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Protective effect of allicin on ischemia-reperfusion injury caused by testicular torsion-detorsion in rats

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Abstract. – OBJECTIVE: Testicular ischemia-reperfusion induced by testicular torsion-detorsion increases the level of reactive oxygen species, leading to testicular damage. Allicin, one of the most active ingredients in garlic, is a significant exogenous antioxidant. In the research, the efficacy of allicin in treating testicular ischemia-reperfusion injury was assessed.

MATERIALS AND METHODS: The study included sixty Sprague-Dawley male rats. Three groups with 20 rats per group were created as follows: control group, testicular ischemia/reperfusion-induced group, and testicular ischemia-reperfusion plus treatment with allicin group. The control group underwent a sham operation of the left testis without other interventions. In the testicular ischemia/reperfusion-induced group, rat left testis was subjected to 720° torsion for two hours and then detorsion. In the allicin-treated group, in addition to testicular ischemia-reperfusion, 50 mg/kg of allicin was injected intraperitoneally, starting immediately following detorsion. Testicular tissue samples were obtained to measure the protein expression of xanthine oxidase, which is a major source of reactive oxygen species formation, malondialdehyde level (a reliable marker of reactive oxygen species), and testicular spermatogenic function.

RESULTS: Testicular ischemia-reperfusion significantly increased the expression of xanthine oxidase and malondialdehyde levels in ipsilateral testes while reducing testicular spermatogenic function. The expression of xanthine oxidase and malondialdehyde levels were significantly lower in ipsilateral testes, whereas testicular spermatogenic function in the allicin-treated group was significantly higher compared with those in the testicular ischemia-reperfusion group.

CONCLUSIONS: Our findings indicate that allicin administration improves ischemia/reperfusion-induced testicular damage by limiting reactive oxygen species generation via inhibition of xanthine oxidase expression.

Key Words:
Allicin, Testicular ischemia-reperfusion injury, Testicular torsion-detorsion, Rats, Xanthine oxidase, Reactive oxygen species, Testicular spermatogenic function.

Introduction

Testicular torsion is a urological emergent condition that commonly occurs in young males before 25 years of age[1,2]. It obstructs testicular blood supply and needs urgent surgical detorsion[3]. If not immediately treated, testicular torsion can lead to testicular death. Early surgical detorsion restitutes testicular blood flow and avoids testicular necrosis. Even though detorsion is performed promptly, 10.2%-73.3% of the cases develop into testicular atrophy[4-8]. Ischemia-reperfusion injury is considered to be the main mechanism that causes testicular atrophy[9]. Testicular ischemia-reperfusion leads to exaggerated production of reactive oxygen species, including hydrogen peroxide, superoxide anion, hydroxyl radical, singlet oxygen, hypochlorous acid, and nitric oxide[10,11]. A high concentration of reactive oxygen species induces lipid peroxidation in the cellular membrane, DNA impairment, and protein coagulation, ultimately leading to a reduction in cellular viability[12,13]. Rich unsaturated fatty acids of the testis make it sensitive to reactive oxygen species-related cellular injury[14].

To date, there is no effective clinical drug to ameliorate testicular ischemia-reperfusion injury. Garlic (*Allium sativum L.*) is a bulbous plant belonging to the Liliaceae family[15]. It is native to Central Asia and is widely cultivated in China, Mexico, Egypt, and some European countries[16,17]. Garlic has been widely used as a food, spice, and household remedy for approximately 5000 years[17-19]. Numerous clinical trials[20-28] have shown that garlic is advantageous for treating a variety of human

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diseases, including hypertension, wound, hyperlipemia, knee osteoarthritis, diabetic retinopathy, gingivitis, non-alcoholic fatty liver disease, rheumatoid arthritis, coronary artery disease, and so on. Allicin (diallyl thiosulfinate) is one of the most active ingredients in garlic[29]. Its molecular formula and molecular weight are $C_6H_{10}OS_2$ and 162.27, respectively[30,31]. Accumulated pharmacological data have demonstrated that allicin has a broad spectrum of properties, such as anti-oxidation, anti-inflammatory, antimicrobial, antiviral, antifungal, anti-parasitic, antitumor, antiplatelet aggregatory, anti-hypertensive, immunomodulatory, cardioprotective, nephroprotective, and neuroprotective activities[32-44]. Recent investigations[45-49] have revealed that allicin can attenuate ischemia-reperfusion injury in various organs, including the heart, liver, brain, kidney, and spinal cord. Nevertheless, there is no report regarding the effect of allicin on ischemia-reperfusion injury in the testis. Thus, the aim of the study was to assess the possible protective impact of allicin on ischemia/reperfusion-induced testicular damage using a rat model.

Materials and Methods

Laboratory Rats

Sixty male Sprague-Dawley rats of 8 weeks of age (weighing 250-300 g) were purchased from SLAC Laboratory Animal Company (Shanghai City, China). Animals were kept in a constant environment, including controlled temperature (21°C ± 1°C), 55% ± 5% relative humidity, and 12-hour light/dark schedule. Rats had unlimited access to a standard pellet chow and tap water. Animal experiments in the present study were reviewed and approved by the Experimental Animal Ethics Committee of Zhejiang Chinese Medical University, China (Approval No. 10790).

Rat Model of Testicular Torsion-Detorsion and Allicin Treatment

We randomly divided sixty rats into three groups (20 rats/group): (1) control group, (2) testicular ischemia-reperfusion group, and (3) testicular ischemia-reperfusion + allicin-treated group. To anesthetize rats, 50 mg/kg ketamine (Sigma Chemical Company, St. Louis, MO, USA) was injected intraperitoneally. The operative area was shaved and washed with 10% povidone-iodine solution. The scrotum was entered via an ilioinguinal incision at the left side. After the tunica vaginalis was opened, the left testis was moved outside the incision. In the control group, an atraumatic 11-0 silk suture was placed through the tunica albuginea. Afterward, the left testis was inserted into the scrotal sac, followed by an incision closing. In the testicular ischemia-reperfusion group, testicular ischemia was created by 720° counterclockwise twisting the left testis around the axis of the spermatic cord[50]. Testicular ischemia was kept by fixing the tunica albuginea of the testis to the dartos of the hemi-scrotum using atraumatic silk thread (11-0)[50]. After 120 minutes of ischemia, the fixing silk thread was removed, and the rotated testis was restored to its anatomical position to allow blood reperfusion[50]. In the testicular ischemia-reperfusion + allicin-treated group, an intraperitoneal dose of 50 mg/kg allicin (Targetmol Chemicals Inc., Wellesley Hills, MA, USA) was given immediately after reperfusion. The dose of allicin was chosen according to the results of previous studies[47-49]. After a four-hour reperfusion period, testicular tissues of 10 rats per group were excised to assess xanthine oxidase expression and malondialdehyde concentration. After three-months reperfusion period, testicular tissues of the remaining 10 rats per group were excised to determine testicular spermatogenic function.

Western Blot Assay for Xanthine Oxidase

Xanthine oxidase protein expression was assessed through the protocol as described previously[51]. Briefly, testicular tissue specimen was homogenized on ice for 15 minutes with lysis buffer, including 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 2 mM sodium orthovanadate, 5 μg/ml aprotinin, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. After centrifugation of the stirred sample at 14,000 × g and 4°C for 15 minutes, the supernatant was separated for the determination of protein. The concentration of protein was assessed by spectrophotometry using the Bradford kit (Bio-Rad Laboratories, Hercules, CA, USA). Total protein (30 μg) extracts in each sample were mixed with loading buffer and denatured by heating at 100°C for 3 minutes. Then, protein extracts were fractionated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature with 5% skimmed milk in Tris-Buffered Saline containing 0.1% Tween-20.

Afterward, overnight incubation of the membrane with the appropriate primary antibodies for anti-xanthine oxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (internal reference; Sigma Chemical Company) at 4°C was done. After the membrane was washed three times for 20 minutes each using Tris-Buffered Saline containing 0.1% Tween-20, horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for incubation with the membrane at room temperature for 1 hour. The process of washing the membrane was repeated, and enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to visualize protein band signals on the membrane. The quantification of band intensity was performed using image lab software (Bio-Rad Laboratories, Hercules, CA, USA). The intensity ratio of the xanthine oxidase protein band to the β-actin protein band represented a relative level of xanthine oxidase protein expression.

Malondialdehyde Concentration

Testicular tissue was homogenized in ice in malondialdehyde lysis buffer at a ratio of 1/10 (w/v). The homogenized product was centrifuged at 5,000 × g for 15 minutes at 4°C. The supernatant portion was kept for malondialdehyde assay. Malondialdehyde concentration in the testicular tissue was evaluated using a commercial biochemical kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing City, China), following the manufacturer's instructions. The testing principle of the kit is based on the reaction between malondialdehyde and thiobarbituric acid[52]. Malondialdehyde reacts with thiobarbituric acid at high temperatures to form a pink product. The maximum absorbance of the pink product at 532 nm wavelength was determined spectrophotometrically. The measured concentration of malondialdehyde was expressed as a nanomole per milligram of protein.

Evaluation of Testicular Spermatogenic Function

Testicular weight, the diameter of the seminiferous tubule, germinal cell layer number, and Johnsen's score were utilized to assess testicular spermatogenic function[53]. A testicular tissue sample was harvested from experimental rats, weighed on a scale, and preserved in Bouin's fixative for histopathological evaluation. Dehydration of the fixed tissue specimen was performed in increased alcohol series (80%, 95%, and 100%). The tissue specimen was cleared using multiple xylol washes, infiltrated using paraffin at 60°C, and then, embedded in paraffin as a cubic block. The embedded tissue was cut into sections of 5 μm thickness, and the section was placed on a glass slide. Subsequently, the tissue section was dewaxed by immersion in xylol, hydrated by immersion in descending grades of alcohol, and stained using the hematoxylin-eosin (H&E, Sigma Chemical Company, St. Louis, MO, USA) technique. Histological evaluation was done under a light microscope at ×200 magnification by a pathologist who was blinded to the experimental protocol. Analysis was performed on 20 randomly selected round seminiferous tubules in each sample. A microscope-adaptable micrometer was employed to measure the diameter of the seminiferous tubule. The germinal cell layer number was evaluated by counting the number of epithelial cell layers from the basal membrane to the lumen of the seminiferous tubule. The Johnsen's scoring method was performed to assess the appearance of spermatogenic cells in each seminiferous tubule[54]. Each tubule was assigned a Johnsen's score of 1-10. Normal spermatogenesis with many mature sperms, regular germinal epithelium, and an open tubular lumen was assigned a score of 10. The lack of tubular cells was assigned a score of 1.

Statistical Analysis

Continuous data were stated as arithmetical mean ± standard deviation. The GraphPad Prism statistical software, version 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the analysis of data. One-way analysis of variance was used for data comparisons among three groups along with the Student-Newman-Keuls post-hoc test. Comparisons between ipsilateral and contralateral testes within the group were carried out using a two-tailed Student *t*-test. Statistical significance was set at a p-value of < 0.05.

Results

Testicular Xanthine Oxidase Protein Expression

As shown in Figure 1, the ipsilateral testicular xanthine oxidase protein expression in testicular ischemia-reperfusion rats was increased significantly compared with the sham-operated control group ($p < 0.05$). The ipsilateral testicular xanthine oxidase protein expression was decreased

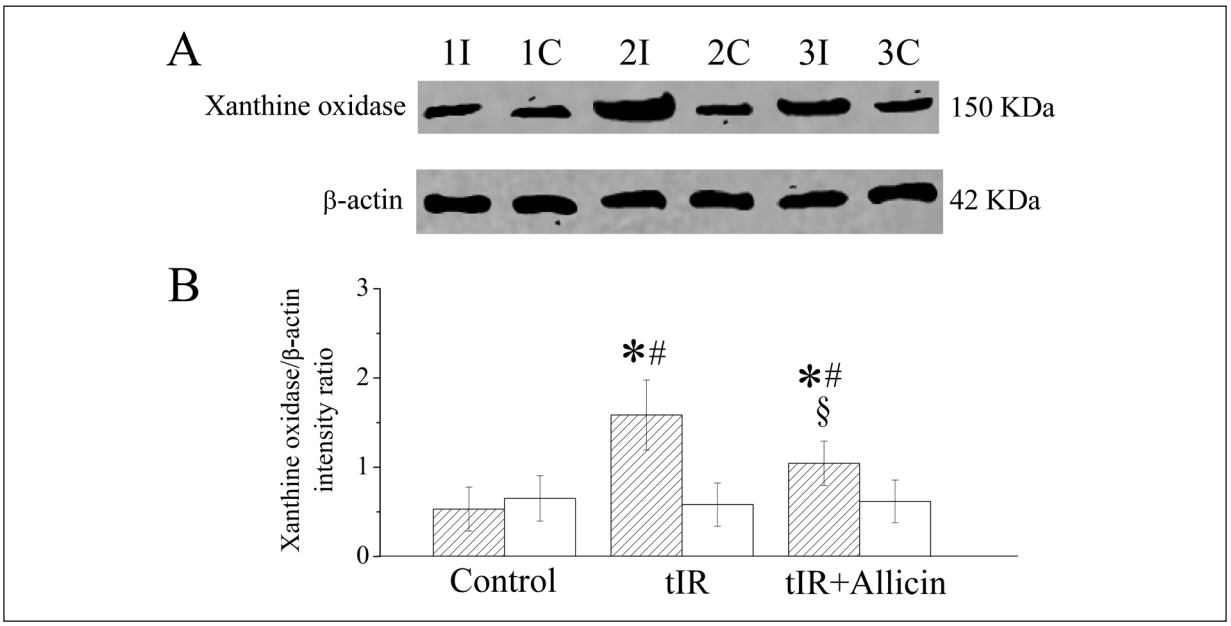

Figure 1. Effect of allicin treatment on protein expression of testicular xanthine oxidase in testicular ischemia-reperfusion (tIR) rat. (**A**) Representative Western blot picture of xanthine oxidase and β-actin (loading control) in testes. 1I and 1C, ipsilateral and contralateral testes in sham-operated control group; 2I and 2C, ipsilateral and contralateral testes in tIR group; 3I and 3C, ipsilateral and contralateral testes in allicin-treated group. (**B**) The intensity ratio of xanthine oxidase protein band to β-actin protein band represented a relative level of xanthine oxidase protein expression. Hatched columns: ipsilateral testes; open columns: contralateral testes. Continuous data were stated as arithmetical mean ± standard deviation (n = 10 rats in each group). $*p < 0.05$, significant difference compared to sham-operated control group; $^{\#}p < 0.05$, significant difference compared to contralateral testes in the same group; $^{\S}p < 0.05$, significant difference compared to ipsilateral testes in the tIR group.

significantly in allicin-treated rats compared with the testicular ischemia-reperfusion group ($p < 0.05$). The contralateral testicular xanthine oxidase protein expression was not significantly different among the three groups ($p > 0.05$).

Testicular Malondialdehyde Content

Figure 2 revealed a significant increase in ipsilateral testicular malondialdehyde content in the testicular ischemia-reperfusion group relative to the sham-operated control group ($p < 0.05$). The allicin-treated group showed decreased malondialdehyde content in ipsilateral testes compared to the testicular ischemia-reperfusion group ($p < 0.05$). None of the three groups indicated a significant difference in contralateral testicular malondialdehyde content ($p > 0.05$).

Testicular Spermatogenic Function

After testicular ischemia-reperfusion, the testicular weight, diameter of seminiferous tubule, germinal cell layer number, and Johnsen's score in ipsilateral testes decreased significantly ($p < 0.05$) compared with the sham-operated control group (Figures 3 and 4). These indexes in ipsilateral testes were significantly increased ($p < 0.05$) in the allicin-treated group compared with testicular ischemia-reperfusion rats. No significant difference was found among the three groups for these indexes in contralateral testes ($p > 0.05$).

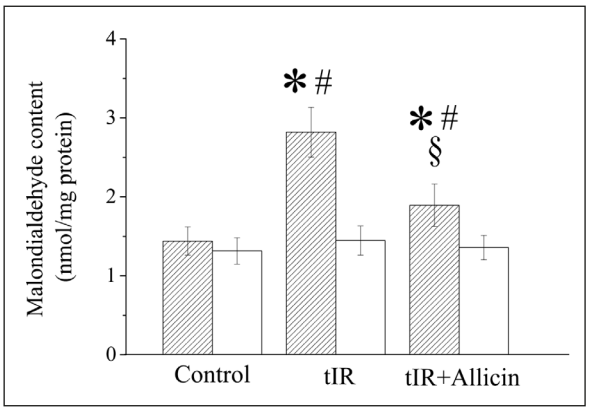

Figure 2. Effect of allicin treatment on testicular malondialdehyde content in testicular ischemia-reperfusion (tIR) rat. Hatched columns: ipsilateral testes; open columns: contralateral testes. Continuous data were stated as arithmetical mean ± standard deviation (n = 10 rats in each group). $*p < 0.05$, significant difference compared to sham-operated control group; $^{\#}p < 0.05$, significant difference compared to contralateral testes in the same group; $^{\S}p < 0.05$, significant difference compared to ipsilateral testes in the tIR group.

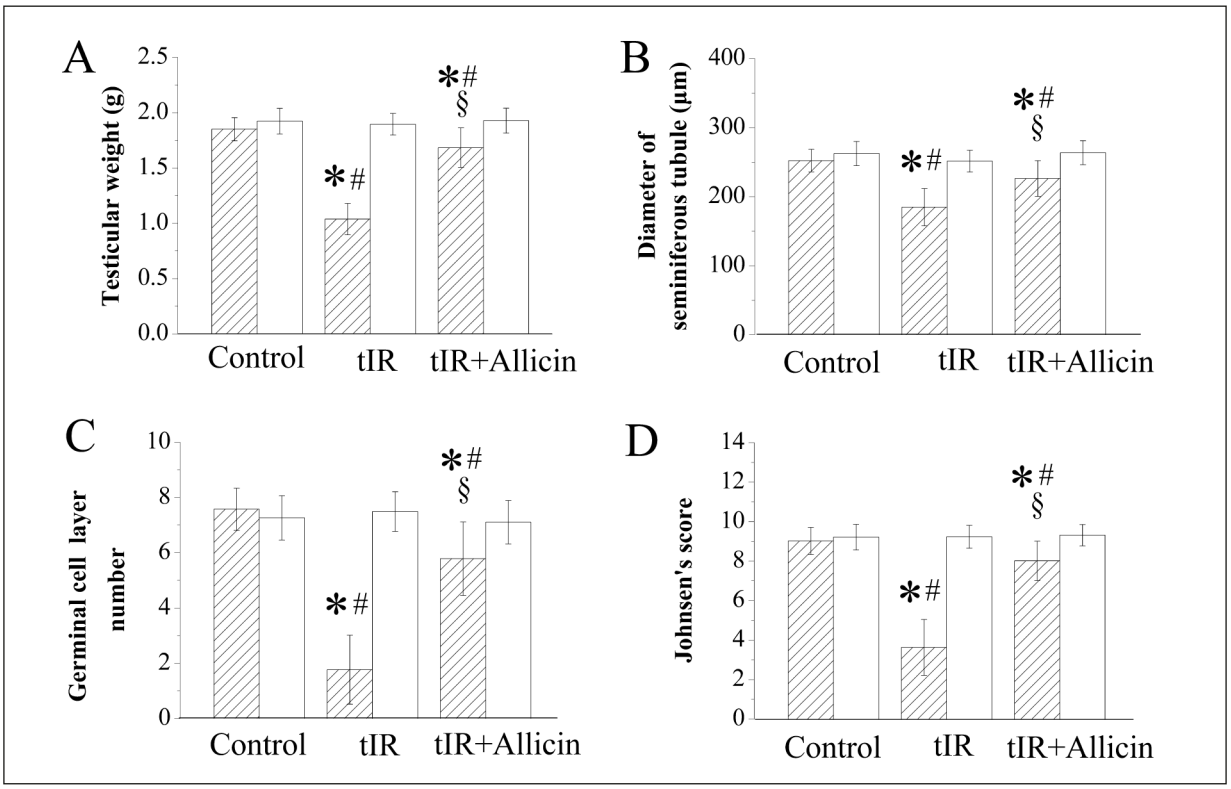

Figure 3. Effect of allicin treatment on (**A**) testicular weight, (**B**) diameter of seminiferous tubule, (**C**) germinal cell layer number, and (**D**) Johnsen's score in testicular ischemia-reperfusion (tIR) rat. Hatched columns: ipsilateral testes; open columns: contralateral testes. Continuous data were stated as arithmetical mean ± standard deviation (n = 10 rats in each group). *$p < 0.05$, significant difference compared to sham-operated control group; #$p < 0.05$, significant difference compared to contralateral testes in the same group; §$p < 0.05$, significant difference compared to ipsilateral testes in the tIR group.

Discussion

Testicular torsion occurs in 1 out of every 4,000 males < 25 years old and it is among urological emergencies that require prompt diagnosis and surgical detorsion[1,2]. If not corrected by surgical detorsion within 6 hours of onset, testicular torsion will result in infarction of testicular tissue[55]. However, it has been reported that 10.2%-73.3% of men with a timely surgical detorsion eventually undergo testicular atrophy[4-8]. In the present study, we found that 2-hour unilateral testicular torsion and 3-month testicular detorsion caused pathological effects in the spermatogenic function of ipsilateral testes, including a marked reduction in testicular weight, the diameter of the seminiferous tubule, germinal cell layer number, and Johnsen's score (Figure 3 and 4).

The damage of the ipsilateral testes after unilateral testicular torsion-detorsion is due to increased production of reactive oxygen species[10,11]. High levels of reactive oxygen species adversely affect cellular lipids, proteins, and nucleic acids and finally induce cellular irreversible injury[12,13]. Based on very reactivity of reactive oxygen species in nature and their extremely short half-life, direct quantitation of reactive oxygen species is very difficult[56]. Excessive formation of reactive oxygen species causes lipid peroxidation of testicular cells[57]. Malondialdehyde is a stable end product of lipid peroxidation that can indirectly reflect reactive oxygen species level[58]. Therefore, malondialdehyde is used as a reliable marker of reactive oxygen species[58]. In this study, there was a sharp reduction in spermatogenic function and a significant increase in malondialdehyde content in ipsilateral testes after testicular ischemia-reperfusion, relative to the control group (Figures 2-4). These results indicate that injury in spermatogenic function following testicular ischemia-reperfusion is attributed to increased production of reactive oxygen species. However, after allicin treatment, malondialdehyde content was markedly lower, and spermatogenic function was markedly higher in ipsilateral testes (Figures 2-4). These findings suggest that therapy with

allicin may improve spermatogenic function by alleviating oxidative stress. Clinical trials[59-64] of allicin have demonstrated that it is effective and safe in the treatment of recurrent aphthous ulceration, hypercholesterolemia, stage II oral submucous fibrosis, *Helicobacter pylori* infection, and Behcet's disease with mucocutaneous lesions. In our experimental study of rat testicular torsion-detorsion, allicin is a promising therapeutic drug for testicular ischemia-reperfusion injury. However, the molecular mechanism by which allicin alleviates oxidative stress is not clear.

Reactive oxygen species are generated from xanthine oxidase during tissular ischemia-reperfusion[65]. In the ischemic state, decreasing adenosine triphosphate (ATP) production leads to conversion from xanthine dehydrogenase to xanthine oxidase[66]. At the same time, the depletion of cellular ATP causes the formation of hypoxanthine[66]. When tissue is reperfused, restoring blood flow provides molecular oxygen[66]. Xanthine oxidase interacts with molecular oxygen and hypoxanthine to generate hydrogen peroxide and superoxide anion[67]. The reaction of hydrogen peroxide and superoxide anion can produce hydroxyl radicals in the presence of iron[68]. Hence, a high level of reactive oxygen species is produced in the ischemia-reperfusion stage. The present study found that testicular ischemia-reperfusion induced a significant increase in ipsilateral testicular xanthine oxidase expression and malondialdehyde content but a decrease in testicular spermatogenic function (Figures 1-4). These results demonstrate that the increase in xanthine oxidase expression following testicular ischemia-reperfusion results in the generation of huge amounts of reactive oxygen species, decreasing testicular spermatogenic function. Furthermore, allicin administration reduced ipsilateral testicular xanthine oxidase expression and malondialdehyde content accompanied by a significant increase in testicular spermatogenic function (Figures 1-4). Our data indicate that allicin ameliorates testicular ischemia-reperfusion injury by suppressing xanthine oxidase expression and attenuating the production of reactive oxygen species.

Whether unilateral testicular ischemia-reperfusion negatively affects the contralateral testis remains obscure. Some investigators found that unilateral testicular ischemia-reperfusion impaired the contralateral testis[69], while other investigators reported that no injury occurred in the contralateral testis[70,71]. In our study, contralateral testicular xanthine oxidase expression, malondialdehyde content, and spermatogenic function

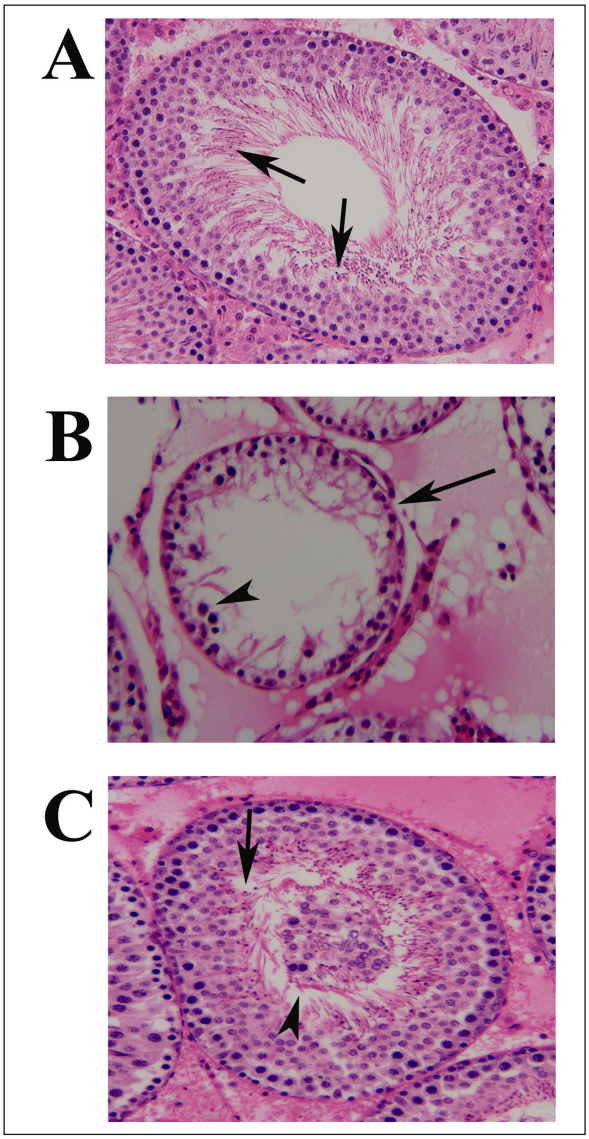

Figure 4. Histopathologic findings in the testes of control, testicular ischemia-reperfusion, and allicin-treated groups. The tissue sections of the testes were stained using the hematoxylin-eosin technique and evaluated under a light microscope at ×200 magnification. **A**, The ipsilateral testes in the control group and contralateral testes in the three groups exhibited normal morphology of the seminiferous tubule. In the seminiferous tubule, there were well-organized germinal epithelial layers and an open tubular lumen. The inner space of the seminiferous tubule was filled with mature sperms (arrows). **B**, Histopathological evaluation of ipsilateral testes from testicular ischemia-reperfusion group revealed an apparent reduction in the diameter of seminiferous tubule (arrow) and disfigured germinal epithelial layers (arrowhead), indicating spermatogenic arrest. No mature sperms were observed in the seminiferous tubule. **C**, The ipsilateral testes of the allicin-treated group showed near normal seminiferous tubular structure and germinal epithelial layers with the presence of many mature sperms (arrow). Nevertheless, some desquamated germinal epithelial cells (arrowhead) accumulated in the tubular lumen. These cells obstructed seminiferous tubule easily.

were not affected by unilateral testicular ischemia-reperfusion (Figures 1-4). Our findings indicate no damage in the contralateral testis after unilateral testicular ischemia-reperfusion.

Conclusions

This is the first experimental study to reveal the beneficial effect of allicin on ischemia/reperfusion-induced testicular injury. Allicin ameliorates testicular tissue damage by reducing xanthine oxidase expression to diminish the production of reactive oxygen species. The outcomes of our study show that allicin is a valuable drug for mitigating testicular ischemia-reperfusion injury.

Authors' Contributions

Si-Ming Wei conceived and designed the study. Si-Ming Wei and Yu-Min Huang contributed to the experimental study. Si-Ming Wei and Yu-Min Huang collected and analyzed the data. Si-Ming Wei and Yu-Min Huang wrote the manuscript. Si-Ming Wei offered a critical revision of the manuscript. All authors read and approved the final manuscript.

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Ethics Approval

Animal experiments in the present study were reviewed and approved by the Experimental Animal Ethics Committee of Zhejiang Chinese Medical University, China (Approval No. 10790).

Informed Consent

Not applicable.

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Data Availability

All data generated or analyzed during this study are included in this article.

Conflict of Interest

The authors declare that they have no conflict of interest to disclose.

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