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Omega-3 polyunsaturated fatty acids ameliorates testicular ischemia-reperfusion injury through the induction of Nrf2 and inhibition of NF-κB in rats

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ABSTRACT

Objective: This study was designed to investigate the protective effect of Omega-3 polyunsaturated fatty acids (n-3 PUFAs) on testicular ischemia-reperfusion (I/R) injury in rats.

Methods: A total of 24 rats were randomly divided into the following three groups: Group A (Control group, n = 8), Group B (I/R group, n = 8), Group C (I/R group treated with n-3 PUFAs, n = 8). Histological examination was used to assess changes in testicular structure. Tissue oxidative stress biomarkers, malondialdehyde (MDA) as well as Superoxide, and antioxidant indexes, including total antioxidant capacity (T-AOC); catalase (CAT); glutathione (GSH); glutathione/glutathione disulfide ratio (GSH/GSSG); superoxide dismutase (SOD) were determined. In addition, nuclear transcription factor erythroid 2-related factor 2 (Nrf2), Nrf2-dependent antioxidant enzymes, such as heme oxygenase-1 (HO-1) and NADPH quinine oxidoreductase-1 (NQO-1), and nuclear factor kappa B (NF-κB) were detected.

Results: Compared to I/R group, n-3 PUFAs could obviously increase the mean seminiferous tubular diameter in the histological examination. After n-3 PUFAs treatment, the level of tissue MDA and Superoxide were significantly decreased, while tissue T-AOC, CAT, GSH, GSH/GSSG and SOD levels were significantly increased, compared to I/R group ($P < 0.05$). Besides, the expression levels of Nrf2, HO-1, and NQO-1 were significantly higher and the NF-κB expression level was significantly lower in the n-3 PUFAs treated group than that in I/R group ($P < 0.05$).

Conclusions: These results provided evidences that n-3 PUFAs ameliorated testes damage caused by testicular I/R injury through its antioxidative capacity and anti-inflammatory effects, involving the activation of Nrf2 and the inhibition of NF-κB.

1. Introduction

Testicular torsion is a urological emergent condition that is mainly seen in 1/4000 males younger than 25 years, which may lead to testicular ischemic injury, infarction, and necrosis, even inevitable orchiectomy sometimes. Long-term follow-up of patients with surgical treatment of testicular torsion, testicular atrophy, function damage and male infertility are observed in the testicular salvage (Anderson and Williamson, 1988; Visser and Heyns, 2003), which is mainly caused by ischemia-reperfusion (I/R) injury. Reactive oxygen metabolites and inflammatory response were reported to contribute to the pathogenesis of I/R injury (Granger and Korthuis, 1995; Carden and Granger, 2000). Previous studies have revealed that the neutrophil infiltration, overproduction of toxic oxygen free radicals and inflammatory response were known as possible cause of testicular I/R injury (Erol et al., 2009; Shimizu et al., 2016; Ergur et al., 2008).

Based on the above reasons, free radical scavenger, antioxidants, anti-inflammatory cytokine, and other drugs were investigated and proved to be effective in preventing adverse effects of I/R injury in the testicular torsion/detorsion model (Shimizu et al., 2016). Fish oil is rich

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sources of omega-3 polyunsaturated fatty acids (n-3 PUFAs), containing eicosapentaenoic acid (EPA) and dexosahexaenoic acid (DHA). Clinical findings have shown that adding EPA and DHA by intake of fish oil could have protective effects on ischemic diseases (Kris-Etherton et al., 2002; Wang et al., 2006). Recent report has also demonstrated that n-3 PUFAs pre-treatment was beneficial for spermatogenesis by decreasing germ cell apoptosis and oxidative stress in a acute doxorubicin (DOX)-induced testicular damage model (Uygur et al., 2014).

Although the nature of the protective mechanisms underlying testicular ischemia by adding n-3 PUFAs are not well understood, some studies have investigated that n-3 PUFAs exerted protection primarily via antioxidative and anti-inflammatory effects. Several studies have suggested n-3 PUFAs protected against ischemic injury in a variety of organs, such as brain and heart, by activating nuclear factor erythroid 2-related factor (Nrf2) (Zhang et al., 2014; Farías et al., 2017). Nrf2 is a cytoprotective regulator of antioxidant defense gene, and is associated with signaling in antioxidant response element (ARE)-mediated regulation of gene expression (Kobayashi and Yamamoto, 2005). Under oxidative stress conditions, the accumulated Nrf2 in the nucleus binds to the ARE promoter and upregulates a battery of antioxidant and cytoprotective genes. These genes include superoxide dismutase (SOD), catalase, hemeoxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase 1 (NQO-1), which confers resistance to the oxidative stress and inflammatory reactions in various disorders (Jaiswal, 2004; Nguyen et al., 2003). It was reported that 4-hydroxy-2-nonenal (4-HNE), an end-product of peroxidation of n-3 PUFAs, could activate Nrf2-mediated gene expression and stimulate GSH biosynthesis in cardiac I/R injury (Zhang et al., 2010). Thus, pharmacological induction of the Nrf2 pathway might constitute a potent protective strategy. However, it is still not known whether Nrf2 contributes to the protection of testicular I/R injury. Meanwhile, previous report of n-3 PUFAs for the treatment of non-alcoholic fatty liver disease revealed that n-3 PUFAs were able to decrease the endogenous lipid production and significantly reduce the expression of pro-inflammatory molecules (TNF-α, IL-1β and IL-6) (Di Minno et al., 2012). Nuclear factor kappa B (NF-κB) is an important transcription factor controlling the expression of pro-inflammatory genes, which could be activated by reactive oxygen species (ROS) in the I/R injury (Rodrigo et al., 2013; Hur et al., 1999). Pharmacological agents that inhibited the activation of NF-κB is associated with anti-inflammatory and anti-apoptotic effects in cerebra and heart I/R injury (Farías et al., 2017; Gu et al., 2012).

However, few studies focused on the protective role of n-3 PUFAs on testicular I/R injury via activating Nrf2 and inhibiting NF-κB. Therefore, the aim of this study was to investigate the protective effect of n-3 PUFAs on testicular I/R injury, and determine the possible mechanism by which n-3 PUFAs modulate Nrf2 and NF-κB signaling pathway and the consequent oxidative stress responses implicated in the development and progression of testicular I/R injury.

2. Materials and methods

2.1. Animals and experimental protocol

Adolescent male Sprague-Dawley (SD) rats (180–230 g), averaging 6 weeks old, were purchased from the Animal Experiment Center of Nanjing Medical University (Nanjing, Jiangsu, China). A total of 24 rats were randomly divided into three groups: Group A (Control group, n = 8), Group B (I/R group, n = 8), Group C (I/R group treated with n-3 PUFAs, n = 8). In Group A, the testes were explored through a scrotal incision and then replaced in the scrotum without torsion. In Group B, the testes were rotated 720° in the clockwise direction for two hours and then testicular reperfusion was carried out by restoring the testes to normal position. Subsequently, orchiectomy was performed four hours later. In Group C, the same testicular torsion process was performed with n-3 PUFAs pre-treated for two weeks. n-3 PUFAs treated group received fish oil (1 g/kg/day, DHA 33%, EPA 22%) for 14 days prior to the testicular I/R injury surgery. The same volume of diluted solution was gavaged in Control and I/R groups. The fish oil was purchased from Shengtianyu Corporation, Ltd. (Wuhan, Hubei, China).

2.2. Surgical procedure

The surgical procedures were performed under chloral hydrate anesthesia (300 mg/kg, intraperitoneally). The testicle and spermatic cord were exposed through the ilioinguinal incision. In the I/R group and n-3 PUFAs treated group, the left testis was rotated 720° in the clockwise direction for torsion. The tunica albuginea was fixed to the scrotum with a 5/0 silk suture to ensure this torsion position. After 2 h of ischemia, detorsion was carried out by restoring the testes to normal position. Subsequently, orchiectomy was performed 4 h later. The right testis underwent the same surgical procedure on each rat. Testis tissues were collected for biochemical analysis, histopathologic examination and western blot analysis.

2.3. Histological examination

The testicular tissue samples were fixed in 10% formaldehyde for 24 h, embedded in paraffin and sectioned at 4–5 μm thickness. The chosen transverse sections from each sample were stained with hematoxylin and eosin. Then the sections were examined under light microscopy (Olympus BX-51, Tokyo, Japan) to evaluate testis structural changes by two investigators blindly. The mean seminiferous tubular diameter (MSTD) was measured with a microscope-adaptable micrometer on 5 different focuses in the same histologic sectionin 20 seminiferous tubules.

2.4. Biochemical measurements

Malondialdehyde (MDA), total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), glutathione/glutathione disulfide ratio (GSH/GSSG), superoxide dismutase (SOD) were determined by using each Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China), as described earlier (Kushwaha and Jena, 2013; Lenoir et al., 2011). For evaluating testicular intracellular reactive oxygen species (ROS) production, intracellular superoxide level assay was detected under a fluorescent microscope (Eclipse Ti-SR, Nikon Co., Japan) according to the method of Benov et al. (1998). The density of the images was detected using a fluorescence spectrophotometer in arbitrary units per millimetre square field.

2.5. Immunohistochemical staining

Paraffin sections from the testes were dewaxed, rehydrated, and washed in phosphate-buffered saline (PBS) (Gibco, Grand Island, USA) three times (5 min each). Slides were microwaved for 20 min, cooled for 1 h at room temperature, and depleted of endogenous peroxidase activity by incubating in 3% H_2O_2 for 15 min. Then the sections were incubated with rabbit anti-mouse Nrf2 and NF-κB antibody (Cell Signaling Technology, USA) overnight at 4 °C. The slides were washed and incubated with a horseradish peroxidase (HRP)-conjugated anti-goat secondary antibody (Cell Signaling Technology, USA) at a 1:100 dilution for 1 h the next day.

2.6. Western blot analysis

Total protein extract of freshly obtained testicular tissues was prepared using a Nuclear Extract Kit (Active Motif, Tokyo, Japan) according to the manufacturer's instructions. After separated upon 7.5% SDS-PAGE, proteins were transferred to 0.45 μm PVDF membrane (Bio-Rad, California, Hercules, USA). The membranes were blocked in 5% nonfat milk in TBST (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 2 h and then incubated overnight at 4 °C with rabbit