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Comparison of combined antioxidants and thymoquinone in the prevention of testis ischemia – reperfusion injury

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SUMMARY

We aimed to compare the preventive effects of combined antioxidants (CA1, 2) with a single antioxidant drug (thymoquinone; TQ) on experimental testis Ischemia/Reperfusion (I/R) injury. Thirty-five adult male Wistar rats were divided into five groups of seven rats each: control, testis I/R, testis I/R + CA1, testis I/R + CA2, and testis I/R + TQ. After 1 h of testicular ischemia, reperfusion was achieved by detorsion for 4 h. Antioxidants were intraperitoneally administered for 30 min prior to reperfusion. All rats were sacrificed 4 h after reperfusion to evaluate the tissue levels of malondialdehyde (MDA) and total antioxidant status (TAS) and the immunohistochemical evaluation of tissue inducible and endothelial nitric acid synthase (iNOS, eNOS) and apoptosis protease-activating factor 1 (APAF-1). MDA levels were lower and TAS values were higher in the I/R + antioxidant groups than in the I/R group ($p < 0.05$). iNOS and eNOS levels in the I/R + antioxidant groups were also lower than those in the I/R group ($p < 0.05$). There were no significant differences between the CA groups and the TQ group according to aforementioned parameters. In addition, tissue APAF-1 values were significantly higher in the I/R group than in the other groups. However, there was a significant difference between the TQ and CA groups in APAF-1 levels, which were highest in the TQ group ($p < 0.05$). Although TQ alone increased TAS values and reduced tissue iNOS and eNOS levels, combined antioxidant treatment may more effectively reduce apoptosis and increase preventive effects in testis I/R injury.

INTRODUCTION

Testicular torsion is a urological emergency that requires immediate mechanical or surgical intervention. It can result in the loss of the testis if the duration of torsion is longer than 4 h. Oxidative stress is known to be involved in the damage observed following torsion. I/R injury leads to the accumulation of reactive oxygen species (ROS), including superoxide anions (O2), hydroxyl radicals (OH), and hydrogen peroxide (H2O2), all of which have a toxic effect on testicular tissue. Furthermore, ROS may cause DNA damage that triggers apoptosis in germ cells (Aitken & Baker, 2013). However, studies of antioxidant therapy have demonstrated that the neutralization of toxic effects because of I/R injury can be achieved (Aitken & Baker, 2013). To date, a number of chemicals and drugs have been successfully used to reduce I/R injury in animal models of testicular torsion, but few of these agents are currently in clinical use (Bozlu *et al.*, 2009).

Thymoquinone (TQ) is the major active constituent of *Nigella sativa* oil and is known to exert an antioxidant effect by increasing the production of antioxidant enzymes [i.e. catalase (CAT), glutathione peroxidase (Gpx), superoxide dismutase (SOD), and nitrite nitrate (NIT)] and decreasing lipid peroxidation (Kanter *et al.*, 2006; Awad *et al.*, 2011). Over the last decade, studies of I/R injury have reported that TQ reverses the toxic effects ROS in the affected organ (Bayrak *et al.*, 2000; Erboga *et al.*, 2016).

The antioxidants, especially combined antioxidants (CA) play an important role in protecting semen from ROS and can improve basic sperm parameters in case of idiopathic oligoasthenoteratozoospermia (Imamovic Kumalic & Pinter, 2014).

To the best of our knowledge, CA usage has not been investigated in the testicular I/R injury model. The aim of this study was to determine whether the cost effective and easily accessible single antioxidant drug (TQ) is superior to CA for the preventive effects of testis I/R injury.

MATERIALS AND METHODS

A total of 35 Wistar albino male rats were divided into five groups of seven animal each: control (C), testis I/R, testis I/R + CA1, testis I/R + CA2, and testis I/R + TQ. All animals were 2 months old and weighed between 250 and 300 g. The rats were maintained on a 12-h light/dark cycle after ethical committee on animal research approval. Institutional Review Board approval was obtained (11/03/2014-378).

Surgical procedures were performed under general anesthesia by intraperitoneal administration of ketamine HCl (50 mg/kg). An ilioinguinal incision was performed, and the left testis was rotated 720° in the clockwise direction. Tunica albuginea was fixed to the scrotum using a 4-0 silk suture. After 1 h of ischemia (Bozlu *et al.*, 2003; Erol *et al.*, 2009, 2010), detorsion (reperfusion) was achieved by counter-rotating the testis to its natural position. Orchiectomy was performed 4 h (Bozlu *et al.*, 2003; Erol *et al.*, 2009, 2010) after the onset of reperfusion. Combined antioxidants (5 mg/kg) were administered intraperitoneally beginning 30 min before reperfusion. One of the combined antioxidant drugs used was CA1 (Eczacibasi – Istanbul, Turkey), which contains L-carnitine (145 mg), acetyl-L- carnitine (64 mg), fructose (250 mg), citric acid (50 mg), selenium (50 mcg), coenzyme Q10 (20 mg), zinc (10 mg), ascorbic acid (90 mg), cyanocobalamin (1.5 mcg), and folic acid (200 mcg). The other combined antioxidant drug used was CA2 (Dorafarma/Catalysis – Turkey), which contains fructose (104 mg), cellulose microcrystalline (Emulsifier E460) (100 mg), Pygeum shell extract (*Pygeum africanum*) (100 mg), L-arginine (50 mg), L-carnitine (40 mg), L-ascorbic Acid (vitamin C) (30 mg), zinc sulfate (7 .2 mg Zn/capsule) (20 mg), vitamin E (D-α-tocopherol) (5 mg), pyridoxine hydrochloride (vitamin B6) (1 mg), sodium selenite (9 μg Se/capsule) (0.02 mg), pteroylmonoglutamic acid (folic Acid) (100 μg), cyanocobalamin (vitamin B12) (0.5 μg), hydroxypropyl methyl cellulose (79.65 mg). Likewise, TQ (20 mg/kg, Sigma, St. Louis, MO, USA) was applied 30 min before detorsion. At the end of the experiments, the rats were killed by pentobarbital overdose (200 mg/kg) and bilateral thoracotomy. MDA, TAS (SOD, CAT, Gpx, and NIT), APAF-1, eNOS, and iNOS levels were measured in the testicular tissue.

The supernatants were stored at −80 °C in Eppendorf tubes. High-performance liquid chromatographic analysis was performed using a Shimadzu HPLC system (Kyoto, Japan) with an MDA kit (Immundiagnostik AG, Bensheim, Germany). Spectrophotometric measurements of TAS (Ran-dox, Crumlin, UK) were performed using a Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer. The results are reported as μmol/g for MDA levels and mmol/g for TAS levels.

Serial sections of 3 μm were collected on poly-L-lysine-coated slides. Tissue sections were deparaffinized in xylene for 10 min. Hydrogen peroxide, phosphate-buffered saline (PBS), and Ultra V block non-specific blocking reagent (ScyTek Laboratories, Logan, UT, USA) were applied as the antigen retrieval solution. APAF-1 antibody (Lab Vision Corp., Neomarkers, Fremont, CA, USA), iNOS, and eNOS were evaluated using iNOS Ab-1 and eNOS Ab-1 antibodies (Lab Vision Corp.), respectively. They were applied at room temperature for approximately 90 min using dilutions in the order to 1 : 100, 1 : 100, and 1 : 100 for APAF-1, iNOS, and eNOS, respectively.

After incubation with the primary reagents, the slides were incubated with Ultra Tek antipolyvalent biotinylated antibodies (ScyTek Laboratories) as the secondary antibody for 15 min. After rehydration, Ultra Tek HRP (ScyTek Laboratories) was added to the specimens. After rehydration with PBS, the DAB chromogenic system (DAB substrate kit, ScyTek Laboratories) was added to the specimens. Finally, slides were counterstained with hematoxylin.

Every section was stained immunohistochemically and analyzed using light microscopy (Olympus BX51, Tokyo, Japan). The number of stained germ cells were counted in 100 seminiferous tubules on circular cross-sections for each group.

Statistical analysis was performed using SPSS Version 15 for WINDOWS, Chicago, IL, USA. All results are expressed as the mean ± SD. Mann–Whitney *U*-test and Chi-square tests were used for statistical analysis of data. p values <0.05 were considered statistically significant.

Table 1 Tissue MDA (mol/μg protein), iNOS, eNOS, and APAF-1 levels (mean ± SD)

	MDA	iNOS	eNOS	APAF1
Group C	38.37 ± 5.67*	0.85 ± 1.57*	0.28 ± 0.75*	0.57 ± 1.51*,**
Group I/R	73.22 ± 8.13	9.00 ± 2.23	9.28 ± 3.14	12.57 ± 2.57
Group CA1	43.55 ± 10.16*	0.85 ± 1.06*	0.57 ± 0.97*	2.14 ± 1.67*,**
Group CA2	41.90 ± 11.73*	1.00 ± 1.73*	0.28 ± 0.75*	3.00 ± 1.73 *,**
Group TQ	51.37 ± 12.40*	1.00 ± 1.29*	0.28 ± 0.75*	5.14 ± 2.60 *

C, control; I/R, ischemia/reperfusion; CA1, combined antioxidant 1; CA2, combined antioxidant 2; TQ, thymoquinone; MDA, malondialdehyde; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; APAF-1, apoptosis protease activating factor 1. Results are mean ± standard deviation. Mann–Whitney *U*-test. *$p < 0.05$ compared with Group I/R. **$p < 0.05$ compared with Group TQ.

Table 2 TAS activities (mmol/μg protein)

	SOD	CAT	GPO	NIT
Group C	0.23 ± 0.02*	201.67 ± 12.97*	1370.28 ± 205.86*	2.78 ± 0.79*
Group I/R	0.10 ± 0.02	150.27 ± 23.78	784.71 ± 139.39	5.08 ± 1.38
Group CA1	0.21 ± 0.06*	199.00 ± 27.30*	1290.71 ± 59.93*	3.05 ± 1.23*
Group CA2	0.22 ± 0.02*	190.74 ± 28.65*	1268.28 ± 169.45*	3.48 ± 0.77*
Group TQ	0.17 ± 0.07*	190.14 ± 15.82*	1033.57 ± 225.80*	3.59 ± 1.40*

C, control; I/R, ischemia/reperfusion; CA1, combined antioxidant 1; CA2, combined antioxidant 2; TQ, thymoquinone; SOD, superoxide dismutase; CAT, catalase; GPO, glutathione peroxidase; NIT, nitrite nitrate. Results are mean ± standard deviation. Mann–Whitney *U*-test. *$p < 0.05$ compared with Group I/R.

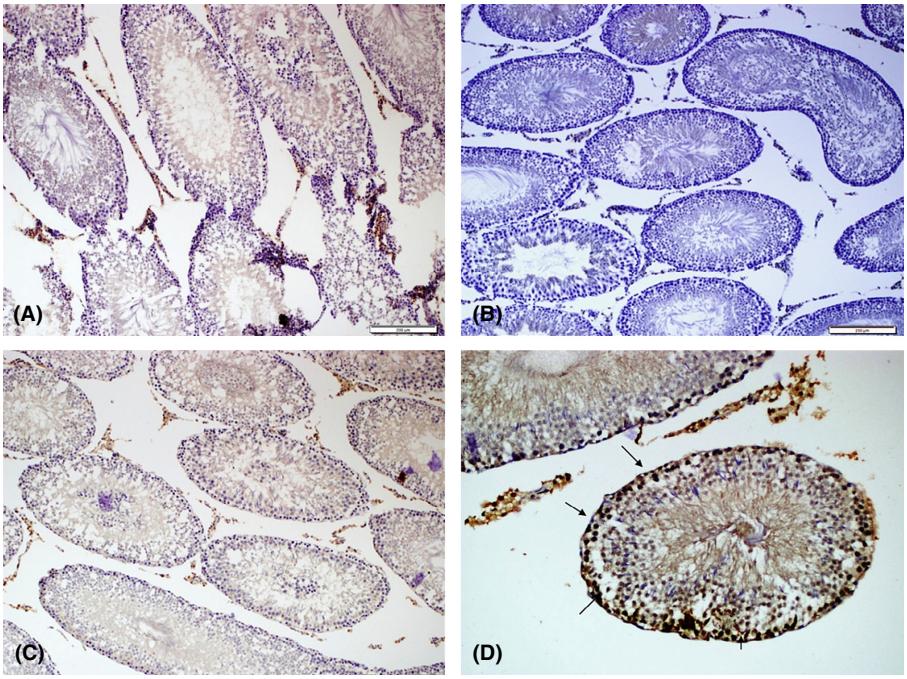

Figure 1 Inducible NOS (iNOS-NOS2) staining of germ cells in seminiferous tubules. (A) Decreased expression of iNOS in the I/R + CA1 group (×100). (B) No expression of iNOS was observed in the I/R + CA2 group (×100). (C) No expression of iNOS was observed in the I/R + thymoquinone group (×100). D) High expression of iNOS in the I/R group (×100).

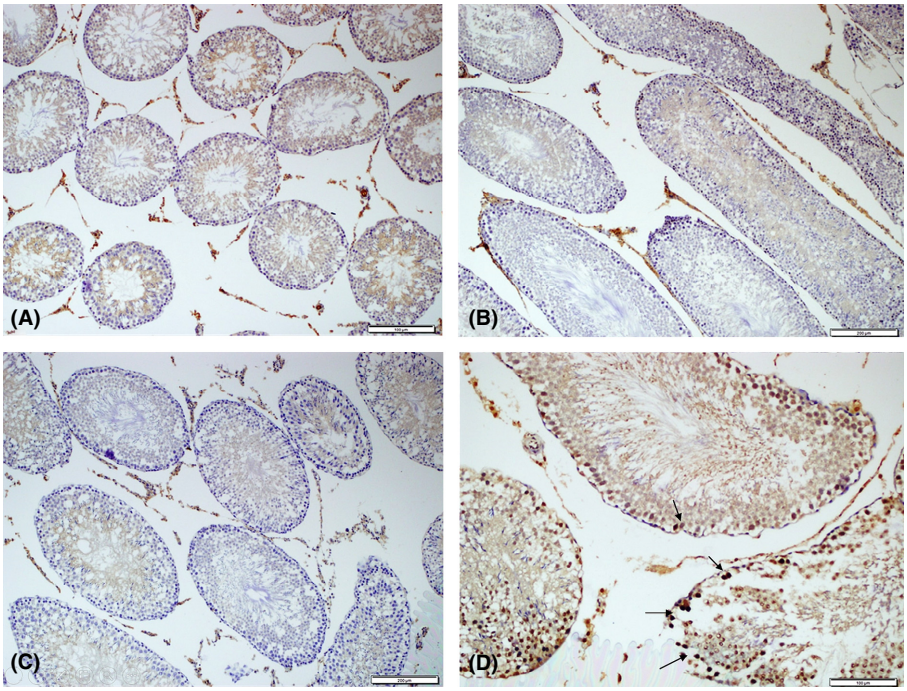

Figure 2 Endothelial NOS (eNOS-NOS3) staining of germ cells in seminiferous tubules. (A) No expression of eNOS was observed in the I/R + CA1 group (×100). (B) No expression of eNOS was observed in the I/R + CA2 group (×100). (C) No expression of eNOS was observed in the I/R + thymoquinone group (×100). (D) Strong expression of eNOS in the I/R group (×100).

RESULTS

All biochemical and immunohistochemical results are shown in Tables 1 and 2. There was no significant difference in MDA levels between the control and antioxidant treatment groups, but the I/R group had a significantly higher MDA level than the control and all treatment groups ($p < 0.05$) (Table 1).

The activity of TAS in the I/R group was significantly lower than those in all treatment groups. All antioxidant enzyme activities were suppressed in the I/R group (Table 2). All differences between the I/R and other groups were statistically significant ($p < 0.05$). In addition, there was no difference among the antioxidant treatment groups in TAS activity ($p < 0.05$).

Immunohistochemical analysis showed that iNOS and eNOS levels were significantly higher in the I/R group (Table 1). However, treatment with antioxidants significantly reduced iNOS and eNOS to levels similar to those of the control group (Table 1, Figs 1 and 2).

Apoptosis protease activating factor 1levels were significantly higher in the I/R group than in the other groups ($p < 0.05$). Nevertheless, TQ did not reduce APAF-1 levels as much as combined

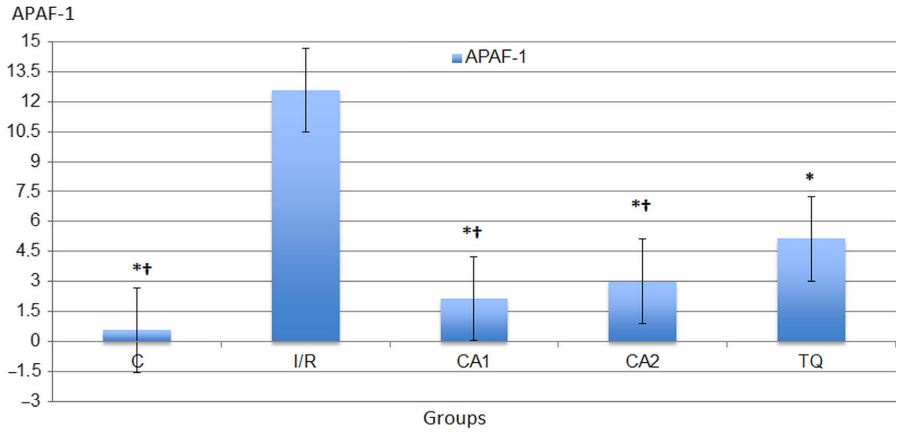

Figure 3 Apoptotic protease activating factor-1 (APAF-1) staining and cell counts in 10000 tubules with respect to each group. The difference between the I/R and other groups was statistically significant ($p < 0.05$). In addition, there was a significant difference between the TQ and CA groups ($p < 0.05$). Mann–Whitney *U*-test; *$p < 0.05$ compared with Group I/R; †$p < 0.05$ compared with Group TQ.

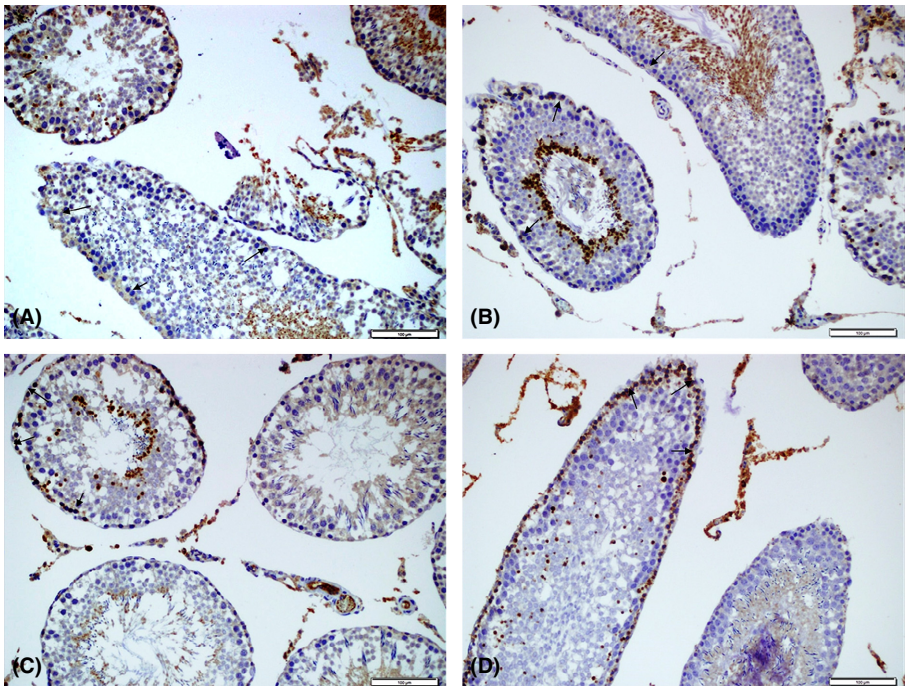

Figure 4 Apoptotic protease activating factor-1 (APAF-1) immunostaining for apoptosis in testis. (A) Weak cytoplasmic staining was observed in the I/R + antioxidant group (×200) (CA1). (B) Weak cytoplasmic staining was observed in the I/R + antioxidant group (×200) (CA2). (C) Moderate cytoplasmic staining was observed in the I/R + thymoquinone group (×200). (D) Strong cytoplasmic staining (×200) in the I/R group (×200).

antioxidants, and this difference was statistically significant (Table 1, Figs 3 and 4).

DISCUSSION

It is well-known that testicular IR injury induce biochemical and morphological changes in rat testicular tissues (Parlaktas *et al.*, 2014). The accumulation of ROS (oxidative stress) is the underlying pathologic mechanism of testicular torsion and is also observed in cases of undescended testis and varicocoele, all of which affect fertility (Unsal *et al.*, 2006). ROS react with proteins, lipids, carbohydrates, and nucleic acids leading to impaired cell function and apoptosis (Cvetkovic *et al.*, 2015). During reperfusion, the testicular tissue can counteract oxidative stress by upregulating antioxidant defenses with antioxidant enzymes (Sarica *et al.*, 1999). The elimination of ROS has been shown to be beneficial in treating I/R injury (Dokmeci, 2006). Previous studies demonstrated that the antioxidant enzyme levels were increased with single antioxidant therapy in testis IR injury (Koc *et al.*, 2005; Salmasi *et al.*, 2005; Cay *et al.*, 2006). Similarly, in our study, TAS activities were significantly higher in the antioxidant groups than the I/R group, but there was no significant difference among the antioxidant treatment groups.

Peroxidation of the lipid in membrane by ROS changes membrane permeability or disrupts membrane integrity and cell integrity (Akgur *et al.*, 1994). MDA is the end product of lipid peroxidation and is generally used as an indicator of radical formation in IR injury. Previous studies demonstrated that administration of antioxidants before reperfusion caused significant reduction in the level of testicular MDA (Abasiyanık & Dağdöndüren, 2004; Ozkan *et al.*, 2004; Erol *et al.*, 2010). In our study, I/R group had a significantly higher MDA level than the antioxidant treatment groups and there was no significant difference between the CA groups and TQ group.

In I/R injury of the rat testis, germ cell-specific apoptosis has been observed with an increase in leukocyte margination and diapedesis (Turner *et al.*, 1997). Once the neutrophils migrate into the interstitium of the testis, they release ROS that may directly cause apoptosis in the germ cell (Lysiak *et al.*, 2001).

IR of the testis stimulated a mitochondrial and caspase 9-dependent pathway to germ cell-specific apoptosis (Lysiak *et al.*, 2007). The mitochondria release cytochrome c which interacts with APAF-1 and activates caspase 9 (Hu *et al.*, 1999). In turn, caspase 9 activates downstream effector caspases that leads to cell death (Steller, 1995). In this study, I/R caused a significant increase in APAF-1 expression in the testis. We found that administering antioxidants and TQ before reperfusion caused a significant decrease in the testicular APAF-1 expression compared with that in IR group. In terms of reducing APAF-1 levels, CA groups were significantly superior to TQ group ($p < 0.05$).

The overexpression of iNOS and eNOS that produces a toxic level of NO has been previously reported in apoptotic germ cells of the mice testis (Lue *et al.*, 2003). Similarly, Hanci *et al.* (2010) and Ustün *et al.* (2008) found significant elevations in iNOS and eNOS levels in ipsilateral testicular tissues after IR injury. We demonstrated that, iNOS and eNOS levels were significantly higher in the I/R group and treatment with antioxidants significantly reduced the levels similar to those of the control group, but there was no significantly difference between the CA groups and TQ group.

The study has some limitations; we did not evaluate the contralateral testes, tissue myeloperoxidase activity, endocrine function of testis, mean seminiferous tubular diameter, mean testicular biopsy score, and germinal epithelial thickness. Finally, no prior study compared TQ to other antioxidant supplements.

In conclusion, the use of combined antioxidants in male infertility can make its potential use in testicular torsion more attractive. The results of the present experimental study show that administration of combined antioxidants may be a novel approach for the therapy of I/R injury of the testis. However, the more cost effective and widely available antioxidant thymoquinone may be recommended for low-income patients. Further studies are needed with a long follow-up period to evaluate the effects of CA1, CA2, and TQ on IR injury.

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