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

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<td>antioxidant, testis, ischemia-reperfusion injury</td>
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Comparison of combined antioxidants and thymoquinone in the prevention of testis ischemia-reperfusion injury

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 5 groups of 7 rats each: control, testis I/R, testis I/R + CA1, testis I/R + CA2, and testis I/R + TQ. After 1 hour of testicular ischemia, reperfusion was achieved by detorsion for 4 hours. Antioxidants were intraperitoneally administered for 30 minutes prior to reperfusion. All rats were sacrificed 4 hours 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 hours. 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 (Lysiak et al. 2003). However, studies of antioxidant therapy have demonstrated that the neutralization of toxic effects due to I/R injury can be achieved (Gharagozloo & Aitken 2011). 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 (Tüfek et al. 2015, Bayrak et al. 2000).

CA1 and CA2, which are combined antioxidants, are the most common drugs used to combat ROS in empirical therapy of male infertility (Gharagozloo & Aitken 2011)). To the best of our knowledge, CA antioxidants 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 a single antioxidant drug (TQ) is superior to combined antioxidants for the preventive effects of testis I/R injury.
Materials and Methods

A total of 35 Wistar albino male rats were divided into 5 groups of 7 animals 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-hour light/dark cycle after ethical committee on animal research approval. Institutional Review Board approval was obtained.

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 hour of ischemia (Erol et al. 2010, Erol et al. 2009, Bozlu et al. 2003), detorsion (reperfusion) was achieved by counter-rotating the testis to its natural position. Orchietomy was performed 4 hours (Erol et al. 2010, Erol et al. 2009, Bozlu et al. 2003) 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 - 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 - Turkey), which contains L-carnitine, fructose, selenium, zinc, ascorbic acid, cyanocobalamin, folic acid, pygeum africanum, L-arginin, α-tocopherol, and pyridoxine hydrochloride. Likewise, TQ (20 mg/kg) 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 (Randox, 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.

For immunohistochemical staining, paraffinized sections were prepared; after deparaffinization, the sections were stained with labeled streptavidin biotin. Germ cell apoptosis was evaluated using the apoptosis protease activating factor 1 (APAF-1) antibody (Lab Vision Corp., Neomarkers, Calif., USA). iNOS and eNOS were evaluated using iNOS Ab-1 and eNOS Ab-1 antibodies (Lab Vision Corp.), respectively. For each group, the number of stained cells in 100 tubules was counted.

Statistical analysis was performed using SPSS Version 15 for Windows. All results are expressed as the mean ± SD. Mann-Whitney U and Chi square tests were used for statistical analysis of data. p values less than 0.05 were considered statistically significant.

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).

TAS activity 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).
APAF-1 levels 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 antioxidants, and this difference was statistically significant (Table 1, figure 1,4). Immunohistochemical analysis showed that eNOS and iNOS 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, figure 2,3).

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 varicocele, 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 (Kawaguchi et al. 2000). Previous studies demonstrated that the antioxidant enzyme levels were increased with single antioxidant therapy in testis IR injury (Cay et al. 2006, Salmasi et al. 2005, Koc et al. 2005). 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 (Erol et al. 2010, Ozkan et al. 2004, Abasiyanik et al. 2004). 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 the present 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 mice testis (Lue et al. 2003). Similarly, Hanci (Hanci et al. 2010 ) and Ustün et al. (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 and also 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.

Acknowledgements, and/or financial disclosures: None

References:


sperm number, and determination of testes size: evidence from null mutant mice. *Endocrinology* 144, 3092-3100.


Table 1: Tissue MDA (µmol/ g protein), iNOS, eNOS and APAF-1 levels

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
<th>iNOS</th>
<th>eNOS</th>
<th>APAF1</th>
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<tr>
<td><strong>Group C</strong></td>
<td>38,37±6,57</td>
<td>0,85±1,57</td>
<td>0,28±0,75</td>
<td>0,57±1,51</td>
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<tr>
<td><strong>Group I/R</strong></td>
<td>73,22±8,13 abc</td>
<td>9,00±2,23 abc</td>
<td>9,28±3,14 abc</td>
<td>12,57±2,57 abc</td>
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<tr>
<td><strong>Group CA1</strong></td>
<td>43,55±10,16</td>
<td>0,85±1,06</td>
<td>0,57±0,97</td>
<td>2,14±1,67</td>
</tr>
<tr>
<td><strong>Group CA2</strong></td>
<td>41,90±11,73</td>
<td>1,00±1,73</td>
<td>0,28±0,75</td>
<td>3,00±1,73 e</td>
</tr>
<tr>
<td><strong>Group TQ</strong></td>
<td>51,37±12,40</td>
<td>1,00±1,29</td>
<td>0,28±0,75</td>
<td>5,14±2,60 fg</td>
</tr>
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</table>

C: control; I/R: ischemia/reperfusion; CA1: combined antioxidants 1; CA2: combined antioxidants 2; TQ: thymoquinone
MDA: malondialdehyde; eNOS: endothelial nitric oxide synthas; iNOS: inducible nitric oxide synthase; APAF-1: apoptosis protease activating factor 1

Results are mean ± standard deviation. Mann Whitney U test

a: p < 0.05 (Group C vs. Group I/R);
b: p < 0.05 (Group CA2 vs. Group I/R);
c: p < 0.05 (Group CA1 vs. Group I/R);
d: p < 0.05 (Group TQ vs. Group I/R);
e: p < 0.05 (Group CA2 vs. Group TQ)
f: p < 0.05 (Group C vs. Group TQ)
\( g; p < 0.05 \) (Group CA1 vs. Group TQ)
Table 2: TAS activities (mmol/g protein)

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

C: control; I/R: ischemia/reperfusion; CA1: combined antioxidants 1; CA2: combined antioxidants 2; TQ: thymoquinone
SOD: superoxid dismutase; CAT: catalase; GPO: glutatyone peroxidase; NIT: nitrite nitrate

Results are mean ± standard deviation. Mann Whitney U test

a: p < 0.05 (Group C vs. Group I/R)
b: p < 0.05 (Group CA2 vs. Group I/R)
c: p < 0.05 (Group CA1 vs. Group I/R)
d: p < 0.05 (Group TQ vs. Group I/R)
e: p < 0.05 (Group C vs. Group TQ)