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Original Research

Long-term protective effect of tadalafil on spermatogenesis following testicular ischemia-reperfusion injury in a rat model

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Abstract

Background: Testicular torsion is a urologic emergency that can lead to testicular atrophy and infertility owing to ischemia-reperfusion injury (IRI). The aim of this study was to evaluate the long-term protective effect of tadalafil, a phosphodiesterase-5 inhibitor used to treat erectile dysfunction, on spermatogenesis in a rat testicular model of IRI. **Methods**: Forty-eight adolescent Sprague–Dawley rats were divided into six groups of 8 each (A–F). Sham operation was performed on group A. Group B underwent surgical 720° torsion of the left testis without any medication. Groups C, D, E and F underwent surgical torsion and administration of tadalafil at varying doses (0.3 and 1.0 mg/kg) and durations (single or daily administration for four weeks). After three hours of torsion, detorsion was performed on all groups except group A. Four weeks after the operation, both testes were evaluated for spermatogenesis using the Johnsen scoring system. To evaluate the protective effect of tadalafil against oxidative stress induced by IRI, the malondialdehyde and superoxide dismutase levels of both testes were analyzed four hours after detorsion using the same experimental protocol as for groups A, B, and C. **Results**: Experimental groups treated with high-dose tadalafil showed higher Johnsen scores for spermatogenesis than the low-dose groups. Groups that received daily tadalafil administration for four weeks showed higher Johnsen scores than those receiving single doses. Furthermore, histopathologic findings and molecular markers related to oxidative stress were markedly improved following tadalafil administration. **Conclusions**: Tadalafil alleviated the oxidative stress and long-term deterioration of spermatogenesis in a rat testicular model of IRI by restoring the antioxidant status.

Keywords: Spermatic cord torsion; Ischemia-reperfusion injury; Phosphodiesterase-5 inhibitors; Animal model; Spermatogenesis

1. Introduction

Testicular torsion is a urologic emergency caused by twisting of the spermatic cord, leading to a reduction in blood flow, arterial obstruction, ischemia and gonadal necrosis [1,2]. It is one of the most common genital diseases and has been estimated to afflict 1 in 4000 adolescent boys aged <25 years [3]. Testicular torsion should therefore be corrected immediately to prevent ipsilateral testicular dysfunction and infertility later in life [4]. Surgical intervention by counter-rotating the testis is the standard treatment method, but can result in ischemia-reperfusion injury (IRI) [5–13]. This can in turn impair spermatogenesis and trigger the production of reactive oxygen species (ROS) [6,14–17].

Salvage rates for testicular torsion have been reported as 30% to 50%, but impaired spermatogenesis occurs in most patients despite considerable efforts [16,17]. Other than surgical reduction, a standard treatment for testicular torsion-induced injury has yet to be developed. Ideally, therapeutic candidates aimed at preventing complications from testicular torsion should attenuate ischemic injury, promote spermatogenesis, and prevent any harmful immune response. Several experimental studies have reported some short-term benefits from various therapeutic agents in terms of reducing oxidative stress. However, the appropriate pharmacologic treatments that could provide long-term benefits for spermatogenesis have yet to be identified.

Phosphodiesterase-5 inhibitors (PDE5is) are commonly used in current urological practice to treat erectile dysfunction [18]. Inhibition of phosphodiesterase enzymes leads to increased tissue cyclic guanosine monophosphate (cGMP) levels, resulting in smooth muscle relaxation. The loosening of smooth muscles in the vessel by PDE5is increases local perfusion, dilates the vessels, and inhibits platelet aggregation. Moreover, some studies have suggested that PDE5is help to prevent IRI [19,20]. Findings

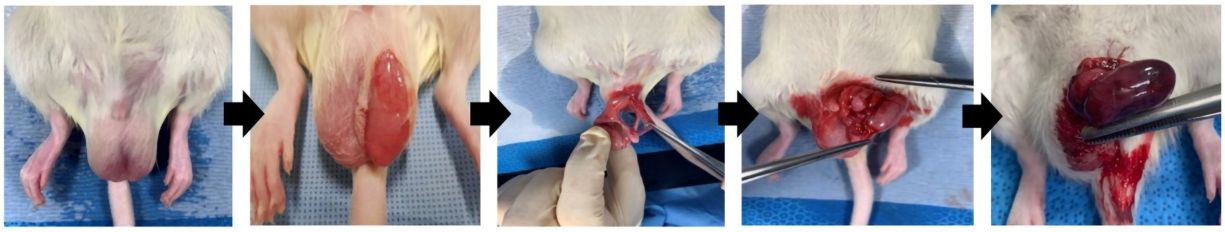

Fig. 1. Testicular torsion experiment. After the left scrotal incision, unilateral testicular torsion on the left is created by a 720° clockwise rotation, followed by fixation to the scrotal wall using a 4-0 silk suture.

from animal models suggest that PDE5i has a protective effect against IRI by regulating the antioxidant activity in several organs [6,20–22]. Recent studies have also investigated the protective effect of several PDE5i agents, including sildenafil, tadalafil, and vardenafil, in a rat testicular torsion model [23–29]. Amongst these, tadalafil has often been used as long-term maintenance therapy, with once-daily administration to maintain a steady-state blood concentration. However, only a few studies have focused on the long-term protective effect of tadalafil on spermatogenesis following IRI.

In the present work we therefore evaluated the protective effect of tadalafil on long-term spermatogenesis in a rat testicular torsion-induced IRI model.

2. Materials and methods

This study was approved by the Institutional Animal Care and Use Committee of the College of Medicine in Yeungnam University (YUMC-AEC2018-029). All surgeries were performed under anesthesia and all possible efforts were made to minimize suffering.

In total, 72 healthy 6- to 8-week-old male Sprague–Dawley rats were used in the study. This age corresponds with the adolescent period in humans [30]. A one-week acclimatization period was allowed before any surgical procedure. All rats had free access to food and water under a 12-hour light/dark cycle. They were anesthetized via one-time intramuscular injection of Rumpun and Zoletil, with the addition of one-third of the initial dose if adequate anesthesia was not attained. The rats were then euthanized using carbon dioxide gas or cervical dislocation. In a previous study with a rat testicular torsion model, we found that testicular damage was aggravated in an ischemic time-dependent manner, and that serious deterioration of spermatogenic activity developed three hours after ischemia [31]. Therefore, three hours of ischemia was selected as the torsion duration period for the present study. The left testes of the rats underwent surgical torsion for three hours, followed by subsequent detorsion. After left scrotal incision, unilateral testicular torsion was created by a 720° clockwise rotation of the left testis, followed by fixation to the scrotal wall using a 4-0 silk suture (Fig. 1). After remaining in a torsion state for three hours, the testis was counter-rotated to its natural position and reinserted into the scrotum.

To evaluate the long-term effects of tadalafil on spermatogenesis, 48 healthy rats (eight per group) were randomly allocated into six groups (A–F) as follows:

Group A: sham operation (surgical incision without testicular torsion/detorsion)

Group B: 3-hour torsion/detorsion (control group)

Group C: 3-hour torsion/detorsion with single intraperitoneal injection of low-dose tadalafil (0.3 mg/kg dissolved in 0.9% sodium chloride) one hour before detorsion

Group D: 3-hour torsion/detorsion with single intraperitoneal injection of low-dose tadalafil (0.3 mg/kg dissolved in 0.9% sodium chloride) one hour before detorsion, followed by once daily oral administration of 0.3 mg/kg tadalafil dissolved in 0.9% sodium chloride for four weeks

Group E: 3-hour torsion/detorsion with single intraperitoneal injection of high-dose tadalafil (1.0 mg/kg dissolved in 0.9% sodium chloride) one hour before detorsion

Group F: 3-hour torsion/detorsion with single intraperitoneal injection of high-dose tadalafil (1.0 mg/kg tadalafil dissolved in 0.9% sodium chloride) one hour before detorsion, followed by once daily oral administration of 1.0 mg/kg tadalafil dissolved in 0.9% sodium chloride for four weeks

For all rats, bilateral orchiectomy and testicular tissue sampling were performed four weeks after the 3-hour torsion/detorsion procedures in order to evaluate spermatogenesis.

In addition, we evaluated the protective effect of tadalafil against oxidative stress by randomly allocating 24 healthy rats (eight per group) into three groups as follows (G–I):

Group G: sham operation (surgical incision without testicular torsion/detorsion)

Group H: 3-hour torsion/detorsion (control group)

Group I: 3-hour torsion/detorsion with single intraperitoneal injection of low-dose tadalafil (0.3 mg/kg dissolved in 0.9% sodium chloride) one hour before detorsion

All rats in groups G–I underwent bilateral orchiectomy and testicular tissue sampling for histopathologic and molecular analysis at four hours after the 3-hour torsion/detorsion procedures.

The excised testes were embedded in paraffin and stained with hematoxylin and eosin. Histopathologic analysis using a light microscope was conducted independently

Table 1. Johnsen scoring system for testicular damage evaluation.

Johnsen score	Description of histologic criteria
10	Full spermatogenesis
9	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium
8	Less than five spermatozoa per tubule, few late spermatids
7	No spermatozoa, no late spermatids, many early spermatids
6	No spermatozoa, no late spermatids, few early spermatids
5	No spermatozoa or spermatids, many spermatocytes
4	No spermatozoa or spermatids, few spermatocytes
3	Spermatogonia only
2	No germinal cells, Sertoli cells only
1	No seminiferous epithelium

by a single pathologist who was blinded to the identity of the experimental groups. The Johnsen scoring system was used to evaluate and compare long-term spermatogenesis between groups A to F [32]. The Johnsen score for each testis was the average value for at least 10 seminiferous tubules. The severity of germ cell injury was classified by the Johnsen score from 1 to 10 points (Table 1).

To analyze and compare the protective effect of tadalafil against oxidative stress in groups G to I, ELISA was used to measure malondialdehyde (MDA) and superoxide dismutase (SOD) levels in both testes four hours after detorsion. Molecular analyses of MDA and SOD used the ratio of left to right testes.

The lipid peroxidation assay kit (BioVision, K739–100, Milpitas, CA, USA) was used for MDA assessment in accordance with the manufacturer's instructions. In brief, 10 mg of testicular tissue was homogenized in MDA (lysis buffer with 3 μL BHT (100×), followed by centrifugation at 13,000× g for 10 minutes to obtain the supernatant. TBA reagent (600 μL) was then added to each sample and the mixture incubated at 95 °C for 60 minutes. After cooling with ice, 300 μL of n-butanol was added and the mixture was centrifuged at 16,000× g for three minutes. N-butanol was then removed and the MDA–TBA adduct placed into a 96-well plate. Absorbance was measured at 532 nm and the MDA content calculated using MDA reference standards.

SOD activity was analyzed using a commercially available kit (Cayman Chemicals, Ann Arbor, MI, USA, Cat. no. 706002). Briefly, approximately 100 mg of testicular tissue was homogenized in 20 mM of HEPES buffer containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. After homogenization, the homogenates were centrifuged at 1500× g for five minutes at 4 °C. The resulting supernatant was diluted with sample buffer (10 × diluted) 50-fold to give absorbances that were within the linear range of the standard curve. The absorbance was read at 450 nm and analysis was carried out according to the manufacturer's instructions.

One-way analysis of variance followed by Tukey's post-hoc test was used for the statistical analyses. The results were representative of at least three experiments, with all values expressed as mean ± SD. p-values of less than 0.05 were considered significant.

3. Results

Fig. 2 shows representative gross findings for the extracted left testes and comparison of the left/right testes ratio for mean maximum longitudinal length between groups A to F. The left/right testes longitudinal length ratio was significantly higher in groups D (0.385 ± 0.040), E (0.472 ± 0.094) and F (0.510 ± 0.010) compared to group B (0.313 ± 0.063). Moreover, tadalafil attenuated testicular atrophy following testicular IRI in a dose- and time-dependent manner.

Fig. 3 shows the histopathologic findings and Johnsen scores for groups A–F. Although the testes in group A showed normal morphology, those in group B showed serious testicular damage, including severe edema, apoptotic bodies, venular ectasia, and lobular coagulative necrosis. In contrast, groups C–F showed improved histopathologic features compared to those seen in group B, again depending on the dose and duration of tadalafil. The mean Johnsen scores for groups A, B, C, D, E, and F were 10 ± 0, 2.3 ± 1.2, 4.1 ± 2.9, 5.3 ± 2.3, 4.4 ± 1.8, and 7.4 ± 1.6, respectively. The mean Johnsen scores for groups D, E, and F were significantly higher compared to that of group B (p = 0.005, p = 0.013, and $p < 0.001$, respectively). Moreover, the mean Johnsen scores were higher in the high-dose tadalafil groups compared to the low-dose tadalafil groups (group C vs. group E: (4.1 ± 2.9) vs. (4.4 ± 1.8), p = 0.836; group D vs. group F: (5.3 ± 2.3) vs. (7.4 ± 1.6), p = 0.047). With regards to the duration of tadalafil administration, groups that received daily administration for four weeks showed higher Johnsen scores compared to groups that received single injection only (group C vs. group D: (4.1 ± 2.9) vs. (5.3 ± 2.3), p = 0.396; group E vs. group F: (4.4 ± 1.8) vs. (7.4 ± 1.6), p = 0.003). The spermatogenic activity of the right testes did not deteriorate in any of the rats and no significant differences in the right testes were observed between any of the groups.

Histologic evaluation of the testicular tissues revealed normal tubular structures within the interstitium in group

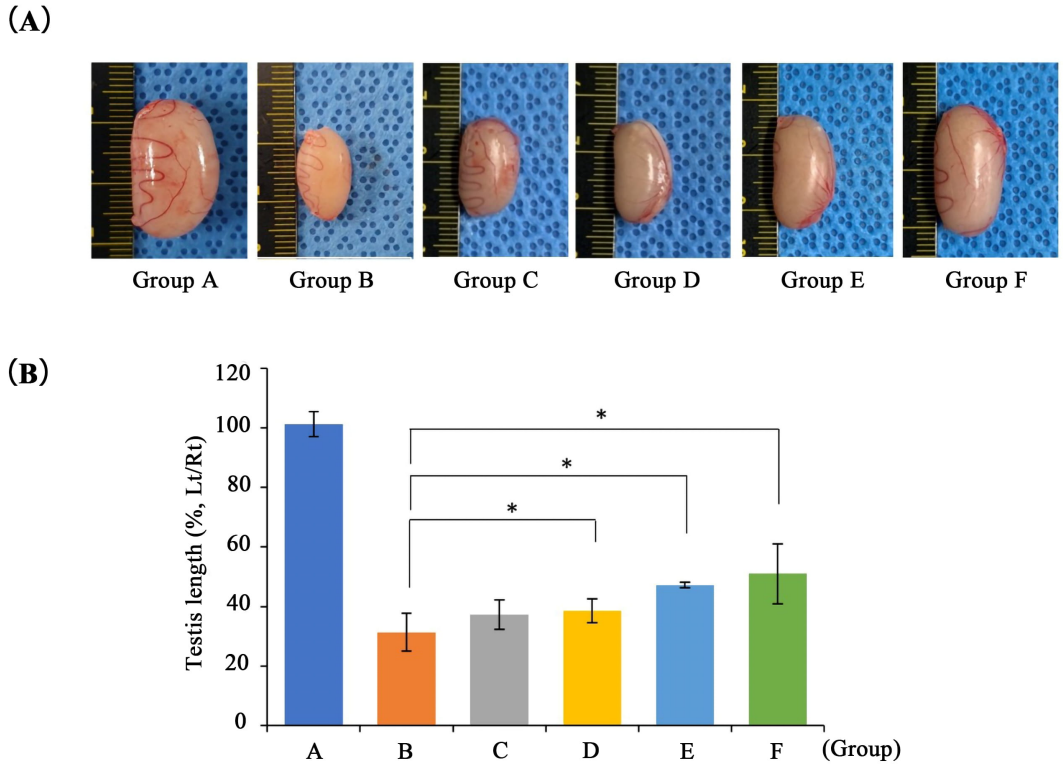

Fig. 2. Representative gross images of the extracted left testes (A) and comparison of the mean maximum longitudinal length ratios of the left and right testicles (B) in groups A to F (*$p < 0.05$). Group A: sham operation; Group B: torsion/detorsion (control group); Group C: torsion/detorsion with single administration of 0.3 mg/kg tadalafil; Group D: torsion/detorsion with daily administration of 0.3 mg/kg tadalafil for four weeks; Group E: torsion/detorsion with single administration of 1.0 mg/kg tadalafil; Group F: torsion/detorsion + daily administration of 0.3 mg/kg tadalafil for four weeks.

G. On the other hand, serious degradation was observed in group H including disorganization of tubular epithelium, interstitial edema, hemorrhage, and less distinct seminiferous tubule borders. In group I, tadalafil administration mitigated the deterioration of tubular histology compared with the findings in group H. Moreover, the levels of oxidative stress-related molecular markers (MDA and SOD) differed amongst groups G–I (Fig. 4). The MDA level was significantly higher in group H (177.3 ± 34.5 %) compared to group G (93.5 ± 23.7 %, $p < 0.001$) or group I (107.3 ± 38.4 %, $p = 0.002$). The SOD level was significantly lower in group H (79.2 ± 10.0 %) compared to group G (102.3 ± 7.5 %, $p < 0.001$) or group I (115.5 ± 9.5 %, $p < 0.001$). In summary, the molecular markers related to oxidative stress and the histopathologic findings both showed marked improvement after tadalafil administration.

4. Discussion

In this study, we investigated the protective effect of tadalafil on a rat testicular torsion-induced model for IRI. Tadalafil attenuated the oxidative stress caused by testicular torsion. Moreover, it had a protective effect against testicular IRI for long-term spermatogenesis in a time- and dose-dependent manner. To the best of our knowledge, this is the first study to report on the protective effect of PDE5i on testicular IRI in terms of the dose and duration. The clinical implication of our data is that tadalafil administration to patients with testicular torsion may have both early and long-term benefits against IRI.

Twisting of the spermatic cord, or testicular torsion, is a critical urologic condition in adolescent men that requires prompt diagnosis. Emergency surgical detorsion has been the standard management to prevent histologic damage and protect reproductive ability [2]. Several studies have identified possible mechanisms for the progression of this condition to testicular atrophy and altered spermatogenesis [5–12]. The main pathophysiologic consequences of testicular torsion are ischemia and reperfusion caused by twisting of the spermatic cord followed by detorsion [13]. Reperfusion after a period of ischemia generates toxic ROS. Subfertility after unilateral testicular torsion develops in nearly 40% of cases, while semen analysis at long-term follow-up can show abnormalities in more than half of patients with testicular torsion [4,33]. Unfortunately, there is currently no established standard treatment for testicular torsion-induced injury other than surgical detorsion. Hence, new therapeutic approaches are required to attenuate the related pathologic consequences. Although several therapeutic agents

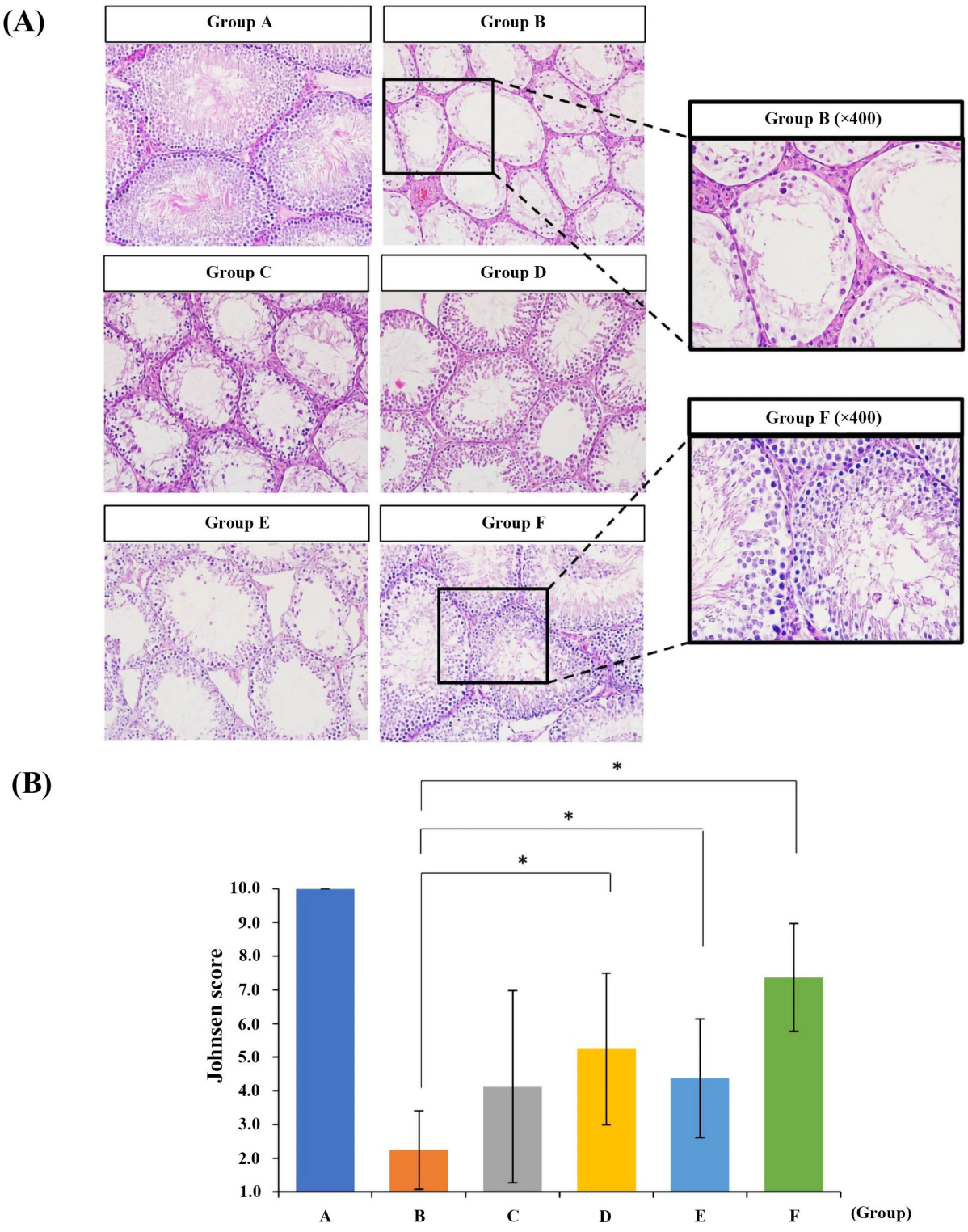

Fig. 3. Representative histopathologic images of the left testes (A) and the Johnsen scores (B) in groups A–F (n = 8 in each group). The magnification of the image is 200× (the magnified image of group B and F is 400×). Group A: sham operation; Group B: torsion/detorsion (control group); Group C: torsion/detorsion with single administration of 0.3 mg/kg tadalafil; Group D: torsion/detorsion with daily administration of 0.3 mg/kg tadalafil for four weeks; Group E: torsion/detorsion with single administration of 1.0 mg/kg tadalafil: Group F: torsion/detorsion + daily administration of 0.3 mg/kg tadalafil for four weeks (*$p < 0.05$).

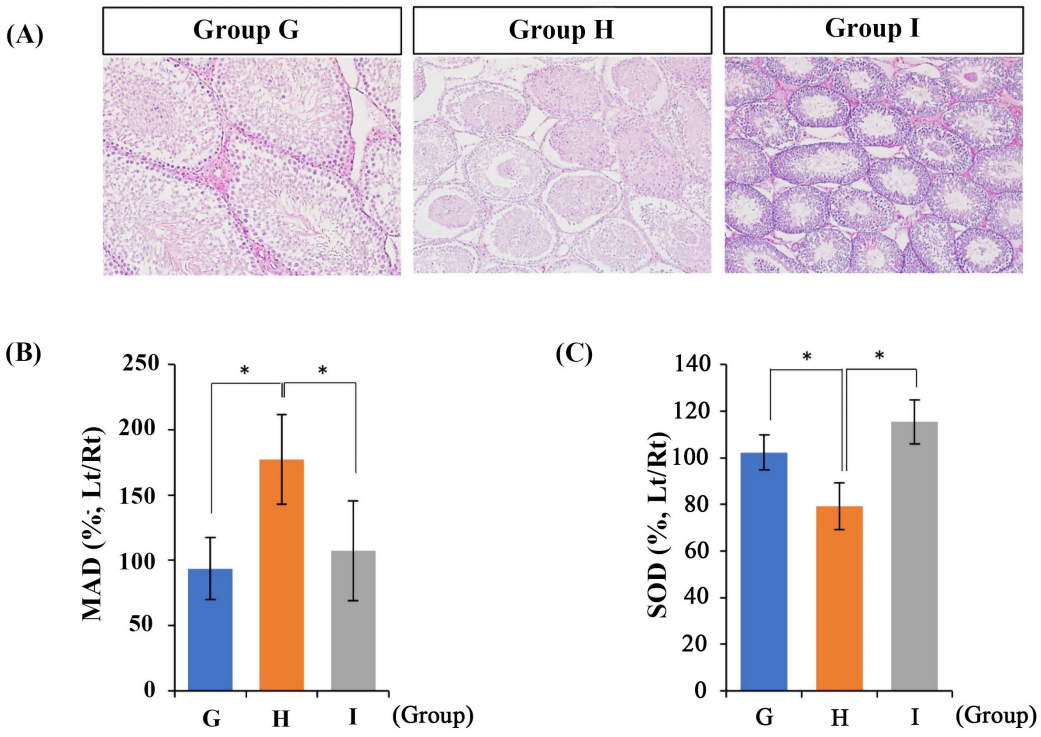

Fig. 4. Representative histopathologic images of the left testes (A) and the oxidative stress-related molecular markers, such as MDA (B) and SOD (C) in groups G–I (n = 8 in each group). The magnification of the image is 100×. Group G: sham operation; Group H: torsion/detorsion (control group); Group I: torsion/detorsion with single administration of 0.3 mg/kg tadalafil one hour before detorsion (*$p < 0.05$).

have shown some benefit in reducing testicular IRI, more suitable pharmacologic treatments must be developed for clinical application.

Several studies using animal models of torsion-induced IRI have attempted to suppress testicular damage through medication and surgical procedures, such as post-conditioning [24–29,34–44]. However, these earlier studies focused mainly on the early changes in molecular markers and on histopathologic findings within hours after IRI. Most studies that evaluated PDE5i as an attenuating medication used similar experimental methods of torsion lasting for 2–4 hours, followed by a detorsion period within four hours before animal sacrifice for bilateral orchiectomy and biochemical and histopathologic analyses. In those studies, oxidative stress biochemical markers such as MDA, SOD and glutathione peroxidase were measured because of their effects in the early phase (i.e., several hours). Although the early phase after detorsion is an appropriate time to assess changes in these factors, its accuracy for the assessment of spermatogenesis is questionable. Testicular torsion generally occurs during puberty, however it is more meaningful to assess spermatogenesis in terms of fertility during adulthood. Moreover, testicular torsion results in testicular epithelial tissue degeneration through apoptosis secondary to ischemia and reperfusion [36]. This composite pathologic cascade is responsible for the testicular atrophy and impaired spermatogenesis observed at later stages. In an animal study, the equivalent adolescence period is therefore desirable for modeling testicular IRI and for subsequent assessment of the histopathologic changes pertaining to spermatogenesis. Previously, we conducted a study on adolescent rats to identify the ischemic duration that would result in serious long-term histopathologic changes after testicular IRI. Histopathologic analysis performed four weeks after testicular IRI confirmed there was serious deterioration in spermatogenic activity after three hours of ischemia, but not after shorter ischemic durations [31]. For the current study we therefore selected three hours of ischemia as the duration of torsion in order to study the long-term effects on spermatogenesis.

Tadalafil is one of the PDE5i frequently used to treat erectile dysfunction. It can maintain a steady-state concentration in blood to repair damaged endothelium and improve erectile function, whereas sildenafil, vardenafil and udenafil are more rapidly absorbed. Therefore, tadalafil is usually taken once daily as a long-term maintenance therapy [18,19]. Inhibition of the PDE5 enzyme can increase cGMP levels and ultimately result in smooth muscle relaxation [18,19]. Several studies using experimental models have reported protective effects of PDE5i therapy against IRI in several organs, including the kidney, heart, intestine, and lung [19,20]. These effects were mediated by inhibi-

tion of inflammation, ROS, and apoptosis. Several other studies also reported that various types of PDE5is may have a protective effect on testicular torsion-induced IRI in animal models [23–29,42]. However, the reported effects of PDE5is on testicular torsion in animal models have been contradictory. In agreement with our results, studies by Ozmerdiven *et al*. [25], Ameli *et al*. [26], Yildirim *et al*. [27] and Wu *et al*. [42] all showed beneficial effects of tadalafil administration in a testicular IRI rat model. They reported increased antioxidant enzyme levels, decreased lipid peroxidation levels, germ cell apoptosis, and attenuated histopathological results in the early phase. Similar results were reported by other studies that investigated the efficacy of sildenafil and vardenafil [23,24,29,45]. On the other hand, some studies have shown controversial results on the effects of PDE5is after testicular IRI. Ustun *et al.* [39] reported that sildenafil and vardenafil caused exaggerated testicular apoptosis and increased nitric oxide synthase levels after one hour of ischemia and two hours of reperfusion injury. In addition, Istanbulluoglu *et al.* [28] suggested that vardenafil worsened the histopathologic changes related to oxidative stress and had no protective effect on testicular IRI in a pig model. We believe the conflicting outcomes reported for PDE5i in testicular IRI may be attributable to the different torsion and reperfusion times, doses of PDE5is, and animal species used in the studies. Although it has been well documented that changes in the testes depend on the duration of torsion, the exact timing of serious testicular deterioration was unclear in previous studies. The present study established three hours as the torsion duration associated with serious damage, while also confirming the protective effect of tadalafil based on an established torsion-induced IRI rat model.

Oxidative stress is caused by an imbalance between the production of oxidants and the level of scavenger, which is part of the defense system. Under normal conditions, ROS is maintained at physiologically low levels in tissues by the endogenous antioxidant defense system, However, an increase in oxidative stress, such as occurs during the course of testicular ischemia and reperfusion, causes ROS generation to exceed the capacity of the defense mechanisms. This contributes to either reversible or irreversible cell injury [46,47]. Several studies have established that testicular torsion-induced IRI increases oxidative stress and decreases the antioxidant scavenger [23–29,42]. MDA is a stable end product of lipid peroxidation generated by ROS and is often used as an indirect indicator of oxidative damage [48,49]. On the other hand, SOD is a key component in cell growth, differentiation, and protection and acts as one of the enzymatic antioxidant defense systems against ROS and oxidative damage [47]. The present results demonstrate that tadalafil can protect spermatogenic activity in the late phase, as well as attenuate these oxidative stress markers in the early phase. This may be explained by the antioxidant properties of tadalafil in a testicular torsion-induced IRI model [19]. Our results are consistent with those of previous studies that investigated the efficacy of tadalafil [25–27,42]. Notably, our study demonstrated that tadalafil can attenuate histopathologic damage following testicular torsion-induced IRI in a time- and dose-dependent manner and in the late phase. Although further studies are needed to elucidate the exact mechanism, our study supports the hypothesis that tadalafil has preventive effects against testicular injury that are dependent on the dose used and on the duration of exposure. Moreover, tadalafil is amenable to dose escalation and long-term use. We believe the preventive effect of tadalafil is due to its persistent antioxidant properties as an efficient scavenger of free radicals.

The findings regarding damage to the contralateral testis after IRI are unclear. Some studies using experimental models reported contralateral testis impairment following ipsilateral testicular IRI [50,51]. The exact mechanism of contralateral damage is not fully understood, but decreased testicular blood flow by reflex sympathetic response, ROS overproduction, and autoimmunization against the spermatogonia have all been proposed [52]. However, several studies have reported no effects of testicular torsion on the contralateral testis [53,54]. Recently, Ozgur *et al*. [24] and Unsal *et al*. [38] observed no pathologic changes in the contralateral testis after ipsilateral testicular IRI, which is consistent with the present findings. The discordant results may be due to the use of different animal models and assessment methods for contralateral damage to the testis. Future studies using standardized animal models should clarify this issue.

Our study had several limitations that should be considered. Tadalafil has a relatively long duration of action and a longer half-life compared to the other PDE5is. Moreover, the recommended initial dose of tadalafil for maintenance therapy in humans is 2.5–5 mg and this can be increased to 20 mg. The high level dosage selected for humans was 0.3 mg/kg tadalafil. Although 1.0 mg/kg tadalafil is a fairly high dose, it was set at this level in order to experimentally confirm the effect of dose escalation. Even though the efficacy was greater at 1 mg/kg compared to 0.3 mg/kg, these doses do not fall within the recommended clinical use limits for humans. Further studies are therefore needed to elucidate the optimal dose and duration of tadalafil administration, while limiting possible adverse effects. There is also concern regarding the long-term use of PDE5is, with a report showing that chronic administration of tadalafil in rats resulted in degenerative changes in the testis and a decline in semen parameters [55]. Therefore, additional studies are required on the possible long-term adverse effects of tadalafil on normal and pathologic testes. Finally, further studies are needed to clarify the mechanism of chronic tadalafil use and to establish a protocol that is clinically applicable for humans.

5. Conclusions

To our knowledge, this is the first study to investigate the effect of varying doses and treatment durations of PDE5is in relation to testicular IRI. In a rat model of testicular torsion-induced IRI, we demonstrated that tadalafil can attenuate histopathological changes and oxidative stress by restoring the antioxidant status. Furthermore, the protective effect of tadalafil against testicular IRI was time- and dose-dependent in regard to its long-term benefit for spermatogenesis. The clinical implication of our results is that PDE5is administration to patients with testicular torsion may provide early and long-term benefits. Further modifications to this promising approach may allow its clinical use in the near future.

Abbreviations

IRI, Ischemia-reperfusion injury; ROS, Reactive oxygen species; PDE5is, Phosphodiesterase-5 inhibitors; cGMP, Cyclic guanosine monophosphate; MDA, Malondialdehyde; SOD, Superoxide dismutase; ELISA, Enzyme-linked immunosorbent assay.

Author contributions

These should be presented as follows: JNL, TGK designed the research study. BK, EHL, BHY performed the research. JYC, PHS provided help and advice on the animal study. GSY, SYC, JWC, YSH, BSK analyzed the data. BK, EHL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal studies were performed after receiving approval from the Institutional Animal Care and Use Committee of the College of Medicine in Yeungnam University (YUMC-AEC2018-029).

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Conflict of interest

The authors declare no conflict of interest.

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