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From Cell Biology to Tissue Engineering

Role of an endothelin type A receptor antagonist in regulating torsion-induced testicular apoptosis in rats

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Summary, Testicular torsion is a well-known medical emergency that can lead to pathological changes in the testicular tissues and male infertility. This investigation was undertaken to gain insight into the effects of an endothelin type A receptor antagonist (BQ123) on torsion-induced germ cell loss. Twenty-eight male Wistar albino rats were divided into four groups. In group I (control group), a sham operation to the left testis was performed. In group II (I/R injury), I/R injury was created by rotating the left testis 720° in a clockwise direction for 2 h and detorsing the testis after 2 h. In group III (I/R injury+BQ123), the rats were subjected to I/R injury and BQ123 injection (1 mg/kg, intravenous). In group IV (control+BQ123), the sham operated rats were subjected to BQ123. The testes of the rats were removed in all groups. Torsion-induced apoptosis and the effects of BQ123 were examined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) technique, immunohistochemistry and western blotting. In group II, the number of TUNEL-positive cells increased after testicular torsion. Immunohistochemistry and western blotting showed that apoptotic proteins (active caspase 3 and Bax) were upregulated, and the anti-apoptotic protein Bcl2 was downregulated in I/R injury. The administration of BQ123 caused a significant decrease in the number of apoptotic cells and the expression of apoptotic proteins ($p<0.05$) when compared with the I/R injury group. No significant effect of BQ123 was observed in the testicular cells of group IV. This animal study provides evidence of the regulatory effects of BQ123 on torsion-induced testicular apoptosis.

Key words: Apoptosis, Endothelin type A receptor antagonist, Rat, Testis, Torsion

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Introduction

Testicular torsion is a common urological emergency, especially among newborns and adolescents. Because inappropriate treatment can lead to infertility, early diagnosis and treatment are quite important in preserving patients' testes and fertility in testicular torsion. Surgery is the appropriate treatment for testicular torsion and provides reperfusion of testicular tissues (Prillaman and Turner, 1997; Kanter, 2010). It is known that testicular torsion and detorsion induce biochemical and morphological changes that are caused by ischemia/reperfusion (I/R) injury in rat testicular tissues (Kanter, 2010; Parlaktas et al., 2014). It has been reported that reactive oxygen species production during I/R injury is one of the main factors in this process (Turner et al., 1997; Filho et al., 2004; Turkili et al., 2012; Parlaktas et al., 2014). Moreover, various I/R models have revealed that both necrosis and apoptosis are mechanisms for I/R-induced testicular damage and cell death (Shiraishi et al., 2000, 2001). Specifically,

testicular ischemic injury caused by torsion leads to significant macroscopic and histological changes in 1 or 2 hrs after torsion (Cho et al., 2011; Kosova et al., 2011). In rat testis, apoptosis is the general mechanism of germ cell degeneration during spermatogenesis, which occurs spontaneously (Desagher and Martinou, 2000). During the mitochondrial apoptotic pathway, permeabilisation of the mitochondrial membrane is triggered by an increased Bax/Bcl-2 ratio (apoptotic regulators), leading to the release of cytochrome C and the apoptosis-inducing factor (AIF). AIF release causes caspase-independent DNA damage, while cytochrome C, apoptotic protease activating factor 1 (Apaf-1) and dATP combine to form the apoptosome, which activates caspase 9 and induces the subsequent downstream activation of caspases 3, 6 and 7 (Lopez-Neblina et al., 2005).

Various chemicals and drugs have been used to protect the testes against I/R-induced testicular damage and cell death (Shiraishi et al., 2000, 2001; Koc et al., 2005; Turkili et al., 2012; Parlaktas et al., 2014). Among them, there is no information that exists about the effects of endothelin type A receptor antagonists on testicular I/R injury-induced apoptosis. Endothelin-1 (ET-1), a 21-amino acid vasoconstrictor peptide, is synthesised and released in endothelial cells of vessels (Yanagisawa et al., 1988). There are two types of receptors for ET-1, namely the endothelin type A receptor (ETAR) and the endothelin type B receptor (ETBR), which are G protein-coupled receptors (GPCRs) (Arai et al., 1990; Sakurai et al., 1990). Via binding to the ETAR, ET-1 induces vasoconstriction. ET-1 is considered to play important roles in the control of blood pressure and cardiac function and also in the genesis and development of cardiovascular diseases, such as atherosclerosis (Kobayashi et al., 2000), cardiac remodelling accompanying chronic heart failure (Sakai et al., 1996), and pulmonary hypertension (Giaid et al., 1993). ET-1 is also synthesised outside the vascular system and may exert diverse effects. For example, it may be involved in controlling central nervous activity and renal function (Takuwa, 1993). High concentrations of ET-1 are found in endocrine organs, such as the pituitary, adrenal, testis, and ovary (Matsumoto et al., 1989; Takuwa, 1993). Apparently, endothelin-1 is synthesised locally in the testis (Sakurai et al., 1991), principally by Sertoli but also by Leydig cells (Fantoni et al., 1993). The intratesticular ET-1 concentration is reported to be approximately 100-fold higher than in plasma (Matsumoto et al., 1989). Testicular interstitial and myoid cells contain ETA receptors (Sakaguchi et al., 1992). ET-1 stimulates Leydig cells for testosterone secretion (Conte et al., 1993), and it also influences Sertoli and myoid cell function *in vitro* (Filippini et al., 1993; Sharma et al., 1994).

The vasoconstrictor effect of ET-1 is mainly mediated through ETA and partly by ETB receptors (Douglas et al., 1992). To reverse proinflammatory actions such as the production of neutrophils, cytokine release from macrophages or monocytes and other deteriorating effects of ET on different tissues, such as myocardium, liver, kidney and skeletal muscle, there is growing evidence that ET can be used as a target for therapeutic intervention (Grover et al., 1993; Herbert et al., 2001; Turkili et al., 2012). This approach resulted in the development of a specific ETA receptor antagonist or mixed ETA/ETB receptor antagonists. BQ123, a cyclic pentapeptide, is a selective antagonist of the ETA receptor and is used frequently in preclinical research and clinical trials of ET (Masaki, 2004). Although various clinical studies have already examined the beneficial effect of BQ123 on different tissues, in the English literature, the effect of the ETA receptor antagonist and the effects of BQ123 on testicular I/R injury-induced apoptosis have not been investigated. To examine this effect, we performed this animal study to investigate apoptotic proteins by immunohistochemistry and western blotting and DNA damage by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) assay in I/R-injured and BQ123-treated rat testis.

Materials and methods

Animals

Male Wistar albino rats, 5- to 6-months-old and weighing between 230 and 270 g, were obtained from the Gaziosmanpasa University Experimental Animal Research Laboratory. The rats were handled in the laboratory according to institutional guidelines as well as the Guide for Care and Use of Laboratory Animals of the National Research Council and the ethic rapor was approved by the local ethics committee (No: 2013 HADYEK-02). All rats were observed for several days to ascertain the health before sample collection. They were kept in a temperature-controlled room (20-23°C), on a 12-h light/dark cycle with food (commercial rat chow) and fresh water available ad libitum.

Testicular torsion

All of the rats were anesthetised with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). The surgical procedures were performed under sterile conditions through standard ilioinguinal incisions. Twenty-eight male rats were divided randomly into four groups, each containing seven rats. The animals in group I (sham-operated control group, n: 7) underwent a sham operation to determine the basal level of apoptosis. In this group, the left testis was exposed through an incision and displaced to the hemiscrotum, and the wound was closed. At the end of the 4-h experimental period, the testes were removed. Group II (I/R injury group, n: 7) was designed to study the effects of I/R injuries on testicular tissues. The left testis was rotated 720° in a clockwise direction and maintained for 2 h by fixing the left testis to the scrotum with a 4-0 silk suture as described elsewhere (Parlaktas et al., 2014). Detorsion

was performed by untwisting the testis and maintaining the position for 2 h. At the end of the experimental period, the testes were removed. In group III (I/R+BQ123, n: 7), the left testicles of the animals were also subjected to the same duration of ischaemia (2 h); however, 30 min before detorsion, one injection of BQ123 (1 mg/kg, intravenous) (Calbiochem, USA) was given to the animals. This dose of BQ123 was titrated in the rats, as previously reported (Aslan et al., 2015). BQ123 was given via tail vein. BQ123 was dissolved in PBS. The animals were weighed before the injection, and the volume of the injection was calculated according to the animal weight. The final volume of the administration was 200 µL. After the same period of detorsion (2 h), the testes were removed. The rats from group IV (sham operated control group+BQ123, n: 7) were also intravenously treated with BQ123 after a sham operation for 2 h. At the end of the experimental procedures, all of the rats were sacrificed and the testicular tissues harvested for various experiments.

Measurement of apoptosis

Apoptosis in the testicular tissue was detected by enzymatic labelling of DNA strand breaks using a TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling) kit (TUNEL system, 1684809, Roche, Mannheim, Germany). Paraffin sections were deparaffinised in xylene, rehydrated and treated in a microwave oven in 10 mM citrate buffer, pH 6.0, twice for 5 min and allowed to cool for 20 min. After three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was inhibited with 3% hydrogen peroxide. The sections were then incubated with equilibration buffer for 10-15 seconds, and TdT enzymatic labelling of nuclear DNA strand breaks was performed in a humidified atmosphere at 37°C for 60 min. The typical labels were revealed by adding an alkaline phosphatase (AP)-converter with subsequent staining with NBT/BCIP solution as the chromogenic substrate. Each step was separated by careful washings in PBS. Counter staining was performed in Mayer's haematoxylin. The average percentage of apoptotic tubules was estimated by examining 100 cross-sections of seminiferous tubules from each specimen. The seminiferous tubules that contained at least one TUNEL-stained nucleus were considered apoptotic (Yazawa et al., 2001). The percentage of apoptotic cells that stained pink was determined.

Immunohistochemical analysis

The testes were fixed in Bouin's fluid for 24 h immediately upon collection, dehydrated, and embedded in paraffin for immunohistochemistry. Immunohisto-chemistry was performed according to the procedure described previously (Cayli et al., 2012). Briefly, serial sections, 5 μm thick, were collected on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 56°C. The tissue sections were deparaffinised in xylene and rehydrated in a graded series of ethanol. The sections were then treated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 5 min twice and allowed to cool for 20 min. After three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by 3% hydrogen peroxide in PBS for 20 min and again washed three times in PBS. The sections were then incubated in a blocking serum (ScyTek Laboratories, USA) for 10 min to block non-specific binding. Subsequently, the sections were incubated for 1 h at room temperature with rabbit polyclonal active-caspase 3 (ab13847, 1:100, Abcam, UK), rabbit polyclonal anti-Bax (ab7977, 1:100, Abcam, UK) and rabbit polyclonal anti-Bcl2 (ab101568, 1:200, Abcam, UK) in a humidified chamber. The sections were washed three times in PBS and incubated with anti-rabbit (ab6721; 1:1000 dilution; Abcam) secondary antibodies for 60 min at room temperature. After three washes with PBS, bound peroxidase was developed with the 3-amino-9-ethylcarbazol (AEC) (ScyTek Laboratories, USA) chromogen, and the sections were counterstained with Mayer's haematoxylin (ScyTek Laboratories, Utah, USA) and mounted with Permount (Fisher Chemicals, Springfield, NJ, USA) on glass slides. For controls, sections were treated with the appropriate isotype mouse IgG or normal rabbit IgG, depending on the primary antibody used, which was diluted to the same final protein concentration as the primary antibody. Photomicrographs were taken with a Leica microscope (Leica DM2500, Nussloch, Germany).

Evaluation of the immunohistochemistry

The evaluation of the immunohistochemical labelling was performed using H-SCORE analyses, as previously described (Cayli et al., 2012). The intensities of Bax, active caspase 3 and Bcl2 immunoreactivities were semi-quantitatively evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). For each tissue, an H-SCORE value was derived by calculating the sum of the percentages of the cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the Formula H-SCORE: $\sum Pi(i+1)$, where 'i' represents the intensity scores and 'Pi' is the corresponding percentage of the cells. In each slide, five randomly selected areas were evaluated under a light microscope (40x objective), and the percentage of cells for each intensity within these areas was determined at different times by two investigators who were not informed about the type and source of the tissues. The average score of both observers was used.

Western blotting

Total testicular proteins were extracted using

modified radioimmunoprecipitation assay buffer (RIPA; 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L sodium chloride [NaCl]; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]; 1 mg/mL each of aprotinin, leupeptin, and pepstatin; 1 mmol/L sodium vanadate [Na3VO4]; and 1 mmol/L sodium fluoride [NaF] in 50 mmol/L Tris-Cl, pH 7.4) and quantitated using the Bradford procedure (Bio-Rad, Hercules, California). Samples (40 μg) were separated on a NuPAGE 4% to 12% Novex Bis-Tris gel (Invitrogen) and blotted onto a nitrocellulose membrane through the iBlot transfer system (Invitrogen). The membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h. Subsequently, the membrane was incubated overnight with the primary antibody against Bax (ab7977, 1:500, in 5% nonfat dry milk; Abcam), rabbit polyclonal active caspase 3 (ab13847, 1:400, in 5% nonfat dry milk; Abcam), Bcl2 (ab101568, 1:500, Abcam, UK.) and b-actin (ab8226, 1:3000 in 5% nonfat dry milk; Abcam, UK). The membrane was washed with PBS-T for 1 h and incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies diluted in 5% nonfat dry milk in PBS-T. After three washes with PBS, bound secondary antibodies were visualised by enhanced chemiluminescence substrate (ab133406, Abcam, UK). After chemiluminescence detection, the membranes were incubated with stripping solution (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) for 15 min at RT and reutilised for further detections. The immunoblot bands for active caspase 3, Bax, Bcl2 and b-actin were quantified using an Alpha DigiDoc 1000 gel documentation unit (Alpha Innotech Corporation, California). The optical density (OD) values for active caspase 3, Bax, Bcl2 bands were divided by the OD values of the cognate b-actin bands to normalise the OD values for loading differences.

Statistical analysis

The pairwise multiple comparisons for immunohistochemistry and western blots were analysed with non-parametric ANOVA on ranks (Holm-Sidak Method) followed by the Kruskal-Wallis test. For the TUNEL assay, non-parametric ANOVA on ranks (Tukey test) was performed. The statistical calculations were performed using SigmaStat for Windows, version 3.5 (Jandel Scientific Corp., San Rafael, CA). Statistical significance was defined as $p<0.05$.

Results

Apoptotic evaluation with the TUNEL technique

The percentage of apoptotic seminiferous tubules in the testes was detected by the TUNEL assay (Fig. 1A-D), which was followed by a quantitative comparison (Fig. 1E). Torsion-induced apoptotic cells were most obvious in the intra-tubular elements; however, a few interstitial components were also observed. Interestingly, the apoptotic germ cells were found to be located at the periphery of each tubule (Fig. 1A,B), which are mostly the spermatogonia. However, in the torsion group, apoptotic germ cells were also observed in the seminiferous tubule lumens (Fig. 1B). The percentages of TUNEL-positive seminiferous tubules of the control, group II (I/R injury group), group III (I/R injury+BQ123 group) and group IV (control+BQ123 group) were 2.4±0.4, 28±2.8, 15.6±1.6 and 3.6±0.6%, respectively. Although TUNEL-positive cells were observed in all groups (Fig. 1A-D), the percentage of TUNEL-positive cells was significantly increased in group II (28±2.8%) and decreased in group III (15.6±1.6%). The TUNEL positivity of group IV was similar to that of group I. Additionally; the morphology of the testicular tubules was different in each of the above-mentioned groups (Fig. 1A-D). Specifically, the seminiferous epithelium in group II and group III was most dramatically affected by testicular torsion (Fig. 1B,C).

Immunohistochemical evaluation

To further investigate germ cell apoptosis, we examined tissue sections from groups I, II, III and IV by immunohistochemistry (Fig. 2A-L). Moderate to strong active caspase 3 immunoreactivity was found in the round spermatids in group I (Fig. 2A). Stronger caspase 3 expression in group II, especially in spermatocytes, round and elongated spermatids, was observed (Fig. 2B) compared with the control. In group III, weak to moderate caspase 3 expression was detected in round and elongated spermatids (Fig. 2C). This expression was significantly different than group II, according to the HSCORE analysis ($p<0.05$, Fig. 3). In group IV, moderately active caspase 3 immunoreactivity was observed, which was significantly different than group II but not group I (Fig. 2D).

Immunostaining of the cytoplasmic protein Bax showed predominant expression in spermatocytes and spermatids and to a lesser extent in spermatogonia in group I (Fig. 2E). There was not any immunostaining in Sertoli cells for Bax protein in all of the groups. However, Bax expression was significantly increased in spermatocytes and round spermatids in group II (Fig. 2F). Additionally, elongated spermatids were found to be immunopositive for Bax. In group III, the number of Bax-positive cells and the Bax intensity was reduced (Figs. 2G, 3). The expression of the apoptotic protein Bax was negligible in spermatogonia and elongated spermatids in group III, and it was mostly observed in spermatocytes (Fig. 2G). There was not any immunoreactivity in Sertoli cells. A punctate intracellular labelling characteristic of mitochondria was observed for Bax (Fig. 2E-H), whereas the caspase-3 (Fig. 2A-D) and Bcl-2 (Fig. 2I-L) immunoreactivities were characterised by a diffuse, perinuclear cytoplasmic pattern.

Bcl-2 immunoreactivity was observed as diffuse

patterns of staining in the perinuclear cytoplasm of almost all spermatogenetic cells of the control group (Fig. 2I). In group II, this expression was significantly reduced in spermatocytes and spermatids, and almost no immunoreactivity was observed in spermatogonia and Sertoli cells (Fig. 2J). In group III, Bcl2 immunoreactivity was increased compared with group II (Figs. 2K, 3). In group IV, diffuse Bcl2 immunoreactivity was

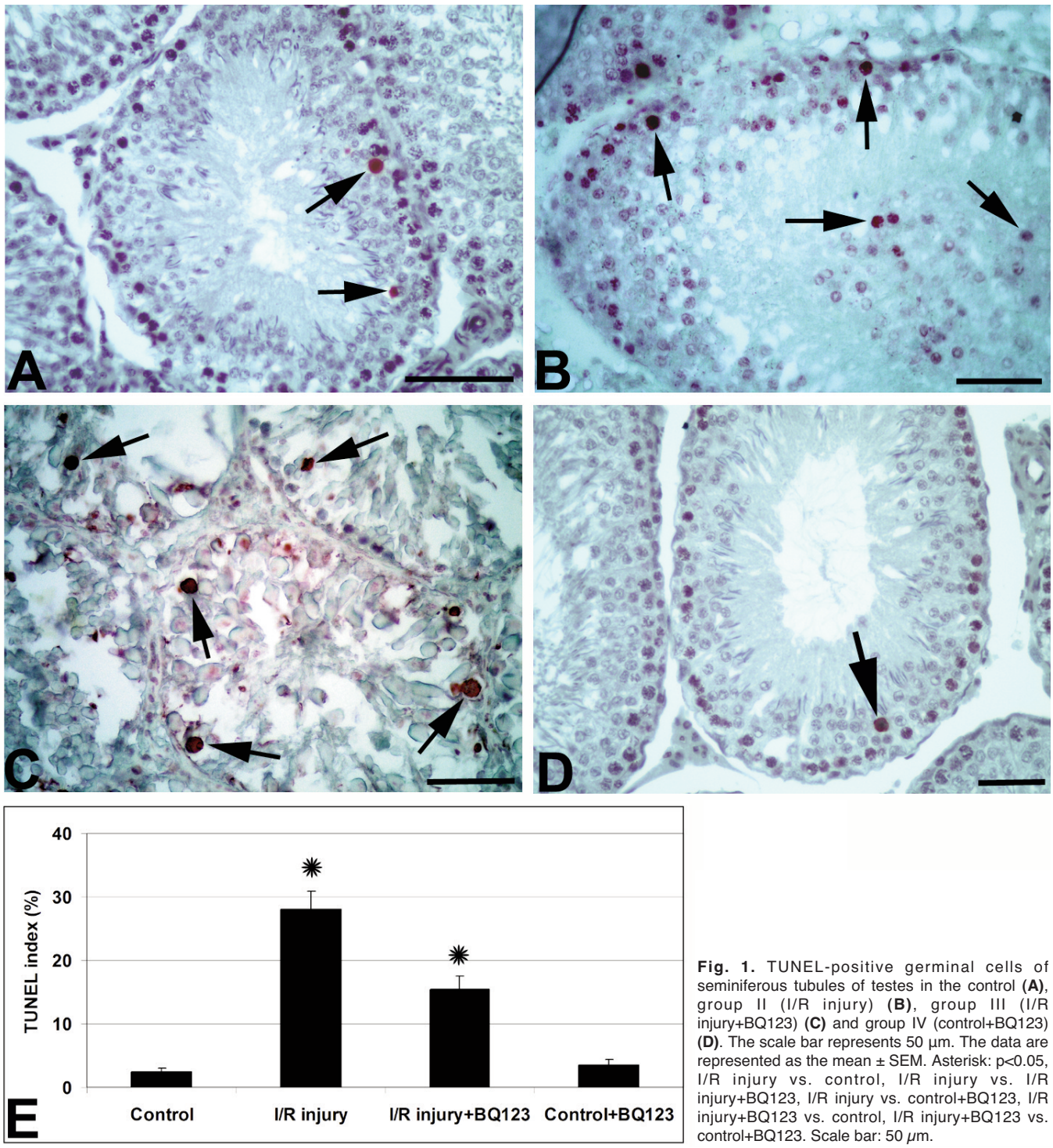

Fig. 1. TUNEL-positive germinal cells of seminiferous tubules of testes in the control **(A)**, group II (I/R injury) **(B)**, group III (I/R injury+BQ123) **(C)** and group IV (control+BQ123) **(D)**. The scale bar represents 50 µm. The data are represented as the mean ± SEM. Asterisk: $p<0.05$, I/R injury vs. control, I/R injury vs. I/R injury+BQ123, I/R injury vs. control+BQ123, I/R injury+BQ123 vs. control, I/R injury+BQ123 vs. control+BQ123. Scale bar: 50 μm.

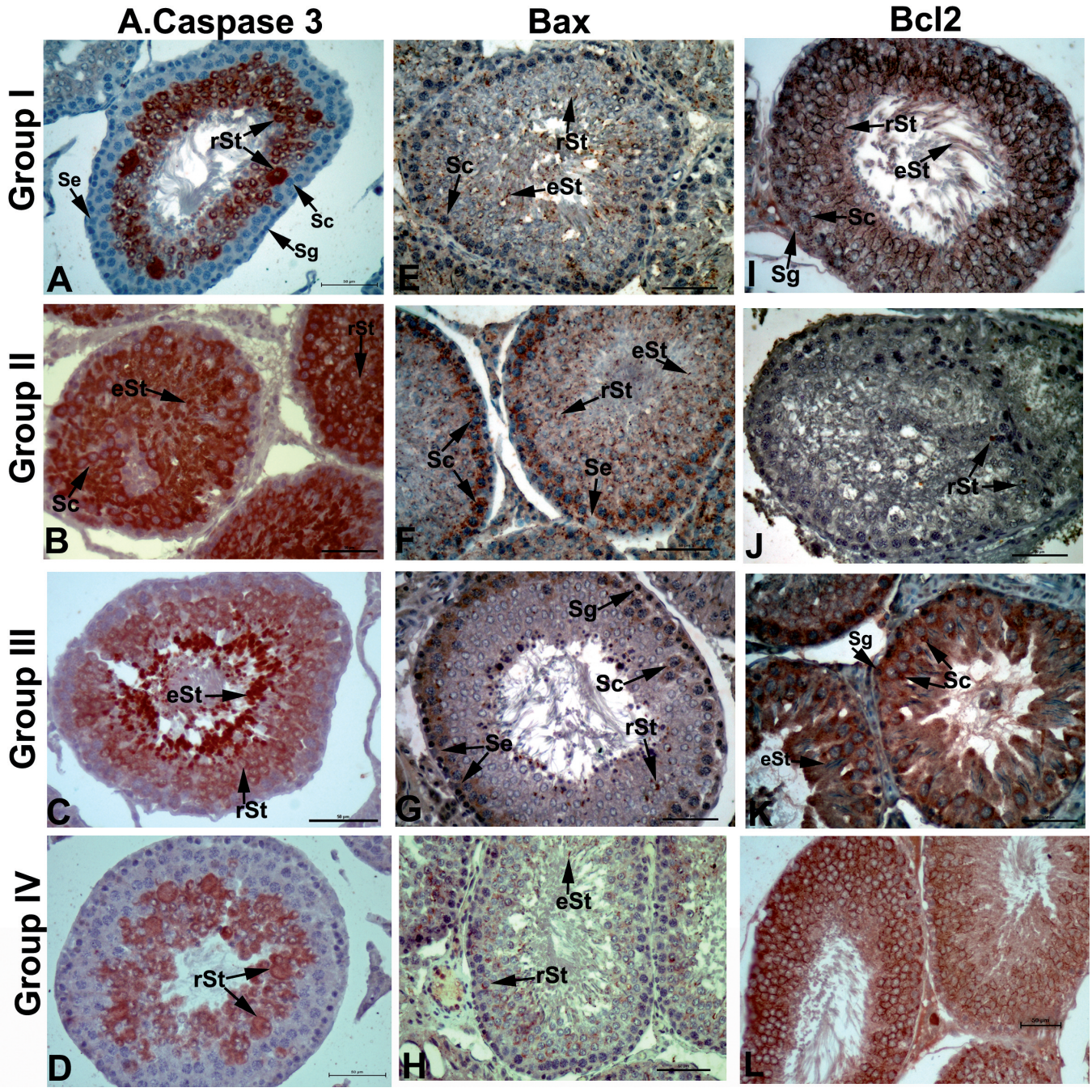

Fig. 2. Immunohistochemical distribution of active caspase 3 **(A-D)**, Bax **(E-H)** and Bcl2 **(I-L)** in Control (group I), I/R injury group (group II), I/R injury followed by BQ123 group (group III) and control+BQ123 group (group IV). **A.** Active caspase 3 is moderately immunolabeled in almost all round spermatids (rSt) in the control testis. No immunolabeling is observed in Sertoli cells (Se), spermatogonia (Sg) and spermatocytes (Sc). **B.** Strong expression of active caspase 3 in rSt, elongated spermatid (eSt) and Sc is displayed. **C.** rSt is moderately active caspase 3-immunopositive, whereas eSt is strongly immunopositive for active caspase 3. **D.** rSt is moderately immunopositive for active caspase 3 in group IV. **E.** Bax was mainly localised in the cytoplasm of Sc, rSt and eSt (arrows) of seminiferous tubules in the control. **F.** Extensive, granular and strong Bax expression in the cytoplasm of Sc, Sg, rSt and eSt in group II. Sertoli cells were immunonegative for Bax in group II. **G.** Moderately stained Sc and rSt are observed (arrows). Sg is observed to be weakly immunopositive for Bax in group III. **H.** rSt and eSt displayed moderate immunopositivity in group IV. **I.** Sg, Sc, rSt and eSt show strongly diffuse cytoplasmic staining for Bcl2 in group I. **J.** Bcl2 is barely detected in spermatogenetic cells, and only rSt shows immunopositivity in group II. **K.** Moderate to strong Bcl2 immunoreactivity is observed in Sg, Sc and eSt in group III. **L.** Moderate to strong Bcl2 immunopositivity is observed in almost all spermatogonetic cells in group IV. Scale bars: 50 µm.

observed in spermatogenetic cells (Fig. 2L).

Western blotting

The western blot analyses revealed a specific band at 17 kDa for active caspase 3, 20 kDa for Bax and 26 kDa for Bcl2 (Fig. 4A). The intensity of the bands was quantified and normalised to the intensity of the beta-actin controls. The active caspase 3 and Bax expression in rat testis was significantly increased in the I/R injury group compared with the control group and significantly decreased in the BQ123-treated group compared with the I/R injury group ($P<0.05$; Fig. 4A,B). Thus, the expression of Bcl2 protein levels is significantly decreased in the I/R injury group compared with the control group and significantly increased in the BQ123-treated group compared with the I/R injury group. There were no differences between group I and group IV in terms of the expression of apoptotic and anti-apoptotic proteins.

Discussion

The most significant complication of testicular torsion is the loss of the testis, which may lead to male infertility if left untreated. Testicular torsion results in ischaemia, and this ischaemic process causes damage to the testicular tissues. After the detorsion procedure, this

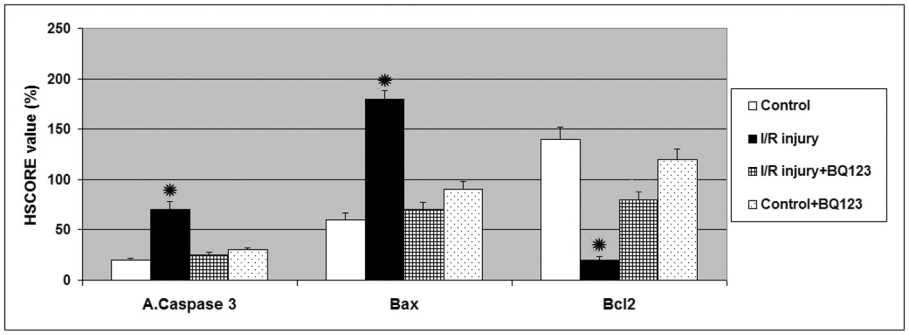

Fig. 3. Comparison of the HSCORE values of active caspase 3, Bax and Bcl2 in the control, I/R injury, I/R injury+BQ123 and control+BQ123 groups. The data are represented as the mean ± SEM. Asterisk: $p<0.05$, control vs. I/R injury, I/R injury vs. I/R injury+BQ123, I/R injury vs. control+BQ123.

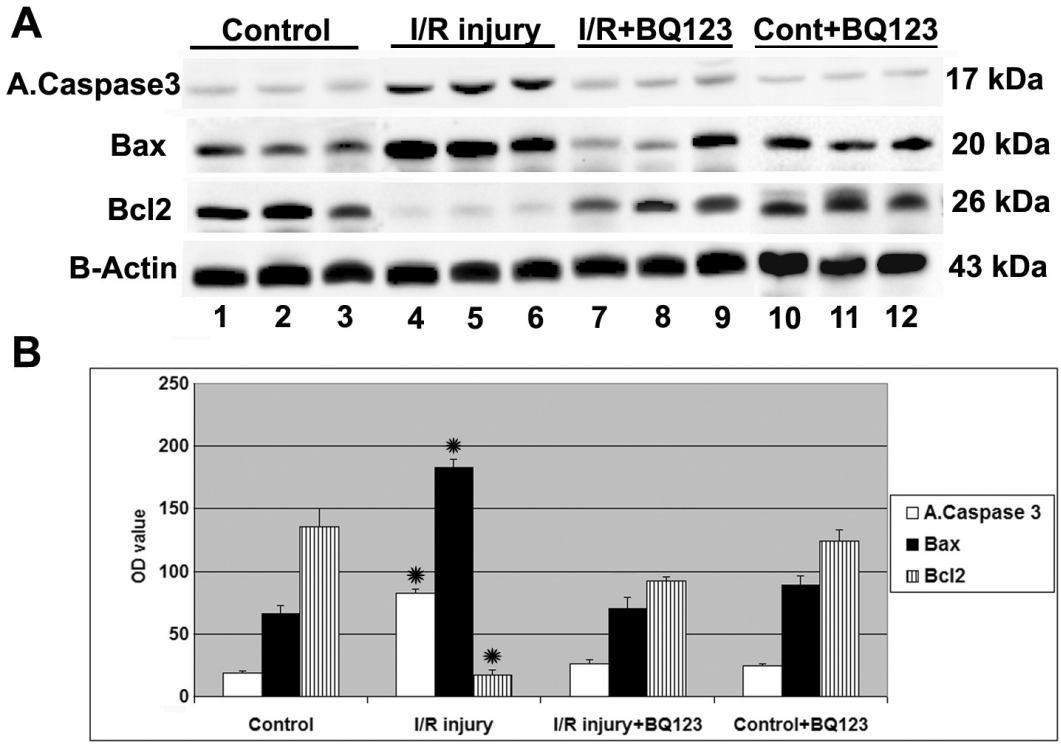

Fig. 4. A. Lysates from control (lanes 1-3), I/R injury (lanes 4-6), I/R injury+BQ123 (lanes 7-9) and control+BQ123 (lanes 10-12) groups were used for immunoblotting. Active caspase 3 (17 kDa), Bax (20 kDa) and Bcl2 (26 kDa) were detected by immunoblotting, and β-Actin (43 kDa) was used as the loading control. **B.** Immunoblot bands were quantified by an optical densitometer. The optical density (OD) values of active caspase 3, Bax and Bcl2 were normalised to the OD values of the b-actin bands. The values are the mean ± SEM. Asterisk: $p<0.05$, I/R injury vs. control, I/R injury vs. I/R injury+BQ123 for active caspase 3, Bax and Bcl2.

damage is elevated by reperfusion injury (Filho et al., 2004; Parlaktas et al., 2014). Reperfusion injury occurs as a result of toxic free radicals, such as superoxide anions, hydroxyl radicals, and nitric oxide or peroxynitrite, and overproduction of these radicals can cause damage to the DNA structure and endothelium and can cause germinal cell apoptosis (Akgur et al., 1993; Lysiak et al., 2003). Therefore, treatment by detorsion may further damage the testis. To date, numerous prior experimental studies have examined the biochemical parameters and germinal cell loss in I/R-injured testis (Turner et al., 1997; Shiraishi et al., 2000, 2001; Lysiak et al., 2003; Filho et al., 2004; Yang et al., 2007; Parlaktas et al., 2014), and many different agents have been studied to protect the testes against such injuries (Shiraishi et al., 2001; Uz et al., 2002; Jeong et al., 2010; Kanter, 2010; Parlaktas et al., 2014). Although numerous experimental animal studies have confirmed the efficacy of various chemicals in reducing the short-term damaging effect of torsion of the testis, different agents have been investigated to reduce short- and long-term testis reperfusion damage. In this context, there is no current information about the effect of BQ123 on the regulation of I/R injury-induced testicular apoptosis. Therefore, we have provided direct evidence that an ETA receptor antagonist plays a significant role in the regulation of I/R injury-induced testicular apoptosis.

The intratesticular ET-1 concentration is reported to be approximately 100-fold higher than in plasma (Matsumoto et al., 1989), and the distribution of ETA receptors in rat testicular interstitial and myoid cells was previously demonstrated (Sakaguchi et al., 1992). Additionally, ET-1 binding sites were also detected in rat Leydig and Sertoli cells, suggesting an autocrine/paracrine role for ET-1 in rat testis (Fantoni et al., 1993). From these studies, the presence and mechanism of ETA receptors in the rat testis are clear.

It is well known that apoptotic cell death plays an important role in limiting the testicular germ cell population following testicular I/R injury, and its dysregulation could be associated with male infertility. In a rat model of testicular I/R, germ cell apoptosis has been reported previously, although the mechanism that regulates this pathway is still under investigation. In a model of I/R-injured rat testis, ROS production followed by I/R injury induces germinal cell apoptosis (Lysiak et al., 2000). Because it is proven that mitochondria are the source for ROS, it is well accepted that the central control point of apoptotic cell death following I/R injury is the mitochondria. For this reason, we have firstly chosen to examine the mitochondrial-associated pathway, which is a predominant pathway to germinal cell apoptosis in the experimental I/R injury model. It is known that apoptosis regulation is determined by the ratio of Bcl-2 family members, inducers (e.g., Bcl-xs, Bax, Bak, Bcl-xb, Hrk, Mcl-1, Bok) or inhibitors (e.g., Bcl-xl, Bcl-2, Bcl-w) (Lopez-Neblina et al., 2005). In the present study, we have employed a rat model of testicular I/R injury to determine whether the activation of the mitochondrial pathway is involved in this injury and to assess whether BQ123 plays a significant role in the regulation of the apoptotic pathway. Bax is well known as a pro-apoptotic protein that translocates to the mitochondria from the cytoplasm, which affects the release of cytochrome c from mitochondria (Beumer et al., 2000). This translocation of Bax is accompanied by an increase in cytochrome c and a second mitochondrial-derived activator of caspase (Smac/DIABLO) in the cytosol, leading to the subsequent activation of caspase 9. Then, active caspase 9 initiates apoptosis by cleaving and thereby activating executioner caspases (caspases 3, 6 and 7). These activities are associated with an increase in germinal cell apoptosis (Lysiak et al., 2007). The data presented here also point to the involvement of Bax, Bcl2 and active caspase 3 in the I/R injury of testis (Fig. 2-4). By 4 h after I/R injury of the testes, both Bax and active caspase 3 expressions were increased, especially in the germ cells (Fig. 2). Immunohistochemical staining of active caspase 3 detects the effector caspase that is downstream of both the mitochondria and death receptor pathways to apoptosis; thus, it is activated in almost all forms of apoptotic cell death. Sections of I/R-injured testis revealed a significant increase in spermatocytes and spermatids for active caspase 3 staining. These data demonstrate that I/R induced apoptosis, which has been shown previously (Lysiak et al., 2000; Lysiak et al., 2007; Bayatli et al., 2013). Furthermore, a decrease in Bcl2 expression was detected after injury, providing evidence of the involvement of Bcl2 in I/R-induced germ cell loss. In addition to the immunohistochemical studies, the expression of apoptotic proteins was also confirmed by western blot studies (Fig, 4).

To elucidate the effect of BQ123 on the torsion-induced apoptotic pathway, the expression of caspase 3, Bax and Bcl2 was also examined after BQ123 treatment in I/R-injured rat testis. The administration of BQ123 revealed a significant increase in the expression of the anti-apoptotic protein Bcl2 and a decrease in the expression of Bax and active caspase 3. The results showed that BQ123 significantly reduced I/R injury-induced germ cell loss, suggesting that the apoptotic effect of I/R injury was mediated by the ETA receptor pathway. One of the reasons for this decrease in the expression of apoptotic proteins in the I/R injury+BQ123 group might be the antioxidant activity of BQ123, which could exert a beneficial effect against pathological alterations caused by free radicals in testicular torsion. Many studies have demonstrated that in the event of I/R injury, the tissue levels of SOD or catalase increase to protect cells from the detrimental effects of reactive oxygen radicals (Sakaguchi et al., 1992; Goyal et al., 2010) Moreover, the protective action of BQ123 was confirmed in other tissues, such as myocardium (Goyal et al., 2010), whereas only one study examined the effects of BQ123 on testis of diabetic rats (Kosova et al., 2011). Future biochemical studies will clarify the antioxidant activity of BQ123 in testis.

We have employed TUNEL techniques to identify

apoptotic cells. In this study, the highest number of apoptotic cells were observed in the I/R injury group, which is in accordance with previous studies (Dokmeci et al., 2007; Kanter, 2010). In this study, an increase in apoptosis was noted 2 h after torsion as assessed by in situ TUNEL, immunohistochemistry and western blotting. In previous studies, an increase in apoptosis was detected at 1, 2, 4 and 24 h after repair of 1 h torsion, and the first significant increase was detected 4 h after torsion repair (Turner et al., 1997; Lysiak et al., 2000). They also detected much larger increases at 24 h after torsion repair. Because the authors used a 1-h torsion period instead of 2 h, this length might affect the level of apoptotic protein expression, and critical molecular events might be initiated by this time. Interestingly, Ayan (2015) recently reported a significant increase in the expression of Bax and active caspase 3 proteins 2 h after torsion repair (Ayan et al., 2015). They also showed that the apoptotic index of the torsion group was much higher at 2 h after repair of torsion. Morover, Bayatli (2013) demonstrated the maximum apoptotic protein expression at 2 h after repair of torsion (Bayatli et al., 2013). In this context, the present study, which shows the earliest germ cell apoptosis observed after 2 h of torsion repair, confirms the findings of Ayan (2015) and Bayatli (2013). Nevertheless, an examination of the timing and upregulation of different apoptotic pathways in testicular torsion and repair needs to be studied in the future.

DNA damage revealed by the TUNEL assay significantly decreased after administration of BQ123. Additionally, the morphology of testicular tubules was different in the I/R injury group and I/R injury+BQ123 group, as shown in Fig. 1B,D. Surprisingly, some tubules in the I/R injury+BQ123 group were not completely recovered after BQ123 treatments and showed more destroyed seminiferous epithelium compared with the other groups.

In light of these findings, it can be concluded that BQ123 not only decreases the expression of apoptotic proteins but also diminishes DNA damage in the I/R-injured rat testis. Administration of the ETA receptor antagonist BQ123 results in a reduction of apoptosis and DNA damage, and BQ123 can also be used as an important agent for protecting testis following I/R injury.

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Authorship. SC and SO performed the immunohistochemical and western blot studies, performed the statistical analysis and drafted the manuscript. ZK participated in the immunohistochemical studies. US, FE and TD conceived of the study and participated in its design and coordination and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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