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 organs 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 mg kg⁻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 tubes 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 (GPO) activities in ipsilateral tests (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 GPO activities in ipsilateral tests 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.

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 testicular 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 hygroscopicus, 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
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 orchectomy 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 spectrophotometrically determined 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 (H₂O₂), and CAT activity was quantified based on ability of tissue CAT to decompose H₂O₂ 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 H₂O₂ 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 H₂O₂ to H₂O. 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 (O₂⁻) to H₂O₂ and molecular oxygen (O₂). SOD activity was determined by decreased absorbance at 340 nm during the reaction.
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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 by ipsilateral 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 loosely packed seminiferous tubules

Grade 3: testicular injuries with disordered, sloughed germ cells with shrunken, pyknotic nuclei, and less distinct 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 during apoptosis, 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 in parameters 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 shown in Table 1. There was significant difference in the evaluated antioxidant enzyme levels between the T/D and control groups. The tissue MDA levels in the

| Group     | MDA (nmol g\(^{-1}\) wet tissue) | CAT (IU g\(^{-1}\) wet tissue) | SOD (IU g\(^{-1}\) wet tissue) | 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\(^{-1}\) | 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\(^{***}\)          |
| 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\(^{***}\),\(^{***}\) |

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

\(^{***}\) \( P<0.001 \) compared with the control group

\(^{***}\) \( P<0.001 \) compared with the T/D group

\(^{***}\) \( P<0.01 \) compared with the T/D group

\(^{***}\) \( P<0.05 \) compared with group receiving rapamycin at dose of 0.5 mg kg\(^{-1}\)

\(^{***}\) \( P<0.001 \) compared with groups receiving rapamycin at doses of 0.5 and 1 mg kg\(^{-1}\)

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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 | 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<0.05 compared with control group

***P<0.001 compared with T/D group

†††P<0.001 compared with group receiving rapamycin at dose of 0.5 mg kg⁻¹

‡‡‡P<0.001 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) were significantly lower than T/D animals. These values were significant 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 injection of dose-dependent rapamycin (P<0.001). On the other 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 testes of rats from the T/D group (group 3). These histopathological changes were also present to a similar extent in testes of rats from group 4. However, these histopathological changes were improved significantly in groups 5 and 6. 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 determined. 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).

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

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 spermatozoa was observed within seminiferous tubules (H&E; magnification × 100).

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 index and percentage of seminiferous tubules significantly decreased and only a few apoptotic nuclei were observed (magnification × 200).
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Table 3. Apoptotic germ cell index and percentage of apoptotic tubules in the rat testes determined using the TUNEL assay

| Group             | Mean apoptotic nuclei/tube | Apoptotic tubules (%) |
|-------------------|----------------------------|-----------------------|
| 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***      |

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

Discussion

Testicular torsion is a medical emergency occurring primarily in adolescent males and young men with an incidence estimated to be at least 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 the testis interstitium, ROS over-production, oxidative 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 immunosuppressant 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 1 hr 720° 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 intra-peritoneal 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 anti-inflammatory effects by down regulating TNF-α and decreasing neutrophil chemoattractant, in small bowel 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<sub>ATP</sub> 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 oxidative 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<sub>ATP</sub> channels is one of the common mediators of acute and delayed preconditioning, induced by both pathophysiological stressors (45-47). Opening of mitoK<sub>ATP</sub> during the ischemic post-Conditioning phase leads to the generation of (ROS) (48, 49). ROS then acts as a second messenger to activate the downstream pathway of protective kinases, including protein kinase C and others (50). This small burst of ROS generated by the mitoK<sub>ATP</sub> 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<sub>ATP</sub> 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 mitoKATP 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 mitoKATP 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 mitoKATP 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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