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Apocynin attenuates testicular ischemia–reperfusion injury in rats

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ABSTRACT

Objective: This study was designed to examine the possible protective effect of apocynin, a NADPH oxidase inhibitor, against torsion/detorsion (T/D) induced ischemia/reperfusion (I/R) injury in testis.
Methods: Male Wistar albino rats were divided into sham-operated control, and either vehicle, apocynin 20 mg/kg- or apocynin 50 mg/kg-treated T/D groups. In order to induce I/R injury, left testis was rotated 720° clockwise for 4 hours (torsion) and then allowed reperfusion (detorsion) for 4 hours. Left orchiectomy was done for the measurement of tissue malondialdehyde (MDA), glutathione (GSH) levels, myeloperoxidase (MPO) activity, and luminol, lucigenin, nitric oxide (NO) and peroxynitrite chemiluminescences (CL). Testicular morphology was examined by light microscopy.
Results: I/R caused significant increases in tissue luminol, lucigenin, nitric oxide and peroxynitrite CL demonstrating increased reactive oxygen and nitrogen metabolites. As a result of increased oxidative stress tissue MPO activity, MDA levels were increased and antioxidant GSH was decreased. On the other hand, apocynin treatment reversed all these biochemical indices, as well as histopathological alterations that were induced by I/R. According to data, although lower dose of apocynin tended to reverse the biochemical parameters, high dose of apocynin provides better protection since values were closer to the control levels.
Conclusion: Findings of the present study suggest that NADPH oxidase inhibitor apocynin by inhibiting free radical generation and increasing antioxidant defense exerts protective effects on testicular tissues against I/R. The protection with apocynin was more pronounced with high dose.

Testicular torsion, which occurs mostly in neonatal period and in adolescents, is a urological emergency. The turning of spermatic cord and spermatic structures around themselves causes biochemical and histological changes and ultimately testicular dysfunction [1]. Owing to testicular torsion, blood flow to the testicular tissues is interrupted and this interruption leads to ischemia where energy rich phosphates (ATP) are depleted and their degradation products (hypoxanthine) rise in the ischemic period [2,3]. On the other hand following detorsion, tissues are exposed to reperfusion and in the presence of oxygen, hypoxanthine gives rise to superoxide anions ($O_2^{\bullet -}$), and in return, the hydroxyl radical ($^{\bullet}OH$); also peroxynitrite ($ONOO^-$) can be produced which all cause excessive damage to tissues after the ischemic period [4]. Thus, the mechanism underlying torsion/detorsion (T/D) damage to the testis is representative of ischemia/reperfusion (I/R) injury and is related to the generation of reactive oxygen and nitrogen species (ROS and RNS) [5]. Moreover it has been demonstrated that activated neutrophils adhere to microvascular endothelium and subsequently emigrate and secrete the enzyme myeloperoxidase (MPO) which catalyzes the formation of ROS. Polymorphonuclear leukocytes (PMNs, neutrophils) have also been shown to be a potential source of ROS in I/R injury [6]. In light of above mentioned interaction of ROS and I/R injury, reduction of ROS generation is suggested to be the first clinical application in preventing the post ischemic organ damage.

NADPH-oxidase (NOX) is a major enzyme that uses NADPH to generate superoxide, initial ROS molecule, from oxygen [5]. Apocynin (4-hydroxy-3methoxy-acetophenone), naturally occurring methoxy-substituted catechol, extracted from the roots of *Apocynum cannabinum* (Canadian hemp) and *Picrorhiza kurroa* (Scrophulariaceae) is a well known inhibitor of NOX [7]. Connell et al. demonstrated that apocynin decreases cell death by increasing antioxidant defense in a rat model of rat cerebral artery occlusion [8]. Similarly apocynin was studied in renal I/R injury where increased MDA levels, MPO activities and apoptosis were reversed with this agent through its antioxidant effects [9].

Accordingly, the present study has been designed to determine whether apocynin could attenuate I/R induced testicular injury using biochemical measurements and histological examination.

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1. Materials and methods

1.1. Animals and chemicals

A total of 32 male Wistar albino rats (8 weeks of age, 200–250 g) supplied by the Marmara University (MU) Animal Center (DEHAMER) were used. Rats were housed in an air-conditioned room with 12:12 light:dark cycles, where the temperature (22 ± 2 °C) and relative humidity (65–70%) were kept constant. All experimental protocols were approved by the MU Animal Care and Use Committee.

Apocynin, DMSO (dimethyl sulfoxide), luminol (5-amino-2,3-dihydro-1,4-phtalazinedione), lucigenin (bis-*N*-methylacridiumnitrate), HEPES (*N*-[2-hyroxyethyl] piperazine-*N*′-[4butanesulfonic acid]), *o*-dianisidine, carboxy-PTIO, were obtained from Sigma Chemical Co. (St Louis, MO, USA) and trichloroacetic acid, 2-thiobarbituric acid, 5,5′-dithiobis (2-nitrobenzoic acid) from Merck (Darmstadt, Germany). All other chemicals were of the purest grade commercially available.

1.2. Experimental protocol

After 24 hours of fasting period rats were divided into following groups containing 8 in each: sham operated control (C) group and I/R-injured groups treated ip. with either vehicle (15% DMSO), 20 mg/kg apocynin (Apo20) or 50 mg/kg apocynin (Apo50), 15 min prior to torsion and immediately before detorsion. The dosage of apocynin was based on previous studies [9,10].

To induce ischemia, rats were anesthetized with xylazine (20 mg/kg) and ketamine (50 mg/kg), through a sub-inguinal incision the left testis was brought out and was rotated 720° clockwise and then inserted and fixed into the scrotum with a 4/0 silk suture placed through the tunica albuginea and subcutaneous tissue. The incision was primarily closed with a 4/0 silk suture. After 4 hours, by using the same incision line, testis was counter rotated to the natural position and reinserted into the scrotum to induce reperfusion for 4 hours [11]. In the sham-operated control group, rats had similar surgical procedures without the torsion and detorsion. At the end of the reperfusion period rats were decapitated and testis tissues were taken for biochemical analyses and histological evaluations. Biochemical and histological samples were blindly examined.

1.3. Tissue chemiluminescence assay

Reactive oxygen species (ROS) were determined by the chemiluminescence (CL) technique. Measurements were made at room temperature using Mini Lumat Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer. ROS were quantitated after the addition of luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) or lucigenin (bis-*N*-methylacridiumnitrate) for a final concentration of 0.2 mmol/l. Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min and corrected for wet tissue weight (rlu/mg tissue) [12]. NO levels were determined by luminol-H_2O_2 CL where NO is converted to peroxynitrite with H_2O_2 and measured thereafter. In order to identify peroxynitrite originating from tissues, a NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (0.5 mM carboxy-PTIO) was added to the vials, incubated in darkness for 15 min and counted again for 5 min. The percentage inhibition value indicated peroxynitrite levels present in the tissues [13].

1.4. Measurement of tissue MDA and GSH levels

Tissue samples were homogenized with ice-cold 150 mM KCl (w/v, 10 %) for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and results were expressed as nmol MDA/g tissue [14]. GSH measurements were performed using a modification of the Ellman procedure [15]. GSH levels were calculated using an extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$. Results were expressed in μmol GSH/g tissue.

Table 1
Modified Johnson scoring [17].

Score	Histologic findings
10	Full spermatogenesis
9	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium
8	Less than five spermatozoa per tubule, few late spermatids
7	No spermatozoa, no late spermatids, many early spermatids
6	No spermatozoa, no late spermatids, few early spermatids
5	No spermatozoa or spermatids, many spermatocytes
4	No spermatozoa or spermatids, few spermatocytes
3	Spermatogonia only
2	No germinal cells, Sertoli cells only
1	No seminiferous epithelium

1.5. Measurement of tissue myeloperoxidase activity

Tissue MPO activity was measured in a procedure similar to that documented by Hillegas et al. [16]. Tissue samples were homogenized (w/v 10 %) in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400*g* for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, *o*-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 minutes (Spectrophtometer, Beckman Coulter DU 73, Fullerton, California, US). MPO activity was expressed as U/g tissue.

1.6. Histological analysis

Testes were removed from the rats and fixed with 10% formaldehyde. Thereafter, the testes were dehydrated with subsequent 70, 90, 96 and 100% ethanol and cleared with toluene. After overnight incubation of paraffin in a 60 °C incubator, testes were embedded and blocked in paraffin at room temperature. Aproximately 5 μm thick paraffin sections in midline area of the testis were stained with hematoxylin and eosin (H&E). In each section at least 30 seminiferous tubules were evaluated microscopically at ×200 magnification. The first seminiferous tubules were selected randomly and the others were taken by sliding the section towards the clockwise. Histopathological scoring was evaluated by the modified Johnson scoring method as shown in Table 1 [17].

1.7. Statistics

Statistical analysis was performed using Graphpad Prism 4.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± SEM. Groups of biochemical data were compared by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests with $p < 0.05$ considered statistically significant. Histological scores were compared by Mann Whitney U test.

2. Results

2.1. Biochemical findings

I/R injury caused significant increases in both luminol and lucigenin CL of testicular tissues ($p < 0.001$) demonstrating increase in ROS production (Fig. 1A and B). Both doses of apocynin, 20 mg/kg and