalize both testicular weight, serum testosterone and cholesterol levels with attenuation of oxidative stress parameters along with amelioration of histopathological changes and IL-1 b immunostaining induced by TIR.
Significance: DIA has a protective effect against TIR induced injury in rats mediated by its anti-inflammatory and anti-oxidant activities.

1. Introduction

Torsion of the testis is a serious urological emergency in infants and adolescents that can lead to testicular necrosis and complete damage. The incidence of testicular torsion approximates 1 in 158 males by the age of 25 years, thus early diagnosis and treatment are mandatory to prevent infertility [1].

Testicular injury due to spermatic cord torsion/detorsion is a state of testicular ischemia/reperfusion (TIR) [2]. Spermatic cord torsion in rats causes permanent cessation of spermatogenesis, due to germ cell-specific oxidative stress, apoptosis and inflammation [3].

The TIR stimulates an intra-cellular signaling cascade that results in neutrophil recruitment, an increase in intra-testicular reactive oxygen species, inflammation and germ cell-specific apoptosis [1].

Ischemia leads to reduction in oxygen supply, depletion of cellular energy and accumulation of toxic metabolites, which result in oxidative stress and germ cell death. Reperfusion leads to increase production of reactive oxygen and nitrogen species causing membrane lipid peroxidation. This leads to tissue injury and disorganization of the cell structure and function [4]. Interleukin-1b (IL-lb) is a major mediator in the cascade of inflammatory process in reperfusion. IL-1b serves as one of the chemo-attractants of the leukocytes to areas of inflammation [5].

Diacerein (DIA) is an anthraquinone derivative anti-inflammatory drug developed for treatment of osteoarthritis [6]. DIA inhibits the production of IL-1-converting enzyme (ICE), also named caspase-1 which is the key limiting factor for the secretion and activity of IL-b cytokine. It also inhibits the IL-1b binding to its receptor with reduction of its specific receptor [6]. The role of IL-b was evaluated in hepatic [7], renal [8], cardiac [9], brain [10], lung [11] and intestinal [12] ischemia reperfusion. However, to date the role of DIA is only investigated against myocardial ischemia reperfusion induced injury [13] so the current experiment aimed to evaluate the role of IL-1b blocker (diacerein) in TIR and clarify its mechanism of action.

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2. Materials and methods

2.1. Drugs and chemicals

DIA was from Eva Pharma Company, Egypt, Polyclonal rabbit/anti-rat IL-1b anti-body was from Lab Vision, USA and biotinylated goat anti-rabbit secondary antibody was from Transduction Laboratories, USA. Total cholesterol kit was purchased from Biomed Company, Egypt.

2.2. Animals

Adult male Wistar albino rats weighing 250–300 g were obtained from the animal house, Giza, Egypt. Animals were left in standard housing conditions in cages (3rats/cage) and supplied by laboratory chow and tap water for one week to acclimatize before starting the experiment. The present study was conducted in the Pharmacology department, Faculty of Medicine, Minia University, Egypt. The animal experimental protocol was approved by the faculty board and in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

2.3. Surgical procedure

The TIR induced rats were fasted overnight before the experiments, but were given free access to water. Animals were weighed and anesthetized using i.p injection of urethane hydrochloride (1 g/kg i.p.) [14]. Scrotal incision was done in the left scrotum then the left testis was twisted 1080° in a clockwise direction for 1 h for induction of ischemia. Reperfusion was done via untwisting the testis to its neutral position and maintained for 2 h. At the end of the experiment each rat was weighed then sacrificed. Venous blood was collected from the jugular vein and centrifuged at 5000 rpm for 15 min (JanetzkiT30 centrifuge, Germany) and left testes were excised.

2.4. Grouping

Rats were divided into 4 groups (6 rats per group). Group I, sham group, was subjected to all operative procedures without testicular torsion/detorsion. Group II was sham treated with DIA (50 mg/kg i.m) [15], group III was TIR induced group [16], and group IV was treated with DIA (50 mg/kg i.m 30 min before reperfusion) + TIR.

2.5. Evaluation of the serum parameters

2.5.1. Evaluation of serum total cholesterol

The total cholesterol level was measured using colorimetric kit according to the manufacturer instructions (Biomed, Egypt).

2.5.2. Evaluation of serum testosterone level

Measurement of serum testosterone was done using testosterone ELISA kit (Cayman Chemicals., USA) according to the manufacturer instructions.

2.6. Measurement of oxidative stress markers in testicular tissue

Part of ipsilateral testis was kept at −80 °C. For preparing testicular tissue homogenate for biochemical analysis, testes were homogenized (Glas-Col homogenizer, USA), and a 20% w/v homogenate was prepared in ice-cold potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 4000 rpm for 15 min at 4 °C in cooling centrifuge (Ray Wild TGL-16, Germany) then the supernatant was used for evaluation of malondialdehyde (MDA) and total nitrites (NOx), reduced glutathione (GSH) levels and superoxide dismutase (SOD) activity.

Testicular GSH level and SOD enzyme activity were evaluated as indicators of testicular antioxidant status. Evaluation of the level of GSH is based on that the sulfhydryl groups of GSH react with 5,5-dithiobis-2-nitrobenzoic acid (Ellman's reagent), that gives a yellow colored 5-thio-2-nitrobenzoic acid. The color density was detected at 412 nm colorimetrically using Beckman DU-64 UV/VIS spectrophotometer, USA [17]. Testicular SOD enzyme activity was evaluated depending on the ability of the enzyme to inhibit the phenazinemethosulphate-mediated reduction of nitrobluetetrazolium dye [18].

Testicular MDA and NO_x were evaluated as indicators of testicular oxidative stress. MDA is the major product of lipoperoxidation that was measured as MDA-thiobarbituric acid (TBA) pink colored Schiff base adduct formed by the reaction of MDA with TBA under high temperature and acidic conditions and the adduct is measured colorimetrically at 535 nm [19]. NOx evaluation was based on estimation of nitrite (NO_{2-}) and nitrate (NO_{3-}) levels which are the stable oxidation end products of NOx. Levels of NO_{2-}/NO_{3-}, the indicators of ·NO production, were estimated by the Griess method [20].

2.7. Histopathological examination

Immediately after sacrifice, the ipsilateral testicles were dissected with removal of the adipose tissue, washed with cold normal saline and weighed on Mettler Toledo scale, Swizer Land. Parts of the ipsilateral testicles were immediately immersed in Bouin's fixative for 24 h, dehydrated, and embedded in paraffin blocks. Hematoxylin and eosin (H&E) staining was used to stain 5-μm tissue sections. The testicular tissue biopsy was evaluated randomly with light microscope using an Olympus microscope, Japan. The observer was unaware as to which group the slide had belonged. Screening of sections was done under light microscope magnification ×200, ×400.

Cosentino score [21] (Table 1) was used for quantitation of histopathological changes by evaluation of the degrees of severity of injury of different seminiferous tubules however, the effect of ischemia on the spermatogenesis was evaluated according to Johnsen's scoring system [22] (Table 2).

2.8. Immunostaining of IL-1b in testicular tissue

For the immunohistochemical evaluations, sections were 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. Antigen retrieval was done by treating the sections with citrate buffer, pH. 6.0 for 15 min in microwave oven, and then washed with PBS. Sections were incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity then washed with PBS. Then, sections were incubated with primary antibody (IL-1b) for 1 h at room temperature (Polyclonal rabbit antibody, 7 ml Ready to use, Lab Vision Laboratories; USA) after that, sections were washed with PBS for 5 min. The secondary antibody was added for 30 min then antibody reaction was detected by with 0.05% diaminobenzidine-chromagen (Lab Vision Laboratories; USA) for 5 min. The slides were counter stained with Mayer's hematoxylin solution, differentiated in 0.5% acid alcohol, then washed well in tap water. Lastly, sections were dehydrated in four

Table 1
Histopathological grading system.

Grade	Characteristics
I	Normal testicular architecture with an orderly arrangement of germinal cells
II	Injury showed less orderly, non-cohesive germinal cells and closely packed seminiferous tubules
III	Injury exhibited disordered sloughed germinal cells, with reduced size of pyknotic nuclei and less distinct seminiferous tubule borders
IV	Injury exhibited seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells