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Metformin mitigates oxidative stress and prevents apoptosis by modulating the Nrf2/HO-1 signaling pathway in a rat model of testicular ischemia/reperfusion

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ABSTRACT

Objective(s): This study aimed to investigate the effect of metformin on testicular ischemia-reperfusion (I/R) injury in rats.

Materials and Methods: Eighteen male SD rats were randomly divided into three groups: Sham group, I/R group, and Metformin (Met) group (n=6 per group). The I/R model was established by rotating the left testis 720° clockwise and fixing it for 1 hr, followed by reperfusion for 4 hr. Rats in the Met group were intraperitoneally injected with 300 mg/kg metformin for 30 min before reperfusion. In contrast, the Sham group underwent a similar surgical procedure without testicular rotation. Histopathological examination, biochemical assays (MDA and SOD), TUNEL assay for germ cell apoptosis, and Western blot analysis for Nrf2, HO-1, and Keap1 protein expressions were performed.

Results: Compared with the Sham group, the I/R group exhibited severe testicular tissue damage, including seminiferous tubule atrophy, disordered spermatogenic epithelium, increased MDA levels, decreased SOD activity, elevated germ cell apoptosis index, up-regulated Nrf2 and HO-1 expressions, and down-regulated Keap1 expression. In contrast, pretreatment with metformin in the Met group significantly ameliorated these pathological changes, as evidenced by improved testicular histology, reduced MDA concentration, increased SOD activity, decreased apoptosis, and reversal of the expression of Nrf2, HO-1, and Keap1 compared with the I/R group.

Conclusion: These results indicate that metformin exerts a protective effect against testicular I/R injury, which may be associated with its anti-oxidant, anti-apoptotic properties, and regulation of the Nrf2/ HO-1pathway.

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Introduction

Testicular torsion, a urological emergency predominantly affecting neonates and adolescents, leads to ischemia reperfusion (I/R) injury upon surgical detorsion (1). Critically, the duration of ischemia profoundly impacts outcomes: surgical intervention within 4-6 hr preserves testicular viability in 90–100% of cases, whereas delays beyond 24 hr reduce salvage rates to <10% (2). The incidence of testicular torsion is 1 in 4,000 in male individuals younger than 25 years of age (3). I/R of the testis is characterized by oxidative stress, a central pathological mechanism driving germ cell apoptosis, impaired spermatogenesis, and subsequent infertility (4, 5). These perturbations lead to histological deterioration, including seminiferous tubule atrophy, reduced Johnsen scores, and impaired sperm quality (6, 7). The resumption of blood flow to previously ischemic tissues during detorsion leads to an exacerbated release of reactive oxygen species (ROS), culminating in diminished cellular viability due to lipid peroxidation, protein denaturation, and DNA damage (8, 9). Early intervention not only limits focal damage but also prevents contralateral testicular injury, as oxidative mediators spread via systemic circulation (1, 10). Therefore, timely diagnosis is thus paramount to mitigate irreversible oxidative damage and restore testicular function. Further exploration of ROS-mediated pathways and combinatorial therapies, coupled with improved diagnostic protocols, is critical to preserving testicular function post-torsion. Under oxidative stress conditions, the activation of NF-E2-Related Factor 2 (Nrf2) plays a leading role (11, 12). Nrf2 is the master gene transcription regulator of several antioxidant enzymes like catalase, superoxide dismutase (SOD), glutathione reductase, and heme oxygenase-1 (HO-1)(13). Physiologically, Nrf2 is bound to its negative regulator, Kelch-like ECH-associated protein 1 (Keap1). Keap1 sequesters Nrf2 in the cytoplasm and enhances its ubiquitination and degradation (14). Thus, induction of the Nrf2 pathway might be considered as a potent protective tactic.

Metformin, an insulin sensitizer that decreases hepatic glucose output and increases peripheral glucose uptake, has

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been commonly used in the treatment of type 2 diabetes mellitus. It has been demonstrated that the administration of Metformin may also exert protective effects against testicular I/R injury in rat models, as shown by positive effects on both sperm quality and oxidative stress-related markers (15-17). In STZ-induced diabetic rats with male reproductive damage, a previous study shows that metformin exerts reproductive protection via activating the Nrf2 signaling pathway (up-regulating testicular antioxidant genes like SOD/CAT and their enzyme activities)(18). However, while metformin's protective effect on rat testicular ischemia-reperfusion injury has been reported in existing research, the specific mechanism underlying this protection, particularly whether it also involves the Nrf2 signaling pathway, remains unexplored. Thus, the purpose of this study is to investigate whether the protective effect of metformin on the testicular I/R model is achieved by regulating Nrf2-related signaling pathways.

Materials and Methods

Animals

Animal care and experimental procedures were conducted following the animal research guidelines of the National Institutes of Health and approved by the Animal Ethics Committee for Wuhan Myhalic Biotechnology Co., Ltd (Approval Number: HLK-20250106-001). Eighteen male SD rats aged 6-8 weeks were selected and housed in individual cages with constant temperature and humidity. The rats were allowed to acclimate to the environment for 3-7 days prior to the experiment. The animal room was maintained at 20-26 ˚C and 40-70% humidity, with a 12-hour light/dark cycle. The rats had continuous access to an adequate amount of food and were allowed to consume freely. Sterile water was provided through an uninterrupted water supply system, allowing for unrestricted access to drinking water.

Experimental groups and surgical procedures

After adaptive feeding, the rats were randomly assigned to three groups, with six rats per group. The ischemia-reperfusion time was set up according to a previous study (19). In detail, all animals were anesthetized with isoflurane gas. Mid-scrotal incisions were used for torsion, detorsion, and sham procedures. The left testis was then rotated clockwise by 720° and fixed in place. After one hour, the testis and scrotum were repositioned, and the skin was sutured closed in layers. The testis was then returned to its initial anatomical position for four hours. The sham group consisted of animals that underwent all surgical procedures but did not have their testicles torsioned. The I/R group underwent 720° torsion-induced ischemia for 1 hr, followed by reperfusion for 4 hr. Both groups received the same volume of normal saline for treatment. The Metformin (Met) group experienced 720° torsion-induced ischemia for 1 hr, and 30 min before testicular detorsion, rats received Met at a dose of 300 mg/kg (i.p). The Metformin dosages used in the present study were based on previous studies (15).

Histopathological examination

The testis specimens were embedded in paraffin blocks after fixing in 4% paraformaldehyde for 24 hr. Sections of thickness 4 μm were obtained and stained using hematoxylin and eosin (H&E). Histopathological changes in the seminiferous tubules were observed using an ocular micrometer (Olympus, Tokyo, Japan) at magnifications of ×40 and ×200, and images were captured. This method, as described by YaPing Jiang *et al.* (20), was used to assess testicular injury and spermatogenesis histopathologically using Johnsen's score (21). Scoring was performed on H&E-stained slides in six non-overlapping fields from each rat in all groups.

Biochemical assays

Malondialdehyde (MDA) concentrations were quantified using the Lipid Peroxidation MDA Assay Kit (Beyotime Biotechnology, Cat. No. S0131) following the manufacturer's protocol. The supernatant absorbance was measured at 532 nm using a microplate reader (Thermo Fisher, Multiskan FC), and the results were expressed in nmol/mg of protein. SOD activity was determined using the WST-8-based Superoxide Dismutase Assay Kit (Beyotime Biotechnology, Cat. No. S0101) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, Multiskan FC), and the results were expressed as U/mg protein.

TUNEL assay

Apoptotic cells were detected using the One-Step TUNEL In Situ Apoptosis Kit (Green, Elab Fluor®488; Elabscience, Cat. No. E-CK-A321) according to the method of Osman Hakan K *et al.* (22). Briefly, Fresh tissues were fixed in 4% paraformaldehyde for over 24 hr, dehydrated through a graded ethanol series, and embedded in paraffin using a JB-P5 embedding machine. Sections of 4 μm thickness were cut on a Leica RM2016 microtome, mounted on slides, and dried at 60 ˚C. For staining, sections were dewaxed in xylene, rehydrated in ethanol gradients and distilled water, then treated with proteinase K at 37 ˚C for 30 min. After PBS washes, sections were equilibrated with TdT buffer and incubated with TUNEL reaction mix. Nuclei were counterstained with DAPI, and slides were mounted with antifade medium. Negative controls lacked the TdT enzyme, while positive controls were pretreated with DNase I (1 U/ml). Fluorescent images were captured using a Zeiss LSM710 confocal microscope (TUNEL: 488 nm excitation; DAPI: 405 nm excitation). Apoptotic cells were quantified as the percentage of TUNEL$^+$ nuclei per field (6 fields/sample) using ImageJ.

Western blot assay

The testicular tissues were extracted with RIPA lysis buffer containing protease inhibitors, homogenized on ice, and centrifuged at 12,000 rpm (4 ˚C, 10 min). Protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, P0010) according to the manufacturer's instructions, with absorbance measured at 562 nm, as reported in a previous study (8). Equal amounts of protein 30 μg were mixed with 5×SDS loading buffer, denatured at 95 °C for 5 min, and separated by SDS-PAGE using a 10% separating gel (30% acrylamide, Tris-HCl pH 8.8) and 4% stacking gel (Tris-HCl pH 6.8) on a Bio-Rad Mini-PROTEAN 3 system. Proteins were transferred to PVDF membranes (Millipore, ISEQ00010) using a wet transfer system (Bio-Rad, 170-3930) at 90 V for 1 hr in transfer buffer. Membranes were blocked with 5% non-fat

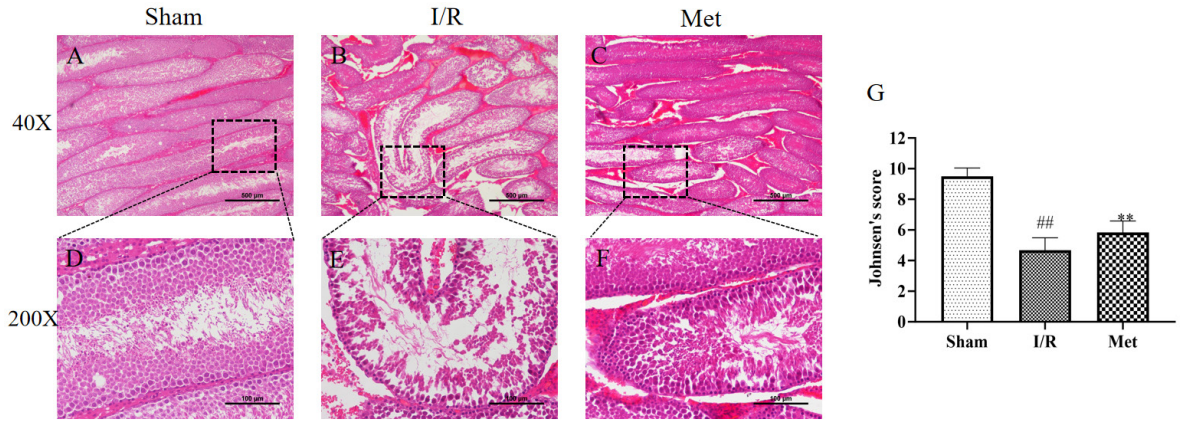

Figure 1. Histological analysis of rat tissue sections (H&E staining) from Sham, ischemia-reperfusion (I/R), and Metformin (Met) groups
Upper row (40× magnification): (A) Sham, (B) I/R, (C) Met. Lower row (200× magnification), (D) Sham, (E) I/R, (F) Met. Scale bars as shown. (G) Johnsen's score. Each value indicates the mean±SD (n=6). ## P<0.01 vs sham group; ** P<0.01 vs I/R group

milk in TBST for 1 hr at room temperature, then incubated overnight at 4 ˚C with primary antibodies (HO-1, Keap1, Nrf2 at 1:2000; GAPDH at 1:5000 in TBST with 1% BSA). After three TBST washes (10 min each), membranes were incubated with HRP-conjugated secondary antibodies (Beyotime, A0216/A0208; 1:5000 in TBST) for 2 hr at room temperature. Signals were detected using ECL substrate (Tanon, 180-5001) and imaged on a Tanon 5200 chemiluminescence system. Protein bands were quantified using ImageJ software.

Statistical analysis

Data were expressed as mean±standard deviation (SD). The analytical results were evaluated using the Statistical Package for the Graph pad v8.03. Control variables were compared among groups using a one-way analysis of variance (ANOVA) with Tukey's honestly significant difference test. Statistical significance was accepted as P<0.05.

Results

Histopathological examination

In the sham group, the seminiferous epithelium maintained a normal germ cell distribution, with clear stratification of spermatogenic cells at different developmental stages (Figure 1A/D). The testes were structurally intact, consisting of seminiferous tubules of varying diameters, each lined with a continuous germinal epithelium, and interstitial Leydig cells were uniformly distributed in the spaces between adjacent tubules. In stark contrast, the I/R group exhibited prominent histopathological impairments in spermatogenesis, characterized by disorganized germ cell developmental stages and disrupted testicular architecture (Figure 1B/E). Key pathological features included a significant reduction in total germ cell count, morphological distortion of interstitial Leydig cells, and marked widening of interstitial spaces; these alterations were readily observable in H&E-stained sections. Consistently, the Johnsen's score of the I/R group (average: 4.67±0.81) was significantly lower than that of the sham group (average: 9.45±0.54) (P<0.01, Figure 1G), confirming the severe impairment of spermatogenic function induced by I/R injury. Among all treatment groups, Met administration exerted the most pronounced protective effect on testicular tissue recovery in I/R-injured rats. In the Met group, spermatogenic cells were tightly and orderly arranged, with clear visualization of cells at all developmental stages (from spermatogonia to mature spermatids) and active differentiation processes. Correspondingly, the Johnsen's score of this group (average: 5.83±0.75) was significantly higher than that of the I/R group (P<0.01, Figure 1G), indicating that Met effectively mitigated I/R-induced testicular pathological damage.

The expressions of MDA and SOD

The concentration of the lipid peroxidation marker MDA was significantly increased in the I/R group compared to the sham group (P<0.01)(Figure 2A). Meanwhile, the enzyme activities of SOD were notably decreased in the I/R group compared to the sham group (P<0.01)(Figure 2B). Compared with the I/R group, treatment with Met decreased MDA levels and increased SOD activity (P<0.01, respectively).

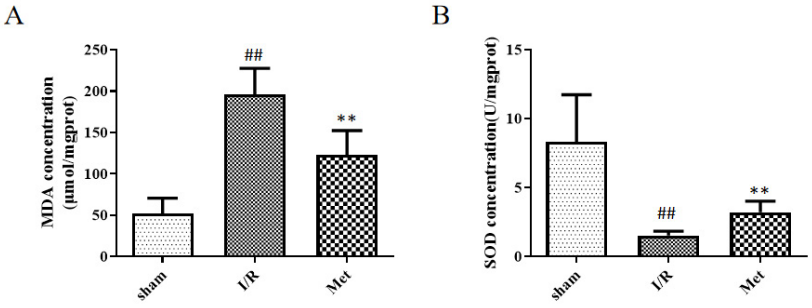

Figure 2. Effects of metformin on the level of malondialdehyde (MDA) (A) and superoxide dismutase (SOD) activity (B) in rat testicular ischemia-reperfusion (I/R) injury
Each value indicates the mean±SD (n=6). ## P<0.01 vs sham group; ** P<0.01 vs I/R group

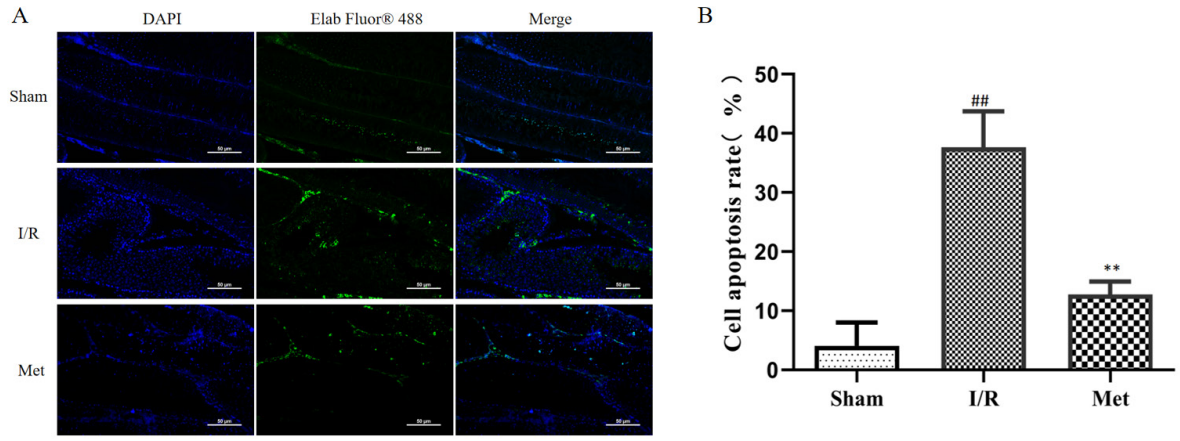

Figure 3. (A) Fluorescence microscopy images showing apoptosis-related staining (DAPI for nuclei in blue, Elab Fluor® 488 for apoptotic signals in green, merge for combined view) in Sham, ischemia-reperfusion (I/R), and Metformin (Met) groups. Scale bars as indicated. (B) Quantitative analysis of cell apoptosis rate in Sham, I/R, and Met groups
Data are mean±SD. ## P<0.01 vs Sham group; ** P<0.01 vs I/R group

Evaluation of germ cell apoptosis using the TUNEL assay

The effect of testicular I/R injury and Met treatment on tissue apoptosis was assessed via TUNEL assay. As shown in Figure 3A, the apoptotic rate of testicular tissue in the I/R group was significantly higher than that in the sham group, indicating that I/R injury strongly promotes testicular cell apoptosis (P<0.01). In contrast, when rats were treated with Met at a dose of 300 mg/kg, the I/R-induced increase in apoptotic rate was effectively reversed: the apoptotic rate in the Met group was significantly lower than that in the I/R group, confirming the anti-apoptotic role of Met in testicular I/R injury (P<0.01; Figure 3B).

Western blot

As shown in Figure 4, compared with the Sham group, the I/R group displayed a marked up-regulation of Nrf2 (P<0.05; Figure 4A) and HO-1 (P<0.01; Figure 4B), accompanied by a significant reduction in Keap1 expression (P<0.05; Figure 4C). Notably, Met intervention partially reversed these I/R-induced changes: relative to the I/R group, the Met group showed statistically significant decreases in Nrf2 and HO-1 expression (both P<0.05), while Keap1 expression was restored to levels comparable to the Sham group (P<0.01 vs I/R group).

Discussion

In the present study, we demonstrated that Metformin affects oxidative stress markers, including MDA levels and SOD activity, as well as TUNEL-detected apoptosis and the structural integrity of testicular tissue following I/R injury. Additionally, Western blotting was employed to examine the expression of pathway proteins potentially involved in its protective mechanism, including Nrf2, HO-1, and Keap1.

A previous study showed that histopathological examination of testicular tissue revealed deterioration, irregularity, and a decrease in the interstitial structure of the testis, the seminiferous tubular structures, and the number of cells in the spermatogenic series, respectively, following I/R (23). Histopathological analyses revealed a decreased Johnsen's score of spermatogenesis (20). Similarly, our experiment detected the same pathological findings in testes subjected to 1 hr of ischemia and 4 hr of reperfusion. Histopathological results confirmed that Metformin reversed the I/R-induced testis injuries, and increased the Johnsen's score. The pathogenesis of I/R injury is a multifactorial process in which multiple contributing factors are implicated. Oxidative stress response has been reported to be a significant factor for I/R testicular damage (1, 2). I/R injury always leads to ROS overproduction; the origin

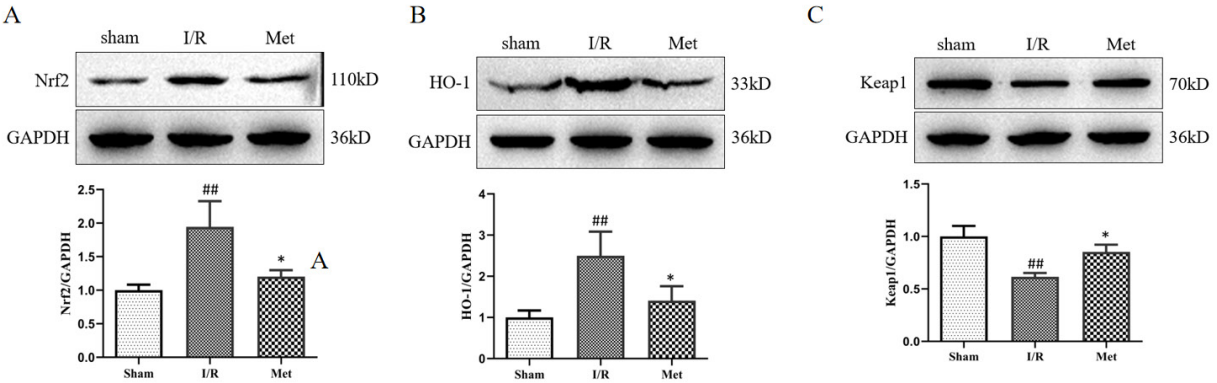

Figure 4. Western blot analysis of Nrf2 (A), HO-1 (B), and Keap1 (C) rat protein expressions in sham, ischemia-reperfusion (I/R), and Metformin (Met) groups. Bands represent immunoblot results of corresponding proteins
Bar graphs show quantitative analysis of relative protein expressions (normalized to GAPDH). ## P<0.01 vs sham group; * P<0.05 vs I/R group

of ROS is mature granulocytes that adhere to the vascular endothelium (23). The most crucial role of granulocytes is the recruitment of neutrophils to the testicular subtunical venules, which induces germ cell-specific apoptosis, further leading to a decrease or loss of sperm stem cells and resulting in infertility (24, 25). ROS are challenging to measure directly because of their high reactivity and short life span. MDA is produced following ROS-induced lipid peroxidation in the cell membrane and is widely used as a sensitive biomarker of ROS (1). SOD is a major enzyme that scavenges harmful ROS in male reproductive organs. Our study found that rats in the testicular Met group had significantly lower MDA levels and higher SOD activity than those in the I/R group, consistent with previous reports. (15-17). Li *et al.* (26) applied the TUNEL assay to reveal the level of apoptosis induced by I/R/R. Apoptosis in testicular cells increased after I/R. Our results were consistent with this previous study; therefore**,** Metformin treatment could reduce the apoptosis induced by I/R.

Nrf2 is a transcription factor that controls the orchestrated expression of phase II enzymes and genes involved in oxidative defense. Under homeostatic conditions, Nrf2 is usually kept inactive in the cytoplasm by its cytosolic inhibitor, Keap1. When oxidative stress occurs in the testicular tissue, Nrf2 dissociates from Keap1 and transfers into the nucleus, thereby regulating the expression of downstream antioxidant proteases, such as HO-1, and further exerts antioxidant stress (20). It is worth noting that the activation of Nrf-2 follows a time-dependent pattern. It is reported that nuclear import of Nrf2, from time of exposure to stabilization, takes roughly 2 hr, followed by the activation of a delayed mechanism that controls switching off of Nrf2 activation (27). A previous study focusing on testicular I/R injury reported that HO-1 shows a clear time-dependent expression pattern (28). Specifically, HO-1 expression increases gradually in the early stage after testicular I/R injury, reaches its highest level on post-injury day 3, and then gradually decreases to levels close to the baseline in the subsequent days (e.g., days 7 and 28). This dynamic change in HO-1 expression is consistent with the pathological progression of testicular I/R injury. Previous studies (29, 30) demonstrated that Nrf2 and HO-1 expression were up-regulated in the I/R group compared with the sham group. Consistent with these findings, our study also observed increased expression of Nrf2 and HO-1, and decreased expression of Keap1, in the testicular I/R group (induced by 1 hour of ischemia followed by 4 hr of reperfusion) relative to the sham group. Metformin exerts protective effects in different disease models by regulating the Nrf2 signaling pathway—activating Nrf2 to enhance antioxidant defense in tissue injury models (e.g., diabetic reproductive damage, cardiac remodeling) and down-regulating/inhibiting Nrf2 to reverse chemoresistance in tumor models (e.g., gastric cancer, hepatocellular carcinoma) (18, 31-33). Consistent with this regulatory pattern of metformin on Nrf2, the results of our study confirm that in the rat testicular I/R injury model, metformin exerts its protective effects of antioxidation and anti-apoptosis by inhibiting the Nrf2/HO-1 signaling pathway.

Conclusion

This study confirms that metformin exerts protective effects against testicular I/R injury in rats. It reverses I/R-induced testicular structural damage (such as seminiferous tubule irregularity and interstitial Leydig cells deterioration). It increases Johnsen's score while reducing oxidative stress (lowering MDA levels and enhancing SOD activity) and germ cell apoptosis. Mechanistically, unlike metformin's activation of Nrf2 in other tissue injury models, it inhibits the overactivated Nrf2/HO-1 pathway (Nrf2 and HO-1 are up-regulated in I/R) here, which is its key protective mechanism. These findings support metformin's potential in clinical management of testicular I/R injury. However, a limitation of this study is that Nrf2 knockout mice were not used to further validate this hypothesis, which will be an important direction for our future research.

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Authors' Contributions

L ZM and W JB designed the experiments; L ZM, Ch RY, and L R performed experiments and collected data; L ZM, Ch RY, and L R discussed the results and strategy; L ZM Supervised, directed and managed the study; L ZM , Ch RY, L R, W JB and Ch AL final approved of the version to be published.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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