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Rapamycin protects testes against germ cell apoptosis and oxidative stress induced by testicular ischemia-reperfusion

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ABSTRACT

Objective(s):Rapamycin is an immunosuppressant compound with a broad spectrum of pharmacological activities. In recent years, it has been used successfully to decrease ischemia-reperfusion injury in several organ systems. The purpose of the present study was to examine the effect of rapamycin on testicular ischemia-reperfusion injury.

Materials and Methods: Seventy-two adult male Wistar rats were divided into six groups: control (group1), sham-operated (Group2), T/D + DMSO as vehicle group (group3), and groups 4–6; respectively received 0.5, 1, and 1.5 $mgkg^{-1}$ of rapamycin , IP 30 min before detorsion. Ischemia was achieved by twisting the right testis 720° clockwise for 1 hr. The right testis of 6 animals from each group were excised 4 hr after detorsion for the measurement of lipid peroxidation, caspase-3, and antioxidant enzyme activities. Histopathological changes and germ cell apoptosis were determined by measuring mean of seminiferous tubules diameters (MSTD) and TUNEL test in right testis of 6 animals per group, 24 hr after detorsion.

Results: Testicular T/D caused increases in the apoptosis, malondialdehyde (MDA), and caspase-3 levels and decreases in the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in ipsilateral testis (P<0.001). The rats treated with rapamycin had significant decreases in the MDA and caspase-3 levels and significant increases in the SOD, CAT and GPx activities in ipsilateral testis compared with the T/D group (P<0.001); germ cell apoptosis was decreased, and MSTD was improved.

Conclusion: Rapamycin administration during testicular torsion decreased ischemia/reperfusion (I/R) cellular damage.

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Introduction

Testicular torsion is a true urologic emergency and a delay in its diagnosis and management can lead to loss of the testicle. Torsion is the most common cause of testicle loss in newborns, children and adolescent boys (1). Testicular torsion/detorsion (T/D) causes morphological and biochemical changes by I/R injury of the testicular tissue. This I/R injury is associated with over generation of reactive oxygen species (ROS) and reactive nitrogen species (2). Rapamycin (sirolimus), an antibiotic derived from *Streptomyces hygroscopius*, is an FDA approved immunosuppressant drug (3). Rapamycin is a well-known specific inhibitor of the serine-threonine kinase mammalian target of rapamycin complex-1 (mTORC1). Recently, Calap-Quintana *et al.* (4) have reported that antioxidant defense mechanism of rapamycin is through the inactivation of mTORC1 signaling. Rapamycin targets several cellular functions such as cell growth, proliferation, and autophagic cell death, and plays a critical role in pathophysiology of cancer (5), diabetes (6), neurological disorders (7), and cardiovascular diseases(8). There is also evidence, indicating that rapamycin inhibited apoptosis by preventing phosphorylation of proapoptotic proteins such as p53 and activation of the mitochondrial cell death pathway (9). In addition, rapamycin enabled to

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enrich$CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells to exert anti-inflammatory effects during IR process (6).By considering these investigations, rapamycin can probably have a protective role against testicular I/R injuries. Thus, here for the first time, the protectiveeffects of rapamycin on testicular T/D injury were evaluated in a rat model. As far as we know, MDA is the final product of lipid peroxidation and is frequently used to define oxidative stress; SOD, GPX, and CAT represent antioxidant enzymes and caspase-3 is a marker of apoptosis; therefore, in this study we evaluated MDA, SOD, GPX, and CAT as markers of oxidative stress and caspase-3 as a marker of apoptosis.

Materials and Methods

Animals and ethics

A total of 72 male Wistar albino rats, weighing 200–250 g, were divided into 6 groups of 12 animals each. Animals were purchased from Department of Pharmacology, Tehran University of Medical Sciences and housed at 23±2 ^oC and 12 hr light/dark cycle with free access to water and standard food. The local ethics committee approved all of the protocols.

Drug and treatment

Rapamycin was dissolved in DMSO and administered intraperitoneally. To rule out the alterations in lipid peroxidation and antioxidant enzymes activities induced by testicular T/D, right testes of six animals in each study group were removed 4 hr after beginning of reperfusion, given that an increase in oxidative stress biomarkers is detectable as early as 4 hr after testicular reperfusion (10, 11). The procedure was repeated for six other animals that had undergone T/D from each group to evaluate germ cell apoptosis 24 hr after reperfusion, when peak level is reached (11-13). The protective effects of rapamycin in testicular T/D were studied by administration of specific doses of the drug (0.5, 1 and, 1.5 mg kg^{-1}, IP).

The studied groups were arranged as follows:

Group 1: Control rats as base line;

Group 2: Sham-operated rats;

Group 3: T/D operated rats, received 2 ml injection of DMSO as vehicle, 30 min after torsion (vehicle group)

Groups 4 to 6: T/D operated rats, received injection of rapamycin at doses of 0.5, 1, and 1.5 mg kg^{-1}, 30 min after torsion(10, 13, 14).

Experimental testicular T/D procedure

Surgical procedures were performed under general anesthesia by intraperitoneal injection of ketamine (50 mg kg^{-1}) and chlorpromazine (25 mg kg^{-1}). Following a vertical incision in scrotal zone, tunica vaginalis was opened and the right testis was twisted (720° in clockwise direction). One hour later, the testis was counter-rotated to the natural position and was inserted into the scrotum. Then, the skin incision was sutured (4–0 nonabsorbable) and animals were kept until harvesting time. In the sham-operated animals, only surgical stress was applied by immediately retracting and replacing the spermatic cord.

Biochemical assays

To evaluate the oxidative stress damage, biochemical assays in tissue were performed following ipsilateral orchiectomy of right testis 4 hr after detorsion. The samples were rapidly stored in -80°C for measurement of tissue malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and caspase-3 levels changes.

Measurement of tissue MDA level

Concentrations of free MDA, an end product, and marker of lipid peroxidation in cell membrane (15), were assayed using thiobarbituric acid reactive substance (TBARS), as described by Ohkawa *et al*(16). In brief, testes were homogenized in 1.15% KCl to make a 10% (w/v) homogenate. Then, 0.9 ml of 1.8% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid solution (pH = 3.5) and 1.5 ml of aqueous TBA solution were regularly added to 0.1 ml of tissue homogenates. The prepared homogenates were centrifuged at 4000 rpm for 10 min. The supernatant was applied to spectrophotometrically determine the MDA level (λ= 532 nm).

CAT activity

CAT activity was spectrophotometricallydetermined in accordance with the method established by Aebi (17). Tissue sections were homogenized in 1% Triton X-100 and were diluted with potassium phosphate buffer. The reaction was initiated following addition of hydrogen peroxide (H_2O_2), and CAT activity was quantified based on ability of tissue CAT to decompensate H_2O_2 by calculating the decrease in absorbance at 240 nm.

GPx activity

GPx activity was measured by modified method of Paglia and Valentine (18). The enzymatic reaction was initiated following addition of H_2O_2, and the alteration in absorbance at 340 nm was applied to measure GPx activity using a spectrophotometer. GPx catalyzed oxidation of glutathione (GSH) by reduction of H_2O_2 to H_2O. This reaction is coupled with oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADPH+.

SOD activity

Using the Paoletti and Mocali method (19), SOD activity level was assayed based on its ability to inhibit NADH oxidation in the reaction mixture and conversion of superoxide anions (O_2) to H_2O_2 and molecular oxygen (O_2). SOD activity was determined by decreased absorbance at 340 nm during the reaction.

Caspase-3 level

The caspase-3 level was measured using ELISA detection kit based on the Biotin double antibody sandwich technology. The colorimetric alteration of samples at 450 nm was applied to measure caspase-3 concentration (ng ml^{-1}) by drawing a standard curve (20).

Histopathological analysis

Histological alterations were analyzed byipsilateral orchiectomy 24 hr after detorsion, following a rapid cervical dislocation. The specimens were fixed in 10% phosphate-buffered formalin, and post-fixed in 70% ethanol, then three 5 µm thick sections were prepared from the upper, lower, and mid portions. After deparaffinization of the sections and staining with haematoxylin–eosin (H&E), slides were evaluated using light microscopy at 100×magnification by two independent reviewers who were blinded to the study design (Figure 1).

To quantify testicular histological injury, the 4-level grading scale of Cosentino's score was used (21):

Grade 1: normal structure with regular arrangement of germ cells

Grade 2: testicular injuries with less orderly, noncohesive germ cells and closely packed seminiferous tubules

Grade 3: testicular injuries with disordered, sloughed germ cells with shrunken, pyknotic nuclei, and less distinction in seminiferous tubule borders

Grade 4: testicular injuries with coagulative germ cell necrosis and intensely packed seminiferous tubules.

Moreover, for each sample, MSTD was calculated by measurement of 10 separate roundest seminiferous tubules using a light microscope-adaptable micrometer.

Evaluation of germ cell apoptosis using TUNEL assay

Immunohistochemical terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) staining method distinguishes cleavage of genomic DNA duringapoptosis, which presents in situ DNA fragmentation in germ cells. Semi quantitative assessment of apoptotic nuclei in specimens was performed using the APO-Brdu-IHC kit according to the manufacturer's instructions.5 µm sections were cut and processed for TUNEL assay. Of each specimen, one hundred seminiferous tubule cross sections were evaluated for the appearance of apoptotic nuclei with intense green staining by manual counting at 200×magnifications under light microscopy by two experts who were unaware of the study design, and the mean number of apoptotic nuclei per tubule cross section was used for statistical analysis. Only circular tubular cross sections cut in bold face were used in these studies.(10, 11, 13).

Statistical analysis

All statistical data and significance tests were performed by using Sigma plot version 12. All data were expressed as mean±SD. The differences between the experimental groups were analyzed using ANOVA. Individual groups were compared using Tukey's multiple comparison tests. $P<0.05$ was considered statistically significant.

Results

None of the study groups showed any significant differences inparameters between control and sham-operated groups.

Biochemical assays

The concentration of testicular MDA and SOD, CAT,GPx, and caspase-3 activities in studied groups are shownin Table 1. There was significant difference in the evaluated antioxidant enzyme levels between the T/D andcontrol groups. The tissue MDA levels in the

Table 1. Testicular levels of MDA and CAT, SOD, GPx, and caspase-3 enzyme activities 4 hr after detorsion

Grop	MDA (nmol g^{-1} wet issue)	CAT (IU g^{-1} wet tissue)	SOD (IU g^{-1} wettissue)	GPx(IU g^{-1} wet tissue)	Caspase-3 activity (ng ml^{-1})
Control	112.15 ± 6.13	364.19 ± 4.94	199.23 ±10.71	741.53±52.26	0.264 ± 0.027
Sham-operated	115.24 ± 21.43	357.62 ± 13.02	1961.39 ± 19.78	713.29 ± 28.98	0.303 ± 0.014
T/D	194.02 ± 11.15^{+++}	251.31 ± 16.53^{+++}	1495.15 ± 25.01^{+++}	512.24 ± 31.99^{+++}	0.567 ± 0.021^{+++}
Rapa 0.5 mg kg^{-}	163.50 ± 6.42***	273.24 ± 9.12	1641.14 ±17.21***	598.49 ± 14.16*	0.470 ± 0.024***
Rapa 1 mg kg^{-1}	137.64 ± 5.47***,†	298.42 ± 12.24*,†	1706.49 ±45.47***	614.10±23.17***	0.336 ± 0.036$^{***,\varphi\varphi\varphi}$
Rapa 1.5 mg kg^{-1}	121.46 ± 14.15***,†††	324.21 ± 20.63***,†	1881.58±50.34***,†††	641.47±12.54***	0.315 ± 0.027$^{***,\varphi\varphi\varphi}$

T/D, torsion/detorsion; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase

$^{+++}P<0.001$ compared with the control group

$^{*}P<0.05$ compared with the T/D group. $^{***}P<0.001$ compared with the T/D group. $^{\dagger}P<0.05$ compared with group receiving rapamycin at dose of 0.5 mg kg^{-1}

$^{\dagger\dagger\dagger}P<0.001$ compared with groups receiving rapamycin at doses of 0.5 and 1 mg kg^{-1}. $^{\varphi\varphi\varphi}P<0.001$ compared with group receiving rapamycin at dose of 0.5 mg kg^{-1}

Table 2. Histological evaluation of the testes using mean seminiferous tubular diameter (MSTD) values and Cosentino's scores 24 hr after detorsion in the studied rats

Group	MSTD (µm)	Grade
Control	291.4 ± 21.52	1
Sham-operated	289.3 ± 8.48	1
T/D	207.0 ± 22.41^{+++}	3
Rapa 0.5 mg kg^{-1}	232.8 ± 5.81	2
Rapa 1 mg kg^{-1}	248.3 ± 19.71***,†	2
Rapa 1.5 mg kg^{-1}	271.8 ± 22.62$^{***,\dagger\dagger\dagger,\varphi}$	2

MSTD; mean of seminiferous tubules diameters
Grades: 1, Minimal or no evidence of injury; 2, Slight injury; 3, Mild injury; 4, Moderate injury
^{+++}P< 0.001 compared with control group
$^{***}P$< 0.001 compared with T/D group
$^{\dagger}P$<0.05 compared with group receiving rapamycin at dose of 0.5 mg kg^{-1}
$^{\dagger\dagger\dagger}P$<0.001 compared with group receiving rapamycin at dose of 0.5 mg kg^{-1}
$^{\dagger}P$<0.05 compared with group receiving rapamycin at dose of 0.5 mg kg^{-1}

rapamycin injected animals (0.5, 1, and 1.5 mg kg^{-1}, IP) weresignificantly lower than T/D animals. These values weresignificant between T/D and rapamycin 0.5 mg kg^{-1}, rapamycin 0.5 and 1 mg kg^{-1}, and rapamycin 1 and 1.5 mg kg^{-1};P<0.001, P<0.05, and P<0.01, respectively. The activity of SOD, CAT, and GPx enzymes in the T/D rats significantly increased following injectionof dose-dependent rapamycin (P<0.001). On theother hand, treatment with rapamycin could not completely normalize the caspase-3 activity, but dose-dependently reduced caspase-3 activity in ischemic/ reperfused tissue (P<0.001).

Histopathological analysis

As expected, the control and sham-operated animals (groups 1 and 2) demonstrated a normal architecture of the seminiferous tubules and interstitium in ipsilateral testes and had intact germinal epithelium with an average thickness of cell layers. There were some histopathological changes such as degeneration, desquamation, and disorganization, reduction in germinal cell counts, interstitial edema, and capillary Congestion in the testis of rats from the T/D group (group 3).These histopathological changes werealso present to a similar extent in testis of rats fromgroup 4. However, these histopathologicalchanges were improved significantly in groups 5 and 6 (Figure 1). Table 2 compares the histopathological parameters in right testes among the 6 experimental studied groups. Significant (P< 0.01) decreases in MSTD and increased Cosentino's scores were observed in the testis of group 3 compared with the groups 1 and 2 (Table 2). MSTD in the testis of groups 4, 5 and 6 was significantly more and Cosentino's score was less than the values in group

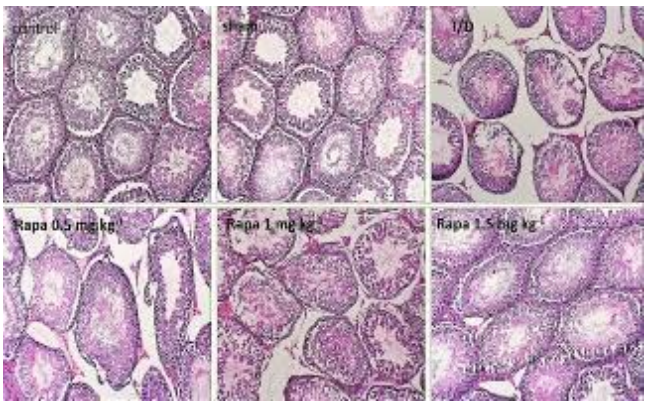

Figure 1. Histological appearances in ipsilateral testes groups: control, sham-operated, T/D, rapamycin 0.5 mg
Kg-1 + T/D, rapamycin 1 mg kg-1 + T/D and rapamycin 1.5 mg kg-1 + T/D. Ischemic alterations and
coagulative necrosis were observed, and the orderly arrangement of germ cells was impaired in the T/D group.
After treatment with rapamycin, spermatogenesis was restarted and orderly structure of germ cells with a
few mature spermatids was observed within seminiferous tubules (H&E; magnification × 100)

3. There were differences between groups 4, 5, and 6. MSTD in the testis of group 6 was more than groups 4 and 5. In other words, the administration of different doses of rapamycin to rats resulted in an improvement in these histopathological parameters dose-dependently

TUNEL assay

Immunohistochemical studies confirm the index of germ cells following TUNEL assay (Figure 2). By double labeling, alterations of the anatomical structures and proportion of the TUNEL-positive nuclei/surrounding normal nuclei (%) were deter-mined. Germ cell apoptosis indices were significantly higher in T/D and rapamycin groups versus control and sham-operated groups; however, rapamycin treatment dose-dependently reduced the apoptosis in rapamycin groups compared with the T/D group. (Table 3).

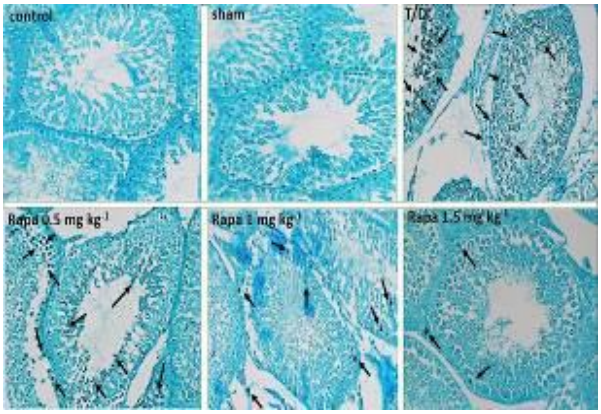

Figure 2. Apoptotic nuclei and seminiferous tubules using TUNEL assay. Apoptotic germ cells significantly increased following T/D. After treatment with rapamycin, especially at dose of 1.5 mg kg-1, apoptosis indexand percentage of seminiferous tubules significantly decreased and only a few apoptotic nuclei were observed (magnification× 200)

Table 3. Apoptotic germ cell index and percentage of apoptotic tubules in the rat testes determined using the TUNEL assay

Group	Mean apoptotic nuclei/tubule	Apoptotic tubules (%)a
Control	0.73 ± 0.81	12.43 ± 3.071
Sham-operated	0.81 ± 0.23	16.73 ± 2.429
T/D	9.93 ± 0.76^{+++}	61.41 ±2.361^{+++}
Rapa 0.5 mg kg^{-1}	6.49 ± 4.63	49.21 ± 1.481
Rapa 1 mg kg^{-1}	5.12 ± 1.42***	42.37 ± 2.345***
Rapa 1.5 mg kg^{-1}	3.21 ± 2.43***	35.83 ± 2.154***,†††

[a]The percentage of tubules in specimens in which at least 1 TUNEL-stained nucleus is observed.
$^{+++}P< 0.001$ compared with control group
$^{***}P< 0.001$ compared with T/D group
$^{†††}P< 0.001$ compared with group receiving rapamycin at doses of 0.5 and 1 mg kg^{-1}

Discussion

Testicular torsion is a medical emergency occurring primarily in adolescent males and young men with an incidence estimated to be as high as 1 in 158 males by the age of 24 years. Surgical detorsion should be done promptly to avoid loss of function of the ipsilateral testis. Despite the unequivocal benefit of reperfusion of blood to ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue even with successful surgical repair, however, testicular atrophy is a common clinical outcome and is a significant urological issue (22). Testicular I/R activates an inflammatory signaling pathway which facilitates transmigration of neutrophils from endothelium into the testis interstitium, ROS over-production, oxidetive stress, cellular dysfunction, and promoting apoptosis (23, 24). To date, a number of chemicals and drugs have been successfully used to reduce the I/R injury in animal models of testicular torsion, but few of them are currently in clinical use(25-28). Rapamycin is a macrolide antibiotic that was initially found to have antifungal effects (29). It has been used for several years as an FDA-approved immunesuppressant to prevent graft/tissue rejection after transplantation (30). By binding with FKBP-12 (FK506-binding protein), rapamycin may inhibit mTOR and prevent further phosphorylation of P70S6K, 4E-BP1, and indirectly, other proteins involved in transcription, translation, and cell cycle control (31). In addition, rapamycin promotes autophagy by inhibiting mTOR and is also widely used as an autophagy inducer (32). Rapamycin also has antitumor effects and inhibits the immune system (33, 34).In this study for the first time, we report that rapamycin could induce a protective effect against I/R injury in the rat testis. It is well established that 1hr 720^{o} testicular torsion leads to a decrease in antioxidant enzyme levels as well as an increase in MDA and caspase-3 levels compared to the sham operated group when measured 4 hr after reperfusion. In addition, germ cell-specific apoptosis significantly increases when assessed by the in situ TUNEL technique 24 hr after detorsion. Based on our study, administration of the specific doses of rapamycin significantly decreased the MDA and caspase-3 levels and increased the activity of antioxidant enzymes in the animals undergoing testicular T/D. Furthermore, the germ cell apoptosis index and percentage of apoptotic seminiferous tubules were significantly reduced following intraperitoneal injection of rapamycin. We found that rapamycin significantly decreases the oxidative stress and our results are also in concurrence with the recent reports which concluded that rapamycin attenuates cisplatin induced oxidative damage (35) and alleviates oxidative stress induced damage in rat erythrocytes (29). Beneficial effects of rapamycin have been shown in I/R models (36-40). Early literature documented its T-cell-independent antiinflammatory effects by down regulating TNF-α and decreasing neutrophil chemoattractant, in small bowl and liver models (39, 40). Its organ protective effects were also shown in kidneys and pancreases by improving microcirculation post I/R (36, 41). Direct cytoprotective effects of rapamycin were demonstrated in cardiac infarct models and *in vitro* cell cultures. Rapamycin has been shown to protect cardiomyocytes against necrosis and apoptosis induced by simulated ischemia and reoxygenation (42, 43). The opening of the mitochondrial K_{ATP} channel and activation of JAK2-Stat3 signaling pathway seemed to play key roles (42, 43). Previous studies illustrated that testicular I/R can induce oxidant/antioxidant imbalance which leads to oxidetive stress inflammation and ultimately germ cell apoptosis (10). Rapamycin acts through inhibition of mTOR, which has been attributed to supporting an antioxidant defense system by inducing autophagy. Rapamycin induced autophagy ensures the continuous removal of ROS-induced damaged/misfolded macromolecules to maintain the protein homeostasis and physiological functionality of the cells and tissues(44).Several studies have shown that opening of mitoK_{ATP} channels is one of the common mediators of acute and delayed preconditioning, induced by both pathophysiological stressors (45-47). Opening of mitoK_{ATP} during the ischemic post-Conditioning phase leads to the generation of (ROS) (48, 49). ROS then acts as a second messenger to activate thedownstream pathway of protective kinases, including protein kinase C and others (50). This small burst of ROS generated by the mitoK_{ATP} channel prior to ischemia acts to prevent the larger, damaging burst during reperfusion/reoxygenation (47, 51, 52). It is reasonable to speculate that the mitoK_{ATP} opening property of rapamycin may lead to a reduced level of ROS generation during reperfusion/reoxygenation, and thus to the protective effects of this drug against inflammation, cell necrosis, and apoptosis. Although it requires further investigation, there are some

explanations of how rapamycin may open mitoK$_{ATP}$channels. First of all, as discussed above, rapamycin-induced mTOR inhibition may enhance compensatory upregulation of upstream survival kinases, such as PI3K and Akt (53, 54). These kinases, in turn, are key mediators in the activation of mitoK$_{ATP}$ channels (50). In addition, it is certainly possible that spatial colocalization of mTOR with the mitochondria allows for physiological regulation of mitochondrial membrane channel activity (55). Moreover, as also discussed above, rapamycin may upregulate NO and several studies have found that NO consequently plays an important role in the opening of mitoK$_{ATP}$ channels(53, 56).

Conclusion

We determined that rapamycin treatment before reperfusion may have the potential to decrease the histologic damage that occurs after testicular torsion. It was found that the most effective dose of different doses of rapamycin administrated was 1.5 mg kg^{-1}. As this drug is used in humans to suppress the immune system, we propose that rapamycin may have the clinical applicability in patients with torsion of the testicle. For this purpose, further clinical studies will be needed.

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