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Protective effect of pterostilbene on testicular ischemia/reperfusion injury in rats

Hyung Joon Kim [a], Jae-Won Lee [a], Bo Ram Hwang [a], Young-Ah Lee [b], Jong-In Kim [a], Yoon Ju Cho [c], Hyun jhung Jhun [d], Jin Soo Han [a,*]

[a] The Institute for the 3Rs & Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[b] Department of Animal Science, Shingu College University, 2 Geumgwang-dong, Jungwon-gu, Seongnam-si, Gyeonggi-do 462-743, Republic of Korea
[c] Department of Pet Science, Seojeong College University, Yongam-ri, Eunhyeon-m yeon, Yangju-si, Gyeonggi-do 482-777, Republic of Korea
[d] Laboratory Animal Research Center, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea

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ABSTRACT

Purpose: To evaluate protective effect of pterostilbene against testicular ischemia/reperfusion (I/R) injury, which results in increased formation of oxidative stress, leading to testicular apoptosis and impaired spermatogenesis.
Methods: Thirty two pubertal male Sprague–Dawley rats weighing 180–220 g were selected and randomly divided into the following four groups: group A (normal control group), group B (sham-operated group), group C (induced I/R injury group), group D (induced I/R injury group receiving pterostilbene treatment). Johnsen's scores and mean seminiferous tubule diameters were evaluated for histopathologic assessment; germinal cell apoptosis was evaluated by the transferase dUTP nick end labeling (TUNEL) assay and immunohistochemistry for caspases. Malondialdehyde (MDA) levels were assessed as an indicator of oxidative stress and total antioxidant capacity (TAC) was measured.
Results: Germ cell apoptosis and MDA level significantly increased whereas TAC significantly decreased in group C; moreover, abnormal morphology and impaired spermatogenesis were observed in group C. In contrast, treatment with pterostilbene inhibited lipid peroxidation and apoptosis induced by ROS and restored the antioxidant capacity in group D.
Conclusions: These results show that treatment with pterostilbene may be a promising therapy for testicular I/R injury.

Testicular torsion is a human urologic emergency induced by torsion of the spermatic cord. It causes ischemia, immediate circulatory changes, testicular dysfunction, and fertility loss. Therefore, it should be reperfused within an appropriate critical time interval for maintaining testicular functions such as production of hormones and sperm.

Although reperfusion is necessary for the survival of testicular cells and tissues, it is associated with reactive oxygen species (ROS) and reactive nitrogen species (RNS) production that induce a cascade of events causing testicular ischemia/reperfusion (I/R) injury [1]. It is assumed that reperfusion itself leads to more severe cell and tissue damage than ischemia-induced reduction in oxygen [2].

Pterostilbene is a natural compound found primarily in blueberries and *Pterocarpus marsupium* (PM) heartwood [3]. It belongs to the group of phytoalexins, which are produced by plants in response to pathogens such as bacteria or fungi [4]. Pterostilbene has been reported to reduce oxidative stress and ROS generation and upregulate the expression of various antioxidant enzymes such as glutathione (GSH), glutathione-S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase [3]. Furthermore, it ameliorates I/R injury in the heart [5]. Therefore, pterostilbene is also expected to ameliorate I/R injury in the testis, however, there have been no reports on the protective effect of pterostilbene on testicular I/R injury thus far.

In the present study, the authors evaluated the protective effects of pterostilbene against testicular I/R injury. Histopathological assessment was conducted after hematoxylin–eosin staining. Additionally, the location and extent of apoptosis were examined by using the transferase dUTP nick end labeling (TUNEL) assay. Immunohistochemistry (IHC) revealed that the protective mechanism of pterostilbene involved caspase-3, 8, and 9 expression. Finally, malondialdehyde (MDA) levels were measured as an indicator of lipid peroxidation and testicular total antioxidant capacities (TACs) were evaluated.

1. Materials and methods

1.1. Experimental rat model

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University and conformed to the National Institutes of Health Guide for the

* Corresponding author: The Institute for the 3Rs & Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Republic of Korea. Tel.: +82 2 2049 6114; fax: +82 2 454 3932.
E-mail address: labvet@konkuk.ac.kr (J.S. Han).

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Care and Use of Laboratory Animals. Thirty-two pubertal male Sprague–Dawley rats (180–220 g) were purchased from KOATECH (Gyeonggi do, Korea) and randomly divided into the following four groups: group A, a normal control group with no surgical stress and I/R; group B, a sham-operated control group with surgical stress and no I/R; group C, an I/R group without treatment; and group D, an I/R group treated with pterostilbene (BP1175, Chengdu, Sichuan, China).

All surgical procedures were performed under deep anesthesia induced by intramuscular injection of xylazine (5 mg/kg) and Zoletil (40 mg/kg). After midline incision of the scrotum, the left testis was exteriorized. In group A, the testis was promptly extracted. In group B, the exteriorized testis was promptly returned to the scrotum, and a 4-0 polyamide monofilament nonabsorbable surgical suture was used to close the tunica albuginea and the midline incision in the scrotum. In groups C and D, the left testis with the spermatic cord was rotated 720° in the clockwise direction and attached to the scrotum with a 4-0 surgical suture in order to maintain the ischemic state. After 5 h of ischemia, the rotated testis was released by rotating it counterclockwise to induce reperfusion [6]. Pterostilbene (50 mg/kg) was simultaneously injected intraperitoneally in group D. The concentration of pterostilbene that showed therapeutic effect during the pilot test was used for the experiments. After the surgery, ketoprofen (5 mg/kg) was injected subcutaneously for reducing pain. In groups B, C, and D, the testis was extracted by using the same anesthetic protocol 24 h after reperfusion.

1.2. Histopathological evaluation of the testis

The testicular tissue was fixed in modified Davidson's fluid (mDF) [7]. Paraffin-embedded tissue was cut into 4-μm-thick slices and attached to slides at 60 °C. Tissue sections were stained with hematoxylin and eosin. Johnsen's scores and mean seminiferous tubular diameters (MSTDs) were used for histopathological evaluation. Ten continuous seminiferous tubules were evaluated, and the mean diameter was calculated. Each seminiferous tubule was assigned a score ranging from 10 to 1 according to the Johnsen's scoring system; the scoring is performed based on the state of germinal epithelium and the presence of germinal cells in the seminiferous tubules [8]. MSTD was calculated from 20 seminiferous tubular diameters [9].

1.3. Evaluation of germinal cell apoptosis

Apoptosis of germinal cells was evaluated with the TUNEL assay using the In Situ Cell Death Detection Kit (Roche Applied Sciences, Mannheim, Germany) following the manufacturer's instructions. After the tissue sections were attached to poly-L-lysine-coated slides at 60 °C, they were deparaffinized and rehydrated. Antigen retrieval was performed using a microwave oven. The slides were boiled in 10 mM citrate buffer, pH 6, in the microwave oven for 10 min. After rinsing with phosphate-buffered saline (PBS) twice, the sections were incubated in the blocking solution for 10 min. After three rinses with PBS, the sections were incubated in the TUNEL reaction mixture at 37 °C for 1 h. Following three more rinses with PBS, 50 μL of converter-POD was added, and the sections were incubated in a humidified chamber for 30 min at 37 °C. After rinsing with PBS three times, the sections were treated with 3,3′-diaminobenzidine (DAB) for 10 s, followed by counterstaining with hematoxylin.

The degree of apoptosis was evaluated based on the apoptotic indices (AI) 1 and 2. AI-1 was defined as the mean number of apoptotic TUNEL-positive cells per positive tubule section in 100 tubules, and AI-2 was defined as the ratio of the number of positive tubules to 100 tubules [6]. The testicular tissues were examined by two qualified pathologists blinded to their origin.

1.4. Evaluation of testicular lipid peroxidation

MDA levels were evaluated with the thiobarbituric acid assay using a commercially available kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Each tissue was resuspended at 100 mg/mL in PBS, and 10 μL of 100× BHT (butylated hydroxytoluene) was added to 1 mL of sample to prevent further oxidation. All samples were homogenized on ice and centrifuged at 10,000*g* for 5 min to collect the supernatant. A dilution series of MDA standards was prepared in the concentration range of 125 to 0 μM by diluting in distilled water. Each MDA-containing sample and standard was assayed in duplicates. One-hundred microliters of samples and standards was added to microcentrifuge tubes. One-hundred microliters of sodium dodecyl sulfate lysis solution was added to both the samples and the MDA standards, and the samples were incubated for 5 min at room temperature. One-hundred microliters of TBA (thiobarbituric acid) reagent was added to both the samples and standards. After incubating for an hour at 95 °C, the tubes were cooled to room temperature in an ice bath for 5 min. All samples were centrifuged at 3000 rpm for 15 min and the supernatants were separated. Supernatants of the samples and standards were transferred to a 96-well microplate. The spectrophotometric scans were performed at 532 nm (Microplate Reader, Tecan Austria GmbH 5082 Grödig, Austria).

1.5. Evaluation of testicular total antioxidant capacity

TAC was evaluated by using a commercially available kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. Each tissue was homogenized in cold PBS and centrifuged at 10,000*g* for 10 min at 4 °C to collect the supernatant. A dilution series of uric acid was prepared in the concentration range of 1 to 0 mM by diluting 2 mM uric acid antioxidant standard in deionized water. Uric acid standards and samples were assayed in duplicates. Twenty microliters of the diluted uric acid standards and samples was added to the 96-well microtiter plate. After adding 180 μL of 1× reaction buffer to each well, initial absorbance was read. The reading was repeated after adding 50 μL of 1× copper ion reagent to each well and incubating for 5 min to initiate the reaction; 50 μL of the 1× stop solution was used to terminate the reaction in each well. The spectrophotometric scans were performed at 490 nm.

1.6. Evaluation of the pattern of immunohistochemical staining for active caspases

Anti-active caspase-3 antibody (ab2302), anti-caspase-8 antibody (ab4052), and anti-caspase-9 antibody (ab32539) from Abcam, Cambridge, UK, were used to evaluate the expression level of caspases. After attaching to a poly-L-lysine-coated slide at 60 °C, tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed using a microwave oven. The slides were boiled in 10 mM citrate buffer, pH 6, in the microwave oven for 10 min. The sections were then incubated in 3% H_2O_2 for 30 min at room temperature. After rinsing in PBS for 5 min, the sections were incubated with the blocking solution for 30 min. After the blocking solution was removed, the primary antibody to active caspase diluted 50:1 was added, and the sections were incubated in a humidified chamber at 4 °C overnight. The sections were then rinsed in PBS for 5 min and incubated with diluted biotinylated "universal" secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min. After rinsing in PBS for 5 min, the sections were incubated with the VECTASTAINN R.T.U Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min. After the DAB substrate (DAKO North America, Carpinteria, CA, USA) was applied to the sections for 10 min, they were counterstained with hematoxylin.

The level of caspase expression was evaluated based on the mean number of positive cells per positive tubule in 100 tubules (PC/PT), the ratio of the number of positive tubules to 100 tubules (PT/CT), and the mean number of positive cells per 100 tubules (PC/CT). The testicular tissues were examined by two qualified pathologists blinded to their origin.