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Targeting soluble guanylate cyclase with Riociguat has potency to alleviate testicular ischaemia reperfusion injury via regulating various cellular pathways

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Abstract

Testicular ischaemia reperfusion (I/R) injury results with serious dysfunctions in testis. This study aims to explore effects of soluble guanylate cyclase (sGC) stimulator Riociguat on experimental testicular I/R injury in rats. Twenty-one male rats were divided into three groups (Control, IR and IRR). The control group was not exposed to any application. Bilateral testis from IR and IRR animals were rotated 720° in opposite directions for 3 h to induce experimental testicular ischaemia. Animals in IR and IRR groups were subjected to 3 h of reperfusion. Isotonic and Riociguat were administered to the animals 30 min prior reperfusion by oral gavage. At the end of experiment, animals were sacrificed and tissue samples were used for analyses. Riociguat treatment significantly decreased tissue malondialdehyde and Luminol levels compared to the IR group ($p < 0.05$). The pathological changes, pro-apoptotic proteins (Bax, Caspase 3, and Caspase 9) and apoptotic index in the IR group were down regulated in Riociguat treated animals ($p < 0.05$). Riociguat treatment was also significantly increased anti-apoptotic Bcl-2 expression, but alleviated tissue injury via modulating pro-inflammatory cytokine IL-1β levels and significantly ($p < 0.05$) down-regulating NF-κB activity. Moreover, mTOR and ERK phosphorylation increased in IR group ($p < 0.05$), but Riociguat treatment reduced protein phosphorylation. Our experiment indicated that targeting sGC might support surgical interventions in testicular I/R injury by modulating oxidative stress, inflammation, and apoptotic protein expression levels, but more detailed studies are required to explore the protective activity of Riociguat and underlying mechanisms in testicular I/R injury.

KEYWORDS
ischaemia reperfusion injury, rat, Riociguat, sGC stimulator, testis

1 | INTRODUCTION

Testicular ischaemia reperfusion (I/R) injury is one of the most important urological conditions that require surgical intervention and is mainly observed in new-borns, young children, and adolescents (Howe et al., 2017). Testicular ischaemia occurs with rotation of spermatic chord around itself and it leads to cease of blood flow to the testis and accumulation of reactive oxygen species (ROS) (Aydogdu et al., 2021). The symptoms of testicular ischaemia are scrotal and abdominal pain, swelling, skin erythema, nausea, vomiting, and fever

(Zvizdic et al., 2021). Although testicular ischaemia can be interfered surgically with de-rotating the twisted spermatic chord in the opposite direction, the secondary underlying mechanism of reperfusion may still require treatment to avoid subfertility, which is a forthcoming result of hypoxia and increased ROS accumulation (Aydın et al., 2021; Dejban et al., 2019; Dolatkhah et al., 2020). It is accepted that the overproduction of ROS is the main reasons for testicular I/R injury (Yousefi-Manesh et al., 2019). The other reasons for testicular I/R injury are the accumulation of inflammatory cytokines such as IL-1β and transcription factors such as NF-κB (Abdel-Gaber et al., 2018; Afolabi et al., 2022). Until today, researchers examined varying treatment strategies such as antioxidants, calcium channel blockers (Jafari et al., 2021; Taheri et al., 2021) vasodilator drugs (Bozlu et al., 2009), plant delivered phenolic compounds (Shokri et al., 2019), anti-fibrotic drugs (Kölükçü et al., 2021), amniotic fluid (Aydogdu et al., 2021) even traditional medical applications such as leech therapy (Davoodi et al., 2021) and varying drugs (Shokoohi et al., 2022) to explore the optimum supplementary treatment to alleviate testicular I/R injury. All of these treatment strategies aim to improve microvascular circulation and reduce oxidative stress. Although varying degrees of success have been reported in previous experiments, the clinical applicability of these examinations still requires more detailed research. For that reason, researchers are intensively performing both clinical and preclinical studies to understand the underlying mechanism of testicular I/R injury and any possible protective drug administration treatment protocol. ROS are a subset of free radicals and, as a general description, imbalance between endogenous antioxidants and free radicals may lead to oxidative stress (Cannavò et al., 2019). However, as a free radical, nitric oxide (NO) can act both harmful or protective in tissue injury depending on the activity mechanism (Wink et al., 2001). That leads the researchers to focus on NO, and some studies report possible protective activity of NO in2014I/R injury in varying organs (Barlas & Hatiboğlu, 2002; Dokucu et al., 2000). Targeting NO to protect tissue is a strategy to provide vascular relaxing and up-regulate blood flow to the organs (Yu et al., 2018). In clinical practice, soluble guanylate cyclase (sGC) stimulators are novel developed drugs that are targeting cGMP indepentdently from NO and result in vasodilation. Recent studies indicate that sGC signalling can activate antioxidant and anti-inflammatory signalling (Bauersachs et al., 2017; Paul et al., 2018; Sravani et al., 2020). However, the potential effects of sGC stimulators on testicular −I/R injury have not been investigated yet. For that reason, we aimed to explore the possible protective potency of sGC stimulator, Riociguat, in testicular −I/R injury with considering oxidative stress, apoptotic process, inflammation and activity of varying cellular signals.

2 | MATERIALS AND METHODS

2.1 | Experimental design

All experimental procedures of this study were performed with approval of Experimental Animal Ethics Committee of Firat University (approval date and number: 01.11.2021-4753). Twenty-one adult 8–10-week-old 300–350-g male Sprague Dawley rats were divided into three groups (Control, IR and IRR) of seven animals in each. Animal numbers were determined by considering previously published testicular I/R injury studies (Dokucu et al., 2000). Until the experiment, the animals were housed in polycarbonate cages at stabilized laboratory unit temperature (21 ± 3°C) with a 12-h light–dark cycle and 50% ± 5% humidity. Animals in the control group did not receive any application during the experiment and were sacrificed at the end of the study. Animals in IR and IRR were exposed to bilateral testicular ischaemia with surgical procedure which described previously (Gozukara et al., 2020). Blood flow to the testes was ceased for 3 h. Animals in IR and IRR groups received 1 ml isotonic and 10 mg/kg Riociguat (Cat no: AS-19299, Key Organics, UK) by oral gavage in the last half hour of ischaemia. At the end of ischemic period, scrotal sutures were removed and bilaterally testes were de-rotated to the contrary directions manually to provide reperfusion. The scrotum of animals in IR and IRR groups was fixed and reperfusion was provided for 3 h. At the end of the experiment, animals were sacrificed with exsanguination and left testes were fixed in 10% neutral buffered formalin for histopathological examinations. The right testes of the animals were encapsulated and seminiferous tubules were isolated. The samples were frozen in liquid nitrogen and stored −80°C for immunoblotting and biochemistry analyses.

2.2 | Biochemical analyses

Testicular malondialdehyde (MDA) analysis and chemiluminescence assay were performed to measure lipid peroxidation end substance and total ROS levels. Measurement of tissue levels of MDA and ROS were carried out as described previously (Şener et al., 2015; Tamer et al., 2019). Lipid peroxidation was evaluated as MDA equivalents using an extinction coefficient of 1.56 × 105 M/cm and results are expressed as nmol MDA/g tissue. Reactive oxygen metabolites (ROM) were measured with luminol assay, which is able to use to detection of hydrogen peroxide, hydroxyl radical and hypochlorite radicals specifically. Measurement performed in a luminometer (Junior LB 9509, EG&G Berthold, Germany) with administration of luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione, Sigma) to the tissue samples. Results of luminol assay are expressed in relative light units (rlu) per mg of tissue.

2.3 | Tissue processing and immunohistochemistry

The left testes of animals were fixed in 10% neutral buffered formalin, a routine tissue processing protocol performed, and the samples were embedded into paraffin blocks. Haematoxylin and eosin (HE) staining, immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL assay) were performed on 5 μm thick sections. HE stained samples were analysed for tissue pathology and Johnsen's biopsy score (Johnsen, 1970). For immunohistochemistry,

antigen retrieval was performed in citrate buffer (pH: 6.0) and all immunohistochemical protocols were performed with a ready to use kit (Cat no: TP-125-HL; Thermo Scientific, Waltham, MA, USA). Primary antibodies of Bax (Cat no: sc-7480; Santa Cruz Biotechnology, Dallas, TX, USA), Caspase 3 (Cat no: sc-56053; Santa Cruz Biotechnology, Dallas, TX, USA) and Caspase 9 (Cat no: sc-56076; Santa Cruz Biotechnology, Dallas, TX, USA) were diluted in 1:100, 1:100 and 1:200 ratios respectively. Sections were counterstained with haematoxylin and mounted with Entellan.

2.4 | TUNEL assay and quantification

Testicular apoptotic index (AI) in animals was obtained with TUNEL assay (Seker et al., 2020). For that purpose, testis sections were deparaffinized and rehydrated through an increasing alcohol series. Sections were washed in distilled water and kept in PBS before TUNEL assay. All procedure were performed with a ready to use detection kit of In Situ Cell Death Detection Kit, Fluorescein (Cat no: 11684795910; Roche Diagnostics, Indianapolis, IND, USA), and all steps were performed according to the manufacturer's directions. Samples were counterstained and mounted with DAPI (Cat no: sc-24941, Santa Cruz Biotechnology, Dallas, TX, USA). For quantification of the AI, randomly selected 100 tubules from each group were evaluated under camera attached fluorescence microscope with 20× magnification. Germinal epithelial cells were considered either positive or negative for DNA fragmentation. In the case of the nuclei of cells were observed with green fluorescence then considered as positive due to DNA fragmentation. If the nuclei were blue due to counterstain DAPI, cells were accepted as negative for DNA fragmentation because of nuclear integrity. The AI in each seminiferous tubule was calculated manually by comparing the number of positive cells to the total number of cells in each tubule, and the results were expressed as a percentage.

2.5 | Western blotting and quantification

Protein lysates were loaded into gel and run for 25 min. Separated proteins were transferred onto a PVDF membrane and blocking was performed for 1 h in 5% skim milk that dissolved in PBS-T. Primary antibodies of IL-1β (Cat no: sc-52012; Santa Cruz Biotechnology, Dallas, Texas, USA), Bcl-2 (Cat no: sc-7382; Santa Cruz Biotechnology, Dallas, TX, USA), NF-κB (Cat no: sc-8008; Santa Cruz Biotechnology, Dallas, TX, USA), mTOR (Cat no: sc-517464; Santa Cruz Biotechnology, Dallas, TX, USA), p-mTOR (Cat no: 2971 S; Cell Signaling Technology Danvers, MA, USA), p44/42 MAPK (Erk1/2) (Cat no: 9107 S; Cell Signaling Technology Danvers, MA, USA), Phospho-p44/42 MAPK (Erk1/2) (Cat no: 4370 S; Cell Signaling Technology Danvers, MA, USA) were diluted 1:1000 and membranes were incubated for 2 h at room temperature. Samples washed in PBS-T and secondary antibody incubations of anti-rabbit (Cat no: ab97051; abcam CB, UK) and anti- mouse (Cat no: ab98808; abcam CB, UK) were performed for 1 h at room temperature. Clarity Western ECL Substrate (Bio-rad, Cat no: #1705061, Bio-rad Laboratories, Hercules, CA, USA) was used for development of band and membranes were monitored under ChemiDoc™ Imaging System (Cat no: 12003153; Bio-rad Laboratories, Hercules, CA, USA). Band intensities were measured with Image J software as described previously (Seker et al., 2022). Total protein measurements of IL-1β, Bcl-2 and NF-κB were performed with comparing protein of interest with loading control β-actin. Phosphorylation ratio of mTOR and ERK were measured with comparing phosphorylated protein to total protein. Each of these total and phosphorylated proteins was normalized with comparing the loading control before measurement of phosphorylation levels.

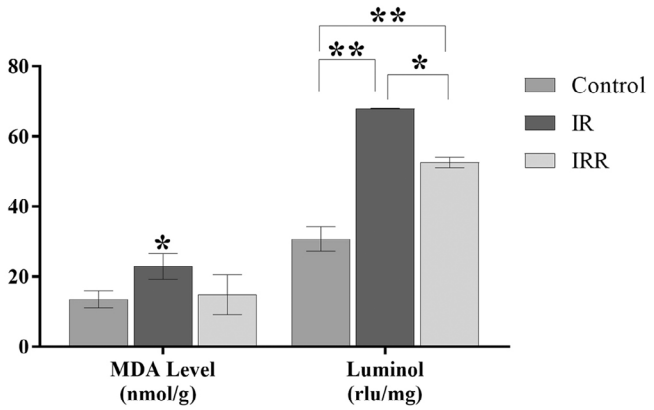

FIGURE 1 Graphical demonstration of biochemical analyses. Different superscripts between columns indicate statistically significance. *$p < 0.05$; **$p < 0.01$

2.6 | Statistical analyses

Statistical analyses were performed in SPSS Version 24. All measured or obtained data were considered whether distributed normally or not. One-way ANOVA used for statistical analyses of parametric tests. For non-parametrical statistics, Kruskal–Wallis test was used. Multiple comparisons were performed with post hoc Tukey and Tamhane's T2 tests for parametric and non-parametric tests respectively. Results are shown as mean ± SD and $p < 0.05$ and $p < 0.01$ considered as significant.

3 | RESULTS

3.1 | Biochemical results

The lowest MDA tissue level was obtained as 13.51 ± 2.49 nmol/g in control group. Tissue level of MDA in IR group elevated significantly to 22.90 ± 3.57 nmol/g and the difference between control and IR groups was significant ($p < 0.05$). In IRR group, the tissue level of MDA was 14.81 ± 5.68 nmol/g and results of this group was similar ($p = 0.959$) to control group but it was significantly different

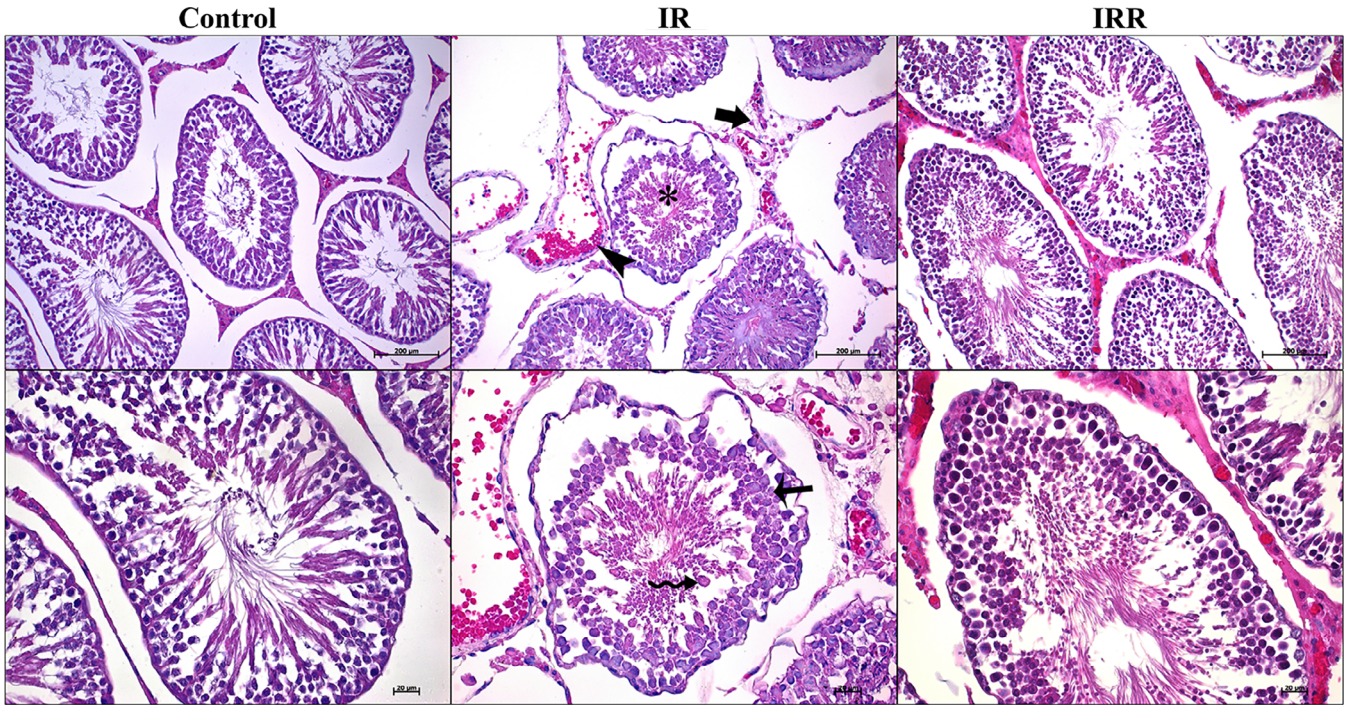

FIGURE 2 Representative micrographs of tissue sections of the groups. Desquamation of germinal epithelium (asterisk), nuclear pyknosis (arrow), edematous interstitium with disrupted interstitial tissue (thick arrow), severe haemorrhage at interstitial tissue (arrow head), desquamated immature cell cluster and protoplasmic droplets in seminiferous tubule lumen (curved arrow). Staining: haematoxylin and eosin, bar: 200 and 20 μm

($p < 0.05$) than the IR group. In luminol assay we observed that the lowest level is measured in control group as 30.73 ± 3.44 rlu/mg and tissue level increased significantly ($p < 0.01$) to 67.95 ± 0.05 rlu/mg in IR group. The tissue level of ROS were alleviated to 52.55 ± 1.55 rlu/mg in IRR group. The difference in IRR group was significant compared to control ($p < 0.01$) and IR groups ($p < 0.05$). Graphical demonstration of the biochemistry results are shown in Figure 1.

3.2 | Histopathological results

Microscopic observations indicated that the seminiferous tubule structure in the control group was normal. In this group, seminiferous tubules were filled with germinal epithelium. There were Leydig cells in interstitial tissue that was located between seminiferous tubules. Seminiferous tubules were surrounded with basement membrane and myoid cells, which were located on the outer surface of the tubules. The lumen of the tubules in this group was filled with developed sperm cells. In IR group, severe pathological changes were observed. Desquamation of germinal epithelial cells, pyknotic germinal epithelium nuclei and multinucleated giant cells were detected widespread. Edematous interstitium and severe haemorrhage was observed as proof of disruption in testis-blood barrier in IR group (Figure 2). The mentioned pathological changes were alleviated in Riociguat-treated group, but this group had slightly pathological outcomes. Johnsen's biopsy score and germinal epithelium thickness in the control group were 9.87 ± 0.34 and 71.03 ± 6.99 μm, but they dramatically ($p < 0.01$) decreased in the IR group to 7.24 ± 1.83 and 50.76 ± 7.56 μm respectively, as a result of desquamation and shrinkage in cell clusters. Johnsen's biopsy score and germinal epithelium thickness in Riociguat treated group were 8.77 ± 0.90 and 67.56 ± 10.07 μm. Results in this group were closer to the control group compared with the IR group, but significantly difference ($p < 0.05$) was still obvious. Graphical demonstration of the histopathological analyses is shown in Figure 3.

3.3 | Immunohistochemistry results

Our microscopic investigations indicated that apoptosis regulator key proteins are expressed in healthy testicular germinal epithelial cells, but there was more intensity observed in the IR group. Mainly desquamated cell clusters were more intense for Bax, Caspase 3 and Caspase 9. Microscopic observations demonstrated that Riociguat pre-treatment before reperfusion down-regulated pro-apoptotic protein expression levels. Statistical analysis of Bax immunopositivity in tissue sections was 3.90% ± 1.20% in the control group. The results were 9.71% ± 2.17% and 6.66% ± 2.66% in IR and IRR groups respectively. The difference between the groups was significant ($p < 0.01$). When Caspase 3 immunopositivity evaluated, the distribution in the control group was 7.74% ± 2.20%. The immunopositivity level in the IR group was 10.95% ± 2.67% and the difference between groups

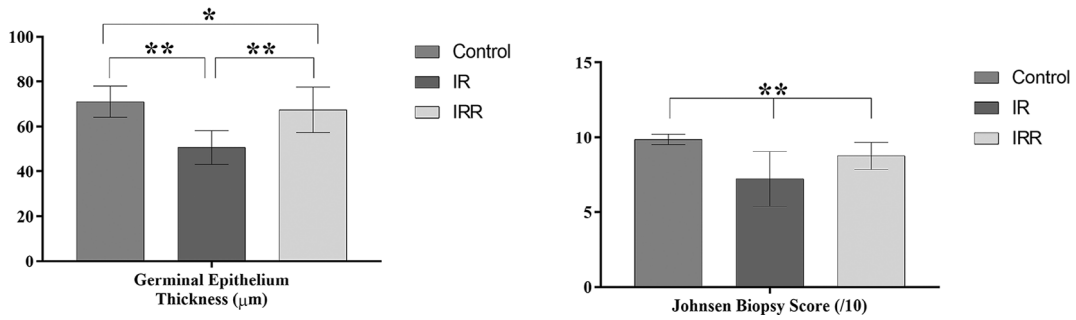

FIGURE 3 Graphical demonstration of the statistical analyses of germinal epithelial thickness and Johnsens's biopsy score. Different superscripts between columns indicate statistically significance. *$p < 0.05$; **$p < 0.01$

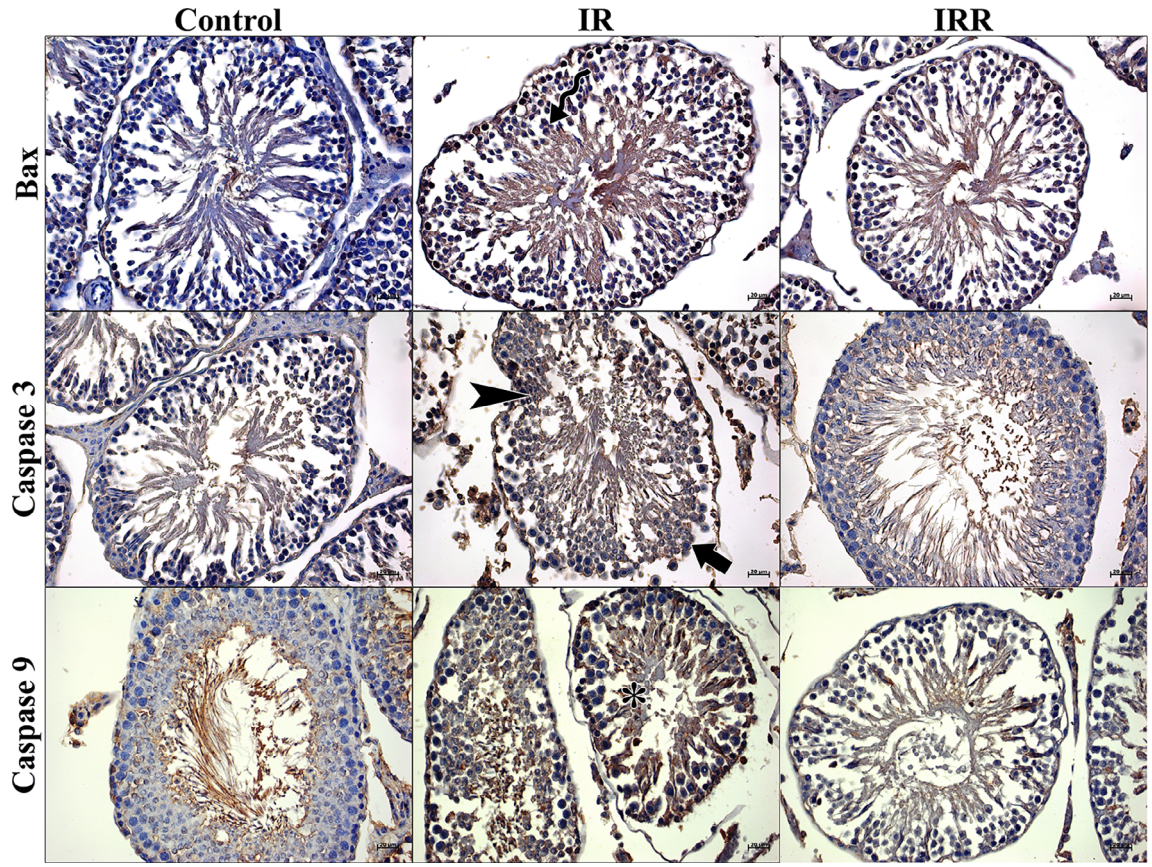

FIGURE 4 Immunohistochemical micrographs of Bax, Caspase 3 and Caspase 9 in control, IR and IRR groups. Intense Bax immunopositivity in germinal epithelium (curved arrow), Caspase 3 immunopostivity in desquamated spermatogonia (thick arrow) and germinal epithelial cell developing stages (arrow head), dense immunopositivity of Caspase 9 in germinal epithelium (asterisk). Bar: 20 μm

was statistically significant ($p < 0.01$). The intensity in IRR group was 8.58% ± 2.19%, and statistical analyses indicated the Caspase 3 tissue level decreased significantly ($p < 0.01$) in the Riociguat treated group compared with the IR group. However, the difference between the control and IRR groups was significant ($p < 0.05$) as well. The intrinsic apoptosis regulator Caspase 9 level in the tissue of the control group was 12.58% ± 2.81% and it increased significantly ($p < 0.01$) in the IR group with 17.73% ± 5.76% tissue distribution density. Caspase 9 immunopositivity range in IRR group was 11.83% ± 3.60%. Analyses indicated that Caspase 9 immunopositivity in control and IRR groups was similar ($p = 0.423$), but there were significant difference ($p < 0.01$) between IR and IRR groups. Representative micrographs of immunostained sections are shown in Figure 4.

3.4 | TUNEL assay results

In our study, we evaluated TUNEL assay positivity in germinal epithelial cells. There were positive signals were obvious, mainly in germinal epithelium. Positive signals were also detected in some testicular

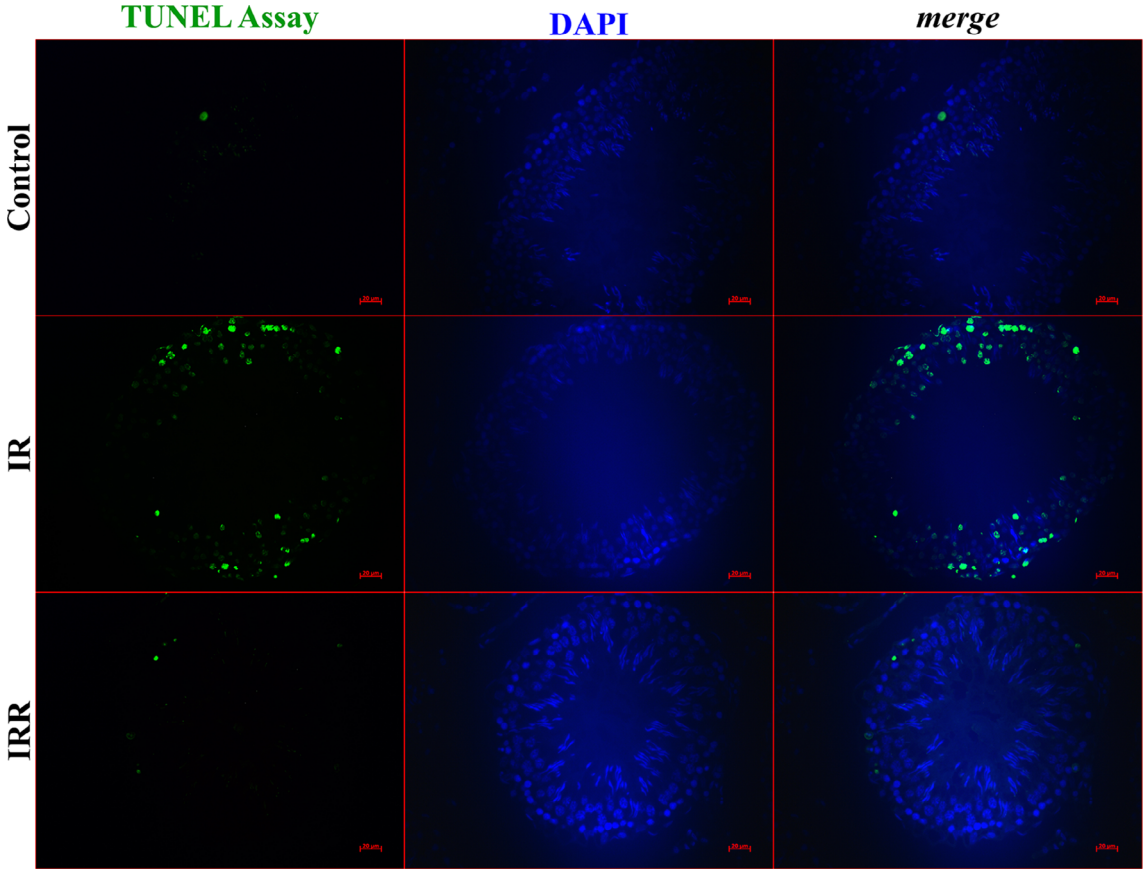

FIGURE 5 Representative micrographs of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay in control, IR, and IRR groups. Staining: TUNEL assay, counterstain: DAPI. Bar: 20 μm

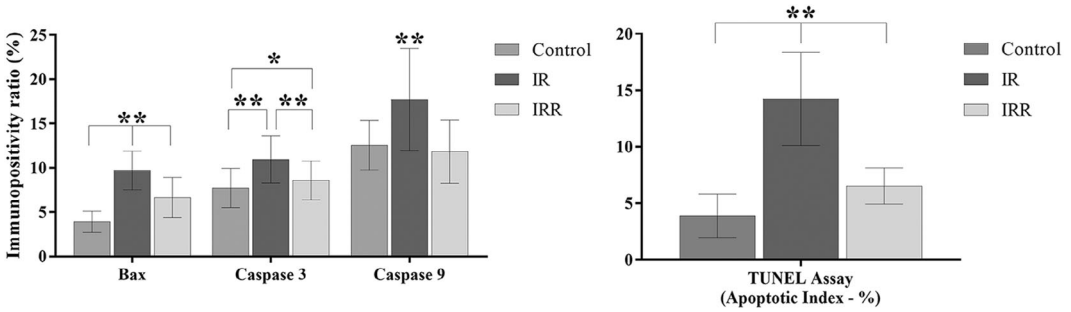

FIGURE 6 Graphical demonstration of statistical analyses of immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labelling assay analyses. Different superscripts between columns indicate statistically significance. *$p < 0.05$; **$p < 0.01$

capillary endothelial cells and interstitial Leydig cells. Statistical analyses of TUNEL assay indicated that AI in germinal epithelial cell in the control group was 3.89% ± 1.91%. AI in the IR group was 14.25% ± 4.15% and the difference between two groups was statistically significant ($p < 0.01$). The ratio of AI in IRR group was 6.53% ± 1.61% and the results of this group was significantly different than both control and IR ($p < 0.01$) groups. TUNEL assay micrographs and graphical representations of the statistical results are shown in Figures 5 and 6.

3.5 | Western blot results

Western blotting band intensity and thickness analyses are shown in Figure 7. Analyses revealed that IL-1β levels increased significantly ($p < 0.05$) in the IR group, while tissue levels in the IRR group were similar to the control ($p = 0.293$) and IR ($p = 0.194$) groups. Anti-apoptotic Bcl-2 protein levels were reduced significantly ($p < 0.01$) in the IR group compared to the control and IRR groups. The lowest NF-κB expression was observed in the control group and it was similar to

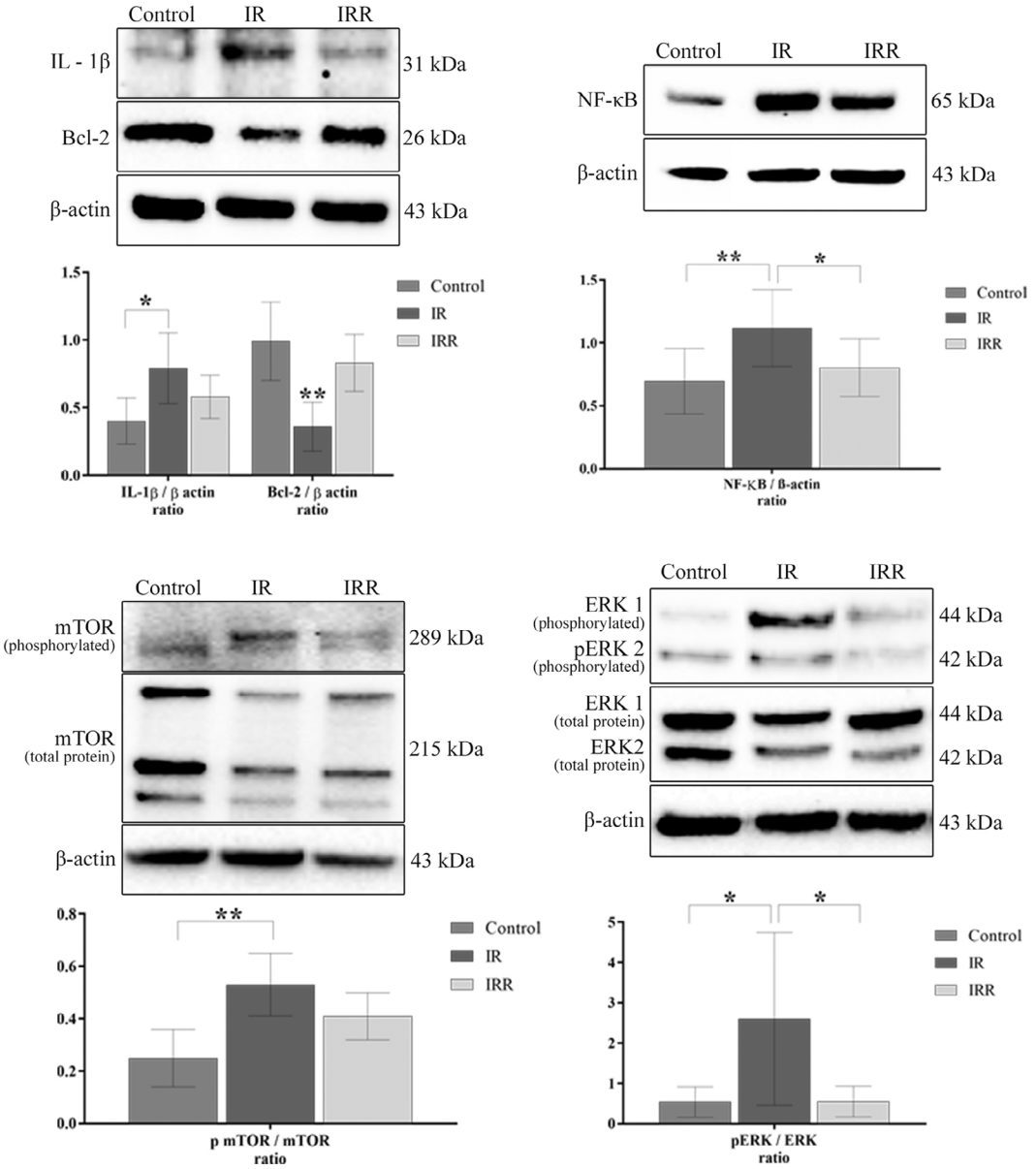

FIGURE 7 Western blotting results and graphical demonstration of statistical analyses. Different superscripts between columns indicate statistically significance. $^{*}p < 0.05$; $^{**}p < 0.01$

the IRR group. The tissue level of NF-κB is significantly up-regulated in IR group compared to control ($p < 0.01$) and IRR ($p < 0.05$) groups. When mTOR phosphorylation results were analysed, there were not any difference between the control and IRR groups ($p = 0.051$), but it was significantly ($p < 0.05$) up-regulated in the IR group compared to control group. Analyses of ERK phosphorylation results indicated that ERK phosphorylation in IR group is significantly ($p < 0.05$) up-regulated compared to control and IRR groups. Our experiment indicated that I/R may up-regulated mTOR and ERK phosphorylation in testicular tissue.

4 | DISCUSSION

Although surgically intervention is the only accepted treatment method, accumulated reactive oxygen species may maintain a tissue degeneration process that is also called as I/R injury. This testicular complication may lead subfertility or infertility in patients after a while. For that reason, researchers aim to explore most appropriate supplementary treatment besides providing reperfusion to the ischemic testis (Davoodi et al., 2020). Most of the previously published articles examined antioxidant substances to suppress the adverse effects of I/R injury on testes (Erol et al., 2017). One of the most commonly observed changes in I/R injury is the accumulation of ROS, the up-regulation of inflammatory cytokine release, germinal epithelial cell pyknosis, cellular desquamation, and the formation of multinucleated giant cells, which is thought to be evidence of a disruption in cell proliferation and maturity (Ayengin et al., 2021; Kohsaka et al., 2022; Seker et al., 2020). We observed severe pathological changes in testis, which were exposed to I/R injury as reported in literature. Today, Johnsen's biopsy is still one of the most accepted testicular tissue

degeneration score systems, and previously published studies reported that targeting oxidative stress or vasodilator pathways may up-regulate Johnsen's biopsy score in I/R exposed testis (Arena et al., 2017; Tanriverdi et al., 2021). In a previously published study, authors reported that vasodilator drugs could increase blood flow to the torsion induced experimental animal testis in an early phase (Savaş et al., 2002). As a matter of fact, vasodilator drugs can reduce testicular ischaemia related testicular pathology with contribution of independent varying process (Beheshtian et al., 2008). Re-oxygenizing drugs such as vasodilators are also reported with their antioxidant activity thus inhibiting germinal epithelial DNA fragmentation and apoptosis. Riociguat has capability to provoke vasodilation through sGC-cGMP signalling. The vasodilation process is regulated through NO-sGC-cGMP signalling and it leads to vasodilation, inhibition of platelet aggregation, anti-inflammatory response and induction of antioxidant enzymes (Nossaman et al., 2012). On the other hand, the novel drug Riociguat has the ability to regulate vasodilation independently from NO with affecting sGC-cGMP signalling. In literature review, we found that Riociguat possess anti-inflammatory activity in various diseases (Toxvig et al., 2019). Riociguat was reported to contribute on anti-inflammatory process via inhibiting the formation of NLRP-1 inflammasome/IL-1β cascade (Donda et al., 2018). When we compare these literatures and our current experiment, we observe consistent results. First of all decrease in tissue MDA and IL-1β level demonstrate possible anti-oxidant and anti-inflammatory activity of this drug. Testicular I/R injury increases germinal epithelial cell apoptosis through not only stimulating pro-apoptotic but also suppressing anti-apoptotic Bcl-2 protein expression (Roshanaee et al., 2022; Unsal et al., 2021). Results of our current study also confirmed that literature information and we observed significantly suppression of Bcl-2 expression in I/R exposed testis. Furthermore, targeting vascular dilation in ischemic organs up-regulates anti-apoptotic Bcl-2 expression, which is probably a result of re-oxygenation as fast as possible (Elmimehr et al., 2021). Despite the apoptotic regulators, cellular NF-κB is suspected to be responsible for the regulation of testicular I/R injury and results of previous experiments reported antioxidant substances inhibit Caspase 3 expression through NF-κB signalling (Afolabi et al., 2022). In our current experiment, we observed that NF-κB is significantly up-regulated in injured testis and that the sGC stimulator Riociguat may have potency to suppress NF-κB activity. The literature demonstrates that NF-κB modulation in oxidative stress dependins on the cell type and disease. The survival or harm contributing activity of this protein complex is stimulated by numerous signalling and the fate is progressed by various mechanisms. When we evaluated our results and consider the literature, it is possible to reach that NF-κB is probably activated in I/R exposed testis with indirect mechanisms by increased ROS level as described previously (Lingappan, 2018). In the end we can conclude that anti-oxidant activity of Riociguat may be responsible to reduce NF-κB due to activating antioxidant enzyme systems.

As a matter of fact, testicular I/R injury is a complicated process and most of the signalling and regulators of this process have not been explored yet. Recent findings indicate that mTOR and ERK are kinases that are possibly contributing to these pathological circumstances. The literature based on mTOR, ERK and I/R injury is still very limited, but when we review literature, a previously performed experiment reported that mTOR phosphorylation is up-regulated as an internal response to tissue injury, and authors of this study concluded that mTOR phosphorylation contributes to tissue degenerations in testicular I/R injury (Javdan et al., 2018). This study also indicated that antioxidant drug administration inhibits not only mTOR phosphorylation but also Caspase 3 expression and DNA fragmentations. There are results of some previously performed experiments indicated that mTOR inhibitor rapamycin administration protected testis tissue against testicular I/R with regulating tissue oxidative stress and apoptotic cell death (Ghasemnejad-Berenji et al., 2017; Ghasemnejad-Berenji et al., 2018). On the other hand, it is possible to achieve some opposing results in other experiments. In one of these, mTOR phosphorylation evaluated, and it was reported that phosphorylation levels were significantly reduced in I/R injury in heart (Wang et al., 2017). The authors also concluded that growth factors have ability to restore mTOR phosphorylation, thus protecting organs. When we compare our results with previously published articles, we observed that mTOR phosphorylation is elevated in I/R exposed testis and it slightly alleviated in Riociguat treated animals. We also believe the conflicting results of mTOR in I/R injury might be related due to multifunctional activity of this kinase. Another kinase, ERK was also reported as a regulatory in apoptosis and it was reported that inhibition of ERK phosphorylation reduced testicular tissue degenerations (Altavilla et al., 2012). When compared our results we also observed a significant increase in ERK phosphorylation in I/R exposed testis and Riociguat treatment reduced this process. We observed that testicular I/R probably provoke ERK phosphorylation, but Riociguat treatment alleviated this imbalance.

5 | CONCLUSIONS

As a conclusion, results of our experiment indicate that targeting sGC with novel drug Riociguat has potency to protect the testis in testicular I/R injury. Targeting vasodilation by sGC might provide oxygenized blood to the ischemic testis, thus have possibility to inhibit the formation of ROS. More advantages of sGC stimulator Riociguat is possibly activation of anti-inflammatory signalling. Furthermore, results of our current experiment and recently published articles reporting possible anti-apoptotic activity of this drug. Although we observed promising results, we believe that more detailed studies are required to understand the underlying mechanism of anti-oxidant, anti-apoptotic, anti-inflammatory and protective activity of this novel drug on I/R injury and the clinical applicability of this drug.

6 | LIMITATIONS OF THE STUDY

PCR analyses of apoptotic proteins, inflammatory cytokine of IL-1β, survival regulatory total mTOR, total ERK and phosphorylated genes would support the results of this study, but our western blotting analyses indirectly indicate gene expression levels of the mentioned pathways with considering protein expression levels. Cleaved Caspase of 3 and 9 would also improve the novelty of this study, but our TUNEL assay results demonstrate double stranded breaks, which also indirectly indicate cleavage and thus activation of mentioned apoptotic proteins. Moreover, evaluating signalling mechanisms such as Akt-PI3K-mTOR and intermediate steps in ERK signalling pathway might provide more detailed information for the underlying mechanism of the protective activity of sGC stimulator Riociguat in testicular I/R injury, but our end product analyses of phosphorylation levels of mTOR and ERK also provide information for the contribution of mentioned signalling mechanisms.

AUTHOR CONTRIBUTIONS

Ugur Seker and Baris Can Guzel: study design; Baris Can Guzel and Saime Betul Baygeldi: experimental protocol; Ugur Seker, Deniz Evrim Kavak, Baris Can Guzel, Saime Betul Baygeldi, Meral Yuksel, Ozlem Unay Demirel, Sevgi Irtegun Kandemir and Dila Sener: Laboratory analyses; Ugur Seker, Deniz Evrim Kavak, Baris Can Guzel, Meral Yuksel and Dila Sener: data collection and analyses; Ugur Seker and Baris Can Guzel: manuscript preparation; Meral Yuksel, Sevgi Irtegun Kandemir and Dila Sener: critical review.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Experiment of this study was performed with the approval of Experimental Animal Ethics Committee of Firat University (approval date and number: 01.11.2021-4753).

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REFERENCES

Abdel-Gaber, S. A., Mohammed, R. K., & Refaie, M. M. (2018). Mechanism mediating the protective effect of diacerein in ischemia-reperfusion-induced testicular injury in rats. *Life Sciences*, *209*, 57–62.

Afolabi, O., Anyogu, D., Hamed, M., Odetayo, A., Adeyemi, D., & Akhigbe, R. (2022). Glutamine prevents upregulation of NF-kB signaling and caspase 3 activation in ischaemia/reperfusion-induced testicular damage: An animal model. *Biomedicine & Pharmacotherapy*, *150*, 113056.

Altavilla, D., Romeo, C., Squadrito, F., Marini, H., Morgia, G., Antonuccio, P., & Minutoli, L. (2012). Molecular pathways involved in the early and late damage induced by testis ischemia: Evidence for a rational pharmacological modulation. *Current Medicinal Chemistry*, *19*(8), 1219–1224.

Arena, S., Iacona, R., Antonuccio, P., Russo, T., Salvo, V., Gitto, E., Impellizzeri, P., & Romeo, C. (2017). Medical perspective in testicular ischemia-reperfusion injury. *Experimental and Therapeutic Medicine*, *13*(5), 2115–2122.

Aydın, A., Sönmez, M. G., Ecer, G., Kılınç, F., Kocabaş, R., Atılgan, A. E., Oltulu, P., & Balasar, M. (2021). The effect of intratesticular dexpanthenol on experimentally-induced testicular ischaemia/reperfusion injury. *Journal of Pediatric Urology*, *17*(4), e441–e447.

Aydogdu, I., Karaca, E., Coban, G., Cay, A., Guler, E. M., Kocyigit, A., Uzun, E., Aydoğdu, Y. E., Metin, H., Miçooğullari, U., Ilbey, Y. O., Keskin, M. Z., & Miçooğullari, U. (2021). An investigation of the effects of amniotic fluid on experimental ischemia/reperfusion damage in rat testes. *Journal of Pediatric Urology*, *17*(6), 761.e1–761.e6.

Ayengin, K., Alp, H. H., Huyut, Z., Yıldırım, S., Altındag, F., & Avci, V. (2021). The effects of CoQ10 supplement on matrix metalloproteinases, oxidative DNA damage and pro-inflammatory cytokines in testicular ischaemia/reperfusion injury in rats. *Andrologia*, *53*(2), e13839.

Barlas, M., & Hatiboğlu, C. (2002). The effect of nitric oxide in testicular ischemia-reperfusion injury. *International Urology and Nephrology*, *34*(1), 81–86.

Bauersachs, J., Butler, J., & Sandner, P. (2017). *Heart failure* (1st ed.). Springer International Publishing AG.

Beheshtian, A., Salmasi, A. H., Payabvash, S., Kiumehr, S., Ghazinezami, B., Rahimpour, S., Tavangar, S. M., & Dehpour, A. R. (2008). Protective effects of sildenafil administration on testicular torsion/detorsion damage in rats. *World Journal of Urology*, *26*(2), 197–202.

Bozlu, M., Acar, D., Cayan, S., Aktas, S., & Tunckiran, A. (2009). Protective effect of trapidil on long-term histologic damage in a rat model of testicular ischemia-reperfusion injury. *World Journal of Urology*, *27*(1), 117–122.

Cannavò, S. P., Riso, G., Casciaro, M., Di Salvo, E., & Gangemi, S. (2019). Oxidative stress involvement in psoriasis: A systematic review. *Free Radical Research*, *53*(8), 829–840.

Davoodi, F., Taheri, S., Raisi, A., Rajabzadeh, A., Ahmadvand, H., Hablolvarid, M. H., & Zakian, A. (2020). Investigating the sperm parameters, oxidative stress and histopathological effects of *Salvia miltiorrhiza* hydroalcoholic extract in the prevention of testicular ischemia reperfusion damage in rats. *Theriogenology*, *144*, 98–106.

Davoodi, F., Taheri, S., Raisi, A., Rajabzadeh, A., Zakian, A., Hablolvarid, M. H., & Ahmadvand, H. (2021). Leech therapy (*Hirudo medicinalis*) attenuates testicular damages induced by testicular ischemia/reperfusion in an animal model. *BMC Veterinary Research*, *17*(1), 1–15.

Dejban, P., Rahimi, N., Takzare, N., Jahansouz, M., Haddadi, N.-S., & Dehpour, A. R. (2019). Beneficial effects of dapsone on ischemia/reperfusion injury following torsion/detorsion in ipsilateral and contralateral testes in rat. *Theriogenology*, *140*, 136–142.

Dokucu, A., Öztürk, H., Özdemir, E., Ketani, A., Büyükbayram, H., & Yücesan, S. (2000). The protective effects of nitric oxide on the contralateral testis in prepubertal rats with unilateral testicular torsion. *BJU International*, *85*(6), 767–771.

Dolatkhah, M. A., Shokoohi, M., Charvandeh, S., Tvrda, E., Shoorei, H., Moghimian, M., & Alihemmati, A. (2020). *Fumaria parviflora* regulates oxidative stress and apoptosis gene expression in the rat model of varicocele induction. *Andrologia*, *52*(11), e13826.

Donda, K., Zambrano, R., Moon, Y., Percival, J., Vaidya, R., Dapaah-Siakwan, F., Luo, S., Duncan, M. R., Bao, Y., Wang, L., Qin, L., Benny, M., Young, K., & Wu, S. (2018). Riociguat prevents hyperoxia-induced lung injury and pulmonary hypertension in neonatal rats without effects on long bone growth. *PLoS One*, *13*(7), e0199927.

Elmimehr, R., Motamed-Sanaye, A., Brazvan, B., Abtahi-Eivary, S. H., Moghimian, M., & Fani, M. (2021). Effects of hypothermia and pentoxifylline on the adnexal torsion/detorsion injuries in a rat testis model. *Andrologia*, *53*(8), e14143.

Erol, B., Sari, U., Amasyali, A., Ozkanli, S., Sogut, S., Hanci, V., Efiloglu, O., Danacioglu, Y. O., Engin, P., Yencilek, F., & Atis, G. (2017). Comparison of combined antioxidants and thymoquinone in the prevention of testis ischemia–reperfusion injury. *Andrology*, *5*(1), 119–124.

Ghasemnejad-Berenji, M., Ghazi-Khansari, M., Pashapour, S., Jafari, A., Yazdani, I., Ghasemnejad-Berenji, H., Saravi, S. S. S., Sadeghpour, S., Nobakht, M., Abdollahi, A., Ansari, J. M., & Dehpour, A. R. (2018). Synergistic effect of rapamycin and metformin against germ cell apoptosis and oxidative stress after testicular torsion/detorsion-induced ischemia/reperfusion in rats. *Biomedicine & Pharmacotherapy*, *105*, 645–651.

Ghasemnejad-Berenji, M., Ghazi-Khansari, M., Yazdani, I., Saravi, S. S. S., Nobakht, M., Abdollahi, A., Ansari, J. M., & Dehpour, A. R. (2017). Rapamycin protects testes against germ cell apoptosis and oxidative stress induced by testicular ischemia-reperfusion. *Iranian Journal of Basic Medical Sciences*, *20*(8), 905–911.

Gozukara, K. H., Ozcan, O., Ozgur, T., Kaya, Y. S., & Tutuk, O. (2020). Protective effects of colchicine on testicular torsion/detorsion-induced ischemia/reperfusion injury in rats. *Urology Journal*, *17*(3), 294–300.

Howe, A. S., Vasudevan, V., Kongnyuy, M., Rychik, K., Thomas, L. A., Matuskova, M., Friedman, S. C., Gitlin, J. S., Reda, E. F., & Palmer, L. S. (2017). Degree of twisting and duration of symptoms are prognostic factors of testis salvage during episodes of testicular torsion. *Translational Andrology and Urology*, *6*(6), 1159–1166.

Jafari, A., Ghasemnejad-Berenji, H., Nemati, M., Pashapour, S., Sadeghpour, S., & Ghasemnejad-Berenji, M. (2021). Beneficial effects of memantine on ischemia/reperfusion injury following torsion/detorsion induced testicular damage in rats: Improvement in histological and biochemical parameters. *Journal of Pediatric Urology*, *17*(4), 441.e1–441.e7.

Javdan, N., Ayatollahi, S. A., Choudhary, M. I., Al-Hasani, S., Kobarfard, F., Athar, A., & Pazoki-Toroudi, H. (2018). Capsaicin protects against testicular torsion injury through mTOR-dependent mechanism. *Theriogenology*, *113*, 247–252.

Johnsen, S. G. (1970). Testicular biopsy score count—A method for registration of spermatogenesis in human testes: Normal values and results in 335 hypogonadal males. *Hormone Research in Pædiatrics*, *1*(1), 2–25.

Kohsaka, T., Yoneda, Y., Yoshida, T., Minagawa, I., Pitia, A. M., Iwasawa, A., & Ikegaya, N. (2022). Relaxin exerts a protective effect during ischemia-reperfusion in the rat model. *Andrology*, *10*(1), 179–189.

Kölükçü, E., Firat, F., Deresoy, F. A., Katar, M., & Atılgan, D. (2021). The effects of pirfenidone on ischaemia–reperfusion injury in testicular torsion-induced rat model. *Andrologia*, *53*(2), e13922.

Lingappan, K. (2018). NF-κB in oxidative stress. *Current Opinion in Toxicology*, *7*, 81–86.

Nossaman, B., Pankey, E., & Kadowitz, P. (2012). Stimulators and activators of soluble guanylate cyclase: Review and potential therapeutic indications. *Critical Care Research and Practice*, *2012*, 1–12.

Paul, T., Salazar-Degracia, A., Peinado, V. I., Tura-Ceide, O., Blanco, I., Barreiro, E., & Barbera, J. A. (2018). Soluble guanylate cyclase stimulation reduces oxidative stress in experimental chronic obstructive pulmonary disease. *PLoS One*, *13*(1), e0190628.

Roshanaee, M. K., Abtahi-Eivary, S.-H., Shokoohi, M., Fani, M., Mahmoudian, A., & Moghimian, M. (2022). Protective effect of minocycline on Bax and Bcl-2 gene expression, histological damages and oxidative stress induced by ovarian torsion in adult rats. *International Journal of Fertility & Sterility*, *16*(1), 30.

Savaş, Ç., Dindar, H., Aras, T., & Yücesan, S. (2002). Pentoxifylline improves blood flow to both testes in testicular torsion. *International Urology and Nephrology*, *33*(1), 81–85.

Seker, U., Kaya, S., Kandemir, S. I., Sener, D., Demirel, O. U., & Nergiz, Y. (2022). Effects of black cumin seed oil on oxidative stress and expression of membrane-cytoskeleton linker proteins, radixin, and moesin in streptozotocin-induced diabetic rat liver. *Hepatology*, *3*, 21–26.

Seker, U., Nergiz, Y., Aktas, A., Akkus, M., Ozmen, M. F., Uyar, E., & Soker, S. (2020). Trolox is more successful than allopurinol to reduce degenerative effects of testicular ischemia/reperfusion injury in rats. *Journal of Pediatric Urology*, *16*(4), 465.e1–465.e8.

Şener, T. E., Yüksel, M., Özyılmaz-Yay, N., Ercan, F., Akbal, C., Şimşek, F., & Şener, G. (2015). Apocynin attenuates testicular ischemia–reperfusion injury in rats. *Journal of Pediatric Surgery*, *50*(8), 1382–1387.

Shokoohi, M., Khaki, A., Abadi, A. R. R., MohammadZadeh Boukani, L., Hassanpour Khodaie, S., Kalarestaghi, H., Khaki, A. A., Moghimian, M., Niazkar, H. R., & Shoorei, H. (2022). Minocycline can reduce testicular apoptosis related to varicocele in male rats. *Andrologia*, *54*(4), e14375.

Shokri, F., Majid Shokoohi, M., Rasht Abadi, A. R., & Kalarestaghi, H. (2019). The ameliorative effect of *Galega officinalis* extract on histological damages, oxidative stress induced by torsion-detorsion in adult rats' ovarian. *International Journal of Women's Health and Reproduction Sciences*, *7*(1), 119–123.

Sravani, S., Saifi, M. A., & Godugu, C. (2020). Riociguat ameliorates kidney injury and fibrosis in an animal model. *Biochemical and Biophysical Research Communications*, *530*(4), 706–712.

Taheri, S., Davoodi, F., Raisi, A., Zakian, A., Rajabzadeh, A., Hablolvarid, M. H., Khezri, A., & Ahmadvand, H. (2021). Co-administration of salvia miltiorrhiza and verapamil inhibits detrimental effects of torsion/detorsion on testicular tissue in rats. *Andrologia*, *53*(6), e14049.

Tamer, S. A., Yıldırım, A., Arabacı, Ş., Çiftçi, S., Akın, S., Sarı, E., Köroğlu, M. K., Ercan, F., Yüksel, M., Çevik, Ö., & Yeğen, B. Ç. (2019). Treatment with estrogen receptor agonist ERβ improves torsion-induced oxidative testis injury in rats. *Life Sciences*, *222*, 203–211.

Tanriverdi, H. I., Şenel, U., Gevrek, F., & Akbaş, A. (2021). Protective effect of famotidine on ischemia–reperfusion injury following testicular torsion in rats. *Journal of Pediatric Urology*, *17*(2), 167.e1–167.e7.

Toxvig, A. K., Wehland, M., Grimm, D., Infanger, M., & Krüger, M. (2019). A focus on riociguat in the treatment of pulmonary arterial hypertension. *Basic & Clinical Pharmacology & Toxicology*, *125*(3), 202–214.

Unsal, V., Kolukcu, E., Gevrek, F., & Firat, F. (2021). Sinapic acid reduces ischemia/reperfusion injury due to testicular torsion/detorsion in rats. *Andrologia*, *53*(8), e14117.

Wang, Z.-G., Li, H., Huang, Y., Li, R., Wang, X.-F., Yu, L.-X., Guang, X. Q., Li, L., Zhang, H. Y., Zhao, Y. Z., Zhang, C., Li, X. K., Wu, R. Z., Chu, M. P., & Xiao, J. (2017). Nerve growth factor-induced Akt/mTOR activation protects the ischemic heart via restoring autophagic flux and attenuating ubiquitinated protein accumulation. *Oncotarget*, *8*(3), 5400–5413.

Wink, D. A., Miranda, K. M., Espey, M. G., Pluta, R. M., Hewett, S. J., Colton, C., Vitek, M., Feelisch, M., & Grisham, M. B. (2001). Mechanisms of the antioxidant effects of nitric oxide. *Antioxidants and Redox Signaling*, *3*(2), 203–213.

Yousefi-Manesh, H., Shirooie, S., Hemati, S., Shokrian-Zeini, M., Zarei, N., Raoufi, M., Farrokhi, V., & Dehpour, A. R. (2019). Protective effects of

modafinil administration on testicular torsion/detorsion damage in rats. *Experimental and Molecular Pathology*, *111*, 104305.

Yu, X., Ge, L., Niu, L., Lian, X., Ma, H., & Pang, L. (2018). The dual role of inducible nitric oxide synthase in myocardial ischemia/reperfusion injury: Friend or foe? *Oxidative Medicine and Cellular Longevity*, *2018*, 1–7.

Zvizdic, Z., Aganovic, A., Milisic, E., Jonuzi, A., Zvizdic, D., & Vranic, S. (2021). Duration of symptoms is the only predictor of testicular salvage following testicular torsion in children: A case-control study. *The American Journal of Emergency Medicine*, *41*, 197–200.

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