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Comparison of multiple doses of cyclosporine A on germ cell apoptosis and epididymal sperm parameters after testicular ischemia/reperfusion in rats

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ABSTRACT

Testicular torsion/detorsion (T/D) is an inflammatory problem in men genital system with infertility effects. Cyclosporine A (CsA) as an immunosuppressant medication, exerts anti-inflammatory properties in tissue injuries. We sought to compare the efficacy of 3 doses of CsA on oxidative stress, apoptosis and epididymal sperm quality after ipsilateral testicular T/D.

Methods: 96 mature male rats were divided into six groups 16 each in: Control group (Group1), Sham operated (Group2), In rest groups, the right testis was twisted 720° in a clockwise direction for 1 h; T/D + 0.1% dimethylsulfoxide) DMSO((Group3), and in groups 4–6; CsA were administered 1, 5, and 10 mg/kg, intravenously (*iv*) 30 and 90 min after torsion, respectively.

Results: Tissue malondialdehyde (MDA) level and caspase-3 activity increased and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities decreased in compared with control group 4 h after detorsion ($p < .001$). In six rats of each group 24 h after detorsion, histopathological changes and germ cell apoptosis were significantly deteriorated by determining mean of seminiferous tubules diameters (MSTD) and TUNEL assay. Moreover, 30 days after T/D, sperm concentration and motility were examined in rest of animals.

Conclusions: Pre- and post-reperfusion CsA diminished MDA and caspase-3levels and normalized antioxidant enzymes activities. Germ cell apoptosis was significantly reduced, as well as, MSTD and long-term sperm insults were improved. Inhibition of mitochondrial permeability transition pore opening is suggested mechanism for cell protection against testicular T/D insults.

1. Introduction

Testicular torsion, due to spermatic cord twisting, is a traumatic inflammatory injury, which causes mitochondrial oxidative stress and germ cell apoptosis with subfertility or infertility results. It commonly affects newborns, children, and adolescent boys, totally one in 4000 males younger than 25 years, annually (Turner et al., 2004; Ghasemnejad-berenji et al., 2017a, 2017b). Ischemia in torsion which is followed by post reperfusion in detorsion, is the main pathophysiological event in torsion/detorsion (T/D) injuries (Ghasemnejad-berenji et al., 2017a, 2017b). Disruption of Ca^{+2} homeostasis and loss of mitochondrial transmembrane potential ($\Delta\Psi m$) in ischemia phase with an increase in intracellular reactive oxygen species (ROS) which arise from invaded neutrophils in reperfusion, aggravates dysregulation of respiratory chain in mitochondria and lead to predisposing of oxidative stress and cell death by mitochondrial permeability transition pore (mPTP) opening (Lysiak et al., 2001; Bozlu et al., 2009). mPTP is a non-specific conductance proteinaceous channel in the inner mitochondrial membrane and its destabilization by accumulation of excessive quantities of Ca^{2+} ions and a variety of compounds in ischaemia/reperfusion (I/R) conditions lead to cell death by apoptosis (Kinnally et al., 2011). It is now widely accepted that the mPTP plays a curtail role in determining the extent of injury in reperfusion after a prolonged period of ischemia and acts like a gatekeeper for apoptosis. In essence, mPTP opening converts mitochondria from ATP providers that energize the cell to cell death agents. Furthermore, the inner membrane permeabilization caused by mPTP opening results in loss of matrix components, impairment of mitochondrial functionality and substantial swelling of the organelle by passing H2O molecules, with outer membrane rupture consequent (Kinnally et al., 2011). According to the recent studies, opening of the mPTP not only prevents mitochondria from synthesizing ATP by oxidative phosphorylation during ischemia, but also allows

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reversal of the F0F1 ATP synthase causing hydrolysis of the ATP produced by glycolysis or any remaining "healthy" mitochondria (Alavian et al., 2014; Azarashvili et al., 2014). If this occurs for any length of time, depleted ATP levels, increased matrix pH and accumulated H2O molecules will precede more necrotic and apoptotic cell death (Kinnally et al., 2011).

Following I/R, peroxidation of lipids in cell membrane by released arachidonic acid can prompt opening of mPTP which can start a sequence of events by releasing of pro-apoptogenic factors like cytochrome *c*, apoptotic induced factor, Smac/Diablo from inter-membrane of mitochondria, lead to DNA fragmentation in nuclei by caspase-dependent pathway and eventually cell death by apoptosis (Penzo et al., 2004; Kinnally et al., 2011). So, overproduction of ROS can disorder mitochondria *via* mPTP directly, or facilitate DNA damage through the activation of caspase indirectly, which can intensify germ cell-specific apoptosis and spermatogenesis in damaged testis (Lysiak et al., 2000). Decreased spermatogenesis, sperm DNA fragmentation, germ cell apoptosis, and infertility remain the most long-term complication in T/D injuries (Sakkas and Alvarez, 2010). Following I/R and disruption in Ca^{+2} metabolism, the involvement of mPTP in cell injuries have been proven in some disorders such as hepatocytes, cardiac failure, neuronal damage in brain, traumatic brain injury and stroke (Pastorino et al., 1993; Di Lisa et al., 2001; Sun et al., 2012). Inhibition of mPTP opening is a therapeutic strategy in ischemic/reperfused cells such as hepatocytes (Pastorino et al., 1993), brain (Friberg and Wieloch, 2002), keratinocyte (Norman et al., 2010) and cardiomyocytes (Wu et al., 2015).

Cyclosporine A (CsA), a potent immunosuppressive, is a specific blocker of the mPTP channel and protects cell following against excitotoxicity and oxygen-glucose deprivation (Wu et al., 2015; Yazdani et al., 2017). Cytoprotective effect of CsA is documented in some cellular models *via* inhibition of mPTP activation and restricting ischemic infarct size. Inhibition of pore activation has been shown in the treatment of numerous I/R-induced *in vivo* and *in vitro* cell injuries such as myocardial infarction, hepatocytes oxidative stress and brain ischemia (Brockemeier et al., 1992; Chen et al., 2002; Javadov et al., 2009).

In line these studies and considering that counter-rotation to the natural position by surgery is the early step in torsion insult, we evaluated the effects of specific doses of CsA on biochemical and histopathological changes in testicular I/R injury as an adjunct pharmacological therapy along with detorsion surgical repair for rescuing testes in T/D conditions.

2. Materials and methods

2.1.1. Animals and ethics

Ninety-six adult Wistar rats weighing 220–250 g were supplied from Tehran Univ. of Medical Sciences and were fed in 23 ± 2 °C and12 h light/dark cycle. Authors considered all standard protocols accordance the Local Ethics Committee (NO. 92.02.10-66) and with the 1964 Declaration of Helsinki and its later amendment.

2.1.2. Drugs and treated groups

CsA (Novartis Pharma, Basel, Switzerland) was dissolved in 0.1% DMSO as vehicle. Ketamine HCL (Gedoon Richter Ltd., Budapest, Hungry) and Chlorpromazine (Darou Pakhsh Pharmaceutical Co. Tehran, Iran) were used as anesthesia agents. All prepared solution injected, freshly.

Right testes of six animals in each group were removed 4 h after beginning of testicular detorsion to rule out the alterations in lipid peroxidation and antioxidant enzymes activities induced by testicular T/D (Lysiak et al., 2000). This action was performed in line the fact that indicated of early enhancement of biomarkers of oxidative stress is detectable 4 h after testicular reperfusion (Turner et al., 1997). The procedure was repeated for six other animals undergone T/D from each group to evaluate MSTD and germ cell apoptosis 24 h after reperfusion, that reaches its pick level (Turner et al., 1997). Finally, for four remaining rats in each group, determination of sperm functions was done 30 days after treatment (Kurcer et al., 2010).

Specific doses of compound (1, 5 and 10 mg/kg) were administrated *iv*, 30 and 90 min after testicular torsion. The studied groups were arranged as follows:

Group 1: Control animals served as intact group, Group 2: sham operated animals, received DMSO as vehicle; Group 3: testicular T/D operated rats, received 2 injections vehicle: 30 and 90 min after torsion, respectively; Groups 4 to 6: testicular T/D operated rats, received 2 injections of CsA at doses of 1, 5 and 10 mg/kg, similarly (Yazdani et al., 2017).

Torsion – – – – 30 min – – – **vehicle or CsA** – – – – – 30 min – – – – **Detorsion** – – – – 30 min – – – **vehicle or CsA** ; and then, the biochemical analyses were performed 4 h after testicular detorsion.

2.1.3. Experimental testicular T/D procedure

Surgical procedures were performed under general anesthesia by intraperitoneal injection with Ketamine HCl (50 mg/kg) and Chlorpromazine (25 mg/kg) (Yazdani et al., 2017). The administrated cocktail provided a reliable dose-response curve and anesthesia was clinically monitored by *Toe Pinch Method* along with the operation. According the method, a positive reflex is indicated by leg retraction or withdrawing of the foot when these areas were firmly pinched by atraumatic forceps or fingernails. The animal is not at a surgical plane of anesthesia if there is leg or body movement, vocalization, or marked increase in respiration. Considering this, we provided a fully anesthetized condition for surgical procedure and animals tolerated. No pain during the stages of the experiment.

Following a vertical incision in the scrotal zone, tunica albuginea was opened and the right testis was twisted (720° in the clockwise direction). 1 h later, the testis was counter-rotated to the natural position and was inserted into the scrotum. Then, the skin incision was suture (4–0 nonabsorbable) and animals were kept until harvesting time. In the sham-operated animals, only surgical stress was occurred by immediately retraction and replacement of the spermatic cord.

2.1.4. Biochemical assays

To assess tissue oxidative stress damage, biochemical tests were accomplished following ipsilateral orchiectomy of right testis 4 h after detorsion. After collecting, the samples were washed by ice-cold saline solution and rapidly snap freezed at −80 °C for determination of MDA, SOD, CAT, GPx and caspase-3 levels by preparing supernatant. Briefly, after weighing, the tissue manually homogenized in 10% *W/V* with ice-cold Tris buffer (10 mM Tris, pH 7.4) for 3 min, and the resulting solution was centrifuged at 4 °C for 20 min at 12,000 rpm, and the gathered solution was used for biochemical analysis (Trounce et al., 1996).

2.1.5. Tissue MDA level

Peroxidation of unsaturated fatty acids in damaged cell membrane indicates the extent of oxidative stress in tissue. Free MDA, ultimate and reactive bi-products of lipid peroxidation, (Motaghinejad et al., 2015) was assayed using thiobarbituric acid reactive substance (TBARS), as described by Ohkawa and Yagi (Ohkawa et al., 1979). 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 sulfate, 1.5 ml acetic acid 20% (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 determination of MDA levels, spectrophotometrically (λ = 532 nm).

2.1.6. SOD activity

Tissue SOD, a primary intra-cellular anti-oxidant, was measured in accordance with Paoletti & Mocali method (Paoletti and Mocali, 1990). In brief, SOD activity level was assayed based on its ability to inhibit NADH oxidation in reaction mixture and conversion of superoxide anions ($O_2^{\cdot-}$) to H_2O_2 and molecular oxygen (O_2). SOD activity was determined by a decrease in absorbance at 340 nm during the reaction.

2.1.7. CAT activity

CAT activity was determined according to Aebi's method (Aebi, 1984). Tissue sections were homogenized in 1% triton X-100 and diluted with potassium phosphate as a buffer. The reaction was initiated following hydrogen peroxide (H_2O_2) addition, and CAT activity was quantified based on the ability of tissue CAT to decompensate H_2O_2 by calculating the decrease in absorbance at 240 nm.

2.1.8. GPx activity

GPx activity was spectrophotometrically evaluated regarding the modified method of Paglia & Valentine (Paglia and Valentine, 1967). The enzymatic reaction was initiated following H_2O_2 addition and the alteration in absorbance at 340 nm was applied to measure GPx activity using a spectrophotometer. GPx catalyzes the oxidation of glutathione (GSH), a reducing reagent, by reduction of H_2O_2 to H_2O. This reaction is coupled to oxidation of NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) to $NADPH^+$.

2.1.9. Caspase-3 level

Active tissue level of caspase-3 was measured applying ELISA detection kit based on the Biotin double antibody sandwich technology (Bioassay technology Laboratory kit, Catalog No. E0280Ra Yangpu District Shanghai, China). The colorimetric alteration of samples at 450 nm was applied to measure caspase-3 concentration (ng/ml) by drawing a standard curve (Namura et al., 1998).

3. Histopathological analysis

3.1.1. Histological preparation

Histological alterations were studied by ipsilateral orchiectomy 24 h after detorsion, following a rapid cervical dislocation. Samples were longitudinally aliquot and fixed in 10% phosphate-buffered formalin, and post-fixed in 70% ethanol, and post-fixed in 70% ethanol and then three 5-μm thickness sections of prepared from the upper, lower, and mid portions.

Following deparaffinization and staining with hematoxylin-eosin (H &E), investigation was performed at 100× magnification with light microscopy under blindfold condition by two experts (Fig. 1).

To illustrate testicular histological injury, the 4-level grading scale of Cosentino score (Cosentino et al., 1986) was used;

Grade 1: normal structure with regular arrangement of germ cells;

Grade 2: testicular injuries with less orderly, non-cohesive 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, MSTD value for each specimen was assessed by measurement of 10 separate roundest seminiferous tubules using an optical microscope equipped with a micrometer.

3.1.2. Evaluation of germ cell apoptosis using TUNEL test

The Semi-quantitative immunohistochemical terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) assay detects DNA fragmentation and is widely used as a specific cytochemical technique for visualizing apoptotic cell death. DNA cleavage in germ cells during apoptosis can produce single-strand breaks (nicks) in high molecular weight DNA. Staining is a method for identification DNA strand breaks by labeling the free 3´–OH termini of genomic DNA during apoptosis, which presents I/R injury in germ cells (Payabvash et al., 2008; Yazdani et al., 2017). Assessment of the apoptotic nuclei in specimens was performed by using the APO-Brdu-IHC® kit according to manufacturer's instructions.

Of each specimen, one hundred seminiferous tubule cross slices were evaluated for presence of apoptotic nuclei with intense brown staining by manual counting at 200× magnifications under light microscopy by two experts who were unaware of the study design (Fig. 2). The mean number of TUNEL positive cells per tubule cross section was obtained and only circular tubular cross sections cut in boldface were (Ghasemnejad-berenji et al., 2018).

3.1.3. Semen analysis

Long-term effects of testicular I/R and CsA effects on semen parameters were evaluated 30 days after operation. Yokoi *et al* method was used for measurement epididymal sperm concentration (Yokoi et al., 2003). Briefly, epididymis completely was cut, squashed by tweezers in physiologic saline and incubated at room temperature for 5 min to allow the movement of spermatozoa from epididymal to fluid. Then, supernatant fluid was diluted 1/100 with a solution containing 5 g sodium bicarbonate, 1 ml formalin (35%), and for sperm dying was added 25 mg eosin per 100 ml of water. The compound was centrifuged at 6000 rpm for 20 min at room temperature. Live sperms remained unstained following staining; whereas, those that showed any pink or red coloration were classified as dead (Yazdani et al., 2017). Then 10 μL of the diluted sperm suspension was transferred to each counting chamber of hemocytometer and the number of alive sperms was counted with a light microscope at the magnification of 200×. Using a standard method which possesses a score ranging from 0% to 100%, the progressive sperm motility percentage was visually recorded under light microscopy (400× magnification). Final motility score estimations calculated by mean value from 4 different fields in each sample.

3.2. Statistical analysis

The results were expressed as arithmetic mean ± SD. The differences in parameters were analyzed with one-way ANOVA. Individual groups were compared using Tukey's multiple comparison tests. Differences with *p-value* < .05 were regarded as statistically significant.

4. Results

In all of study groups showed no significant differences in parameters between control and sham-operated groups.

5. Biochemical assays

The concentration of testicular MDA and SOD, CAT, GPx and caspase-3 activities in all studied groups of animals are shown in Table 1. Significant differences were observed in the evaluated antioxidant enzyme levels between the T/D and control groups in biochemical parameters.

Pre- and post-reperfusion treatment with studied doses of CsA in T/D rats, dose-dependently exerted protective effects by a reduction in lipid peroxidation induced by testicular I/R. The tissue MDA levels in the CsA double injected animals (1, 5 and 10 mg/kg, *iv*) were significantly lower than T/D operated animals ($p < .001$). The activity of SOD, CAT and GPx antioxidant enzymes in the T/D operated animals increased following double injection of CsA. These values were significant at CsA10 mg/kg dose ($p < .001$). On the other hand, CsA

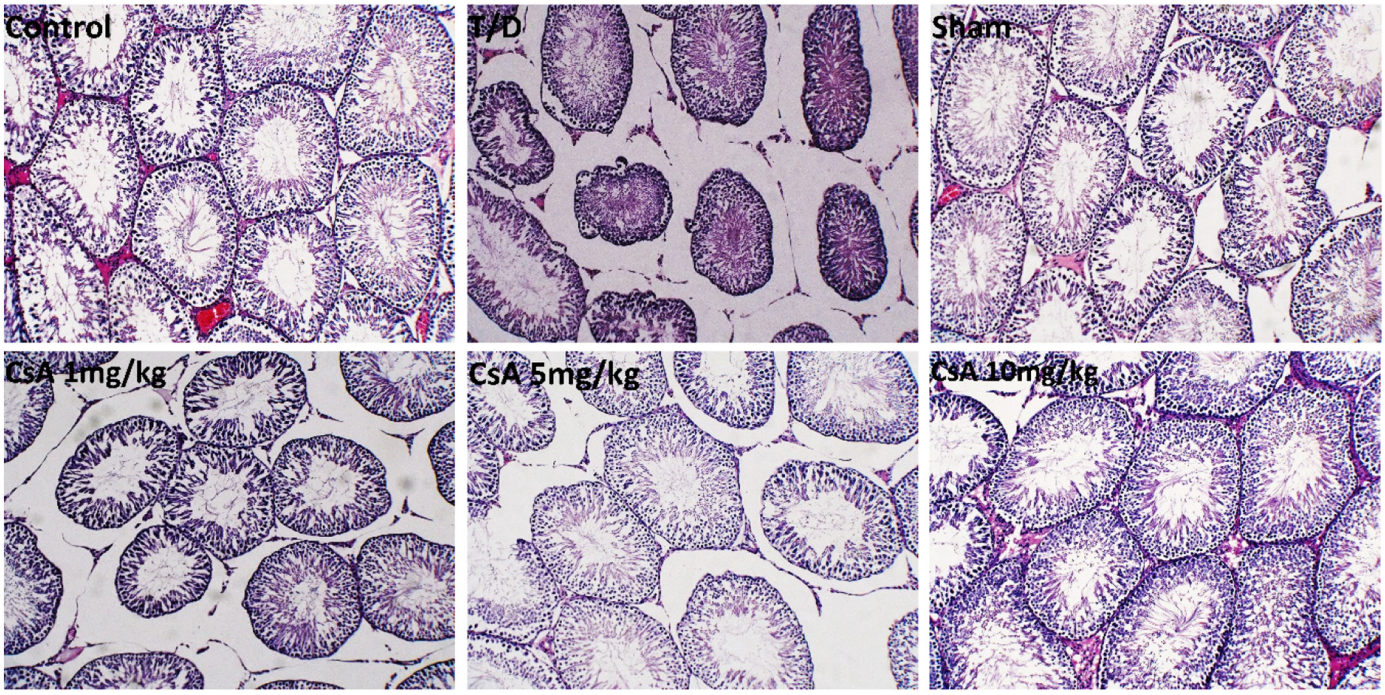

Fig. 1. Histological appearances in ipsilateral testes groups: control, sham-operated, T/D, CsA 1, CsA 5 and CsA 10 mg/kg + T/D. The ischemic alterations and coagulative necrosis were observed, and the orderly arrangement of germ cells was impaired in T/D group. After treatment with CsA, spermatogenesis was restarted and orderly structure of germ cells with a few mature spermatids was observed within seminiferous tubules (H&E; magnification ×100).

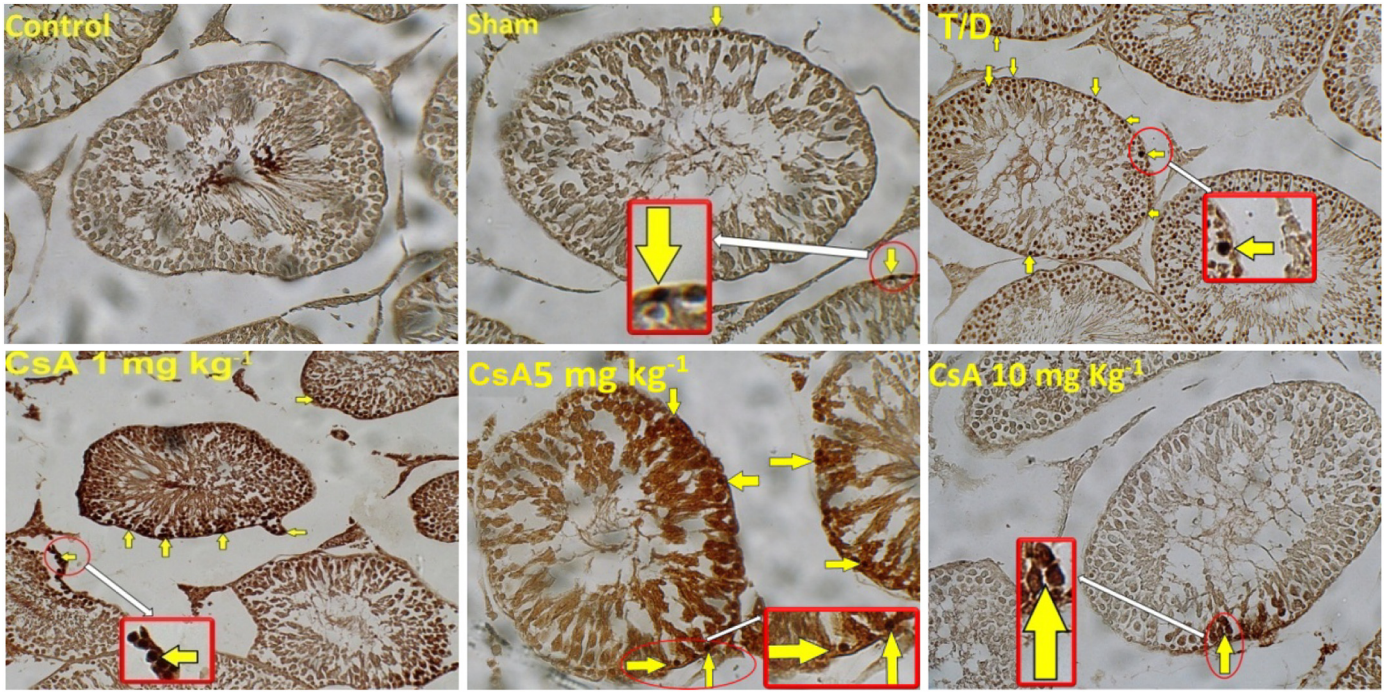

Fig. 2. Apoptotic nuclei and seminiferous tubules. A few apoptotic nuclei were observed in sham-operated group. Apoptotic germ cells significantly increased following T/D. After treatment with CsA, especially at dose of 10 mg/kg, apoptosis index and percentage of seminiferous tubules significantly decreased and only a few apoptotic nuclei were observed (magnification ×200).

could not completely normalize the caspase-3 activity, but administered doses recovered tissue insults; this improve is significant between T/D group and all CsA received groups ($p < .001$).

6. Histological findings

Table 2 compares the histological parameters among the experimental groups. The control and sham-operated animals exhibited a normal morphology of the seminiferous tubules and intact germinal epithelium with a normal thickness of cell layers. Following testicular I/R, a noticeable distortion of tubules with a severe decrease in MSTD was observed (Fig. 1). Testicular I/R diminished spermatogenesis in the T/D operated animals, consequently, lead to a reduction in the mean testicular scores. Treatment with CsA remarkably improved Cosentino's

Table 1
Testicular levels of MDA and s CAT, SOD, GPx and caspase-3 enzymes activities 4 h after detorsion.

Group	MDA (nmol/g wet tissue)	CAT (IU/g wet tissue)	SOD (IU/g wet tissue)	GPx (IU/g wet tissue)	caspase-3 activity (ng/ml)
Control	116.09 ± 5.43***	354.19 ± 9.64***	1955.23 ± 10.70***	706.53 ± 32.26***	0.275 ± 0.024***
Sham-operated	121.62 ± 22.33	347.62 ± 14.02	1920.39 ± 19.78	673.23 ± 28.99	0.308 ± 0.035
T/D	183.02 ± 18.26	264.71 ± 19.53	1581.13 ± 45.01	562.63 ± 31.99	0.523 ± 0.016
CsA 1 mg/kg	155.03 ± 10.08***	276.35 ± 10.28	1722.82 ± 41.54***	615.16 ± 15.44*	0.425 ± 0.024***
CsA5 mg/kg	133.45 ± 5.27***, †	300.71 ± 13.51*, †	1752.49 ± 43.73***	630.20 ± 25.31***	0.363 ± 0.028***, φφφ
CsA 10 mg/kg	129.05 ± 6.10***, †††	326.37 ± 5.18***,†	1830.94 ± 27.87***, †††	653.03 ± 13.17***	0.317 ± 0.022***, φφφ

* $p < 0.05$compared with T/D group.
*** $P < .001$ compared with T/D group.
† $P < .05$ compared with group received CsA at dose of 1 mg/kg.
††† $P < .001$ compared with group received CsA at doses of 1 and 5 mg/kg.
φφφ $P < .001$ compared with group received CsA at dose of 1 mg/kg.

Table 2
Histological evaluation of the testes using MSTD values and Cosentino's scores 24 h after detorsion.

Group	MSTD (μm)	Grade
Control	295.7 ± 11.72***	1
Sham-operated	287.2 ± 9.33	1
T/D	228.7 ± 10.63	3
CsA 1 mg/kg	253.2 ± 17.80***	2
CsA5 mg/kg	269.2 ± 18.05***, †††	2
CsA 10 mg/kg	228.7 ± 10.63	2

Cosentino's score: 1. Minimal or no evidence of injury, 2. Slight injury, 3. Mild injury, 4. Moderate injury.
*** $p < .001$ compared with T/D group.
††† $p < .001$ compared with group received CsA at dose of 1 mg/kg.

score. Spermatogenesis parameters significantly enhanced between T/D and CsA5 mg/kg ($p < .001$), which is significant between CsA1 and 10 mg/kg in treated groups ($p < .001$).

7. Immunohistochemical findings

The results of DNA laddering semi-qualitative study have been exhibited (Fig. 2). By performing double labeling, the TUNEL positive nuclei were compared with surrounding normal nuclei, and the anatomical structures were examined. For apoptotic germ cells computing, only the TUNEL-positive cells with fulfilled morphological criteria were counted. Proliferation of apoptotic cells per seminiferous tubules was significantly increased after T/D (Fig. 3).

Treatment with double injections of CsA at 1, 5 and 10 mg/kg dosages significantly lessened the mean apoptotic nuclei/tubule ($p < .001$) (Fig. 3). The changes in percentages of apoptotic tubules were so similar. The percentages of apoptotic tubules in T/D group

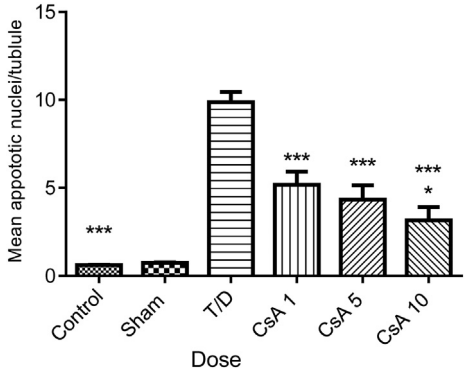

Fig. 3. Apoptotic germ cell index.
*$p < .05$ compared with CsA 5 mg/kg group. ***$p < .001$ compared with T/D group. ($n = 6$).

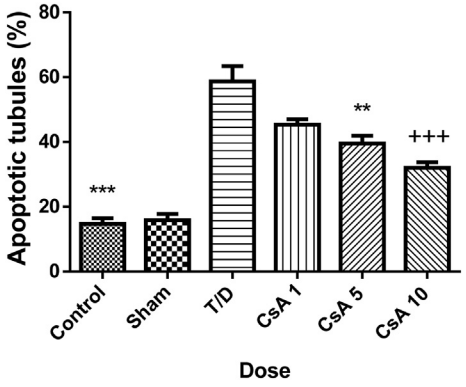

Fig. 4. Percentage of apoptotic tubules which at least 1 TUNEL-stained nucleus is observed in each specimen.
**$p < .01$ compared with CsA 1 mg/kg. ***$p < .001$ compared with T/D group. +++$p < .001$ compared with CsA 5 mg/kg. (n = 6).

were significantly upper than control group ($p < .001$). Double injection of CsA dose-dependently ceased the rate of apoptosis in compared with T/D group. However, the values were higher than control group (Fig. 4).

8. Semen analysis findings

Epididymal sperm concentration and motility were deteriorated in long-term effects of testicular T/D. Double injection of CsA (1, 5 and 10 mg/kg) dose-dependently improved the reduced sperm concentration in compared with T/D group ($p < .05$, $p < .01$ and $p < .001$, respectively) (Fig. 5).

Reduced percentage of sperm motility resulted from testicular T/D did not improved at two injections of CsA 1 mg/kg. This value significantly improved at 5 and 10 mg/kg doses $p < .05$ and $p < .01$, respectively (Fig. 6).

9. Discussion

Testicular torsion is a pathological consequence in urologic system and inappropriate treatment can lead to infertility effects in young men. Surgical detorsion should be done promptly to avoid loss of testis function. Although the exact reasons remain unclear, the primary detrimental event in testicular torsion attributes to ischemia mechanisms followed by reperfusion in detorsion. Despite the unequivocal benefit of blood reperfusion to ischemic tissue by detorsion, reperfusion can elicit a cascade of adverse reactions which deteriorates paradoxically insults even with successful surgical repair (Mogilner et al., 2006; Kurcer et al., 2010; Ghasemnejad-berenji et al., 2017a, 2017b). To date, a number of drugs have been used to reduce the T/D injury during detorsion surgery, but few of them are currently in clinical use. Based on a pervious

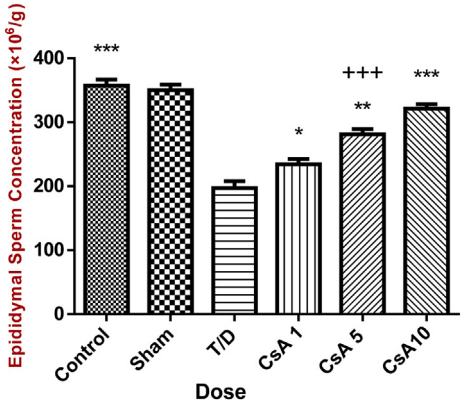

Fig. 5. Epididymal sperm concentration.
*p < .05 compared with T/D group. **p < .01 compared with T/D group. ***p < .001 compared with T/D group. + + +p < .001 compared with CsA 1 mg/kg. (n = 6).

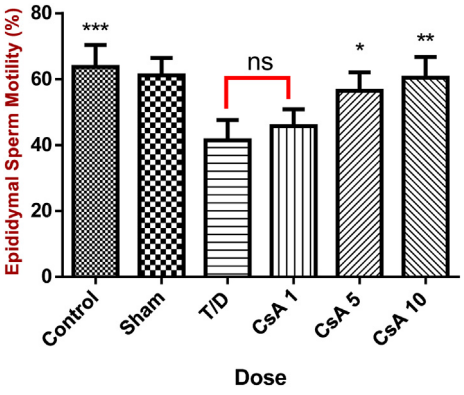

Fig. 6. Epididymal sperm motility.
*p < .05 compared with T/D group. **P < .01 compared with T/D group. ***P < .001 compared with T/D group. (n = 6).

study, immunophilin ligands could induce a protective effects against T/D injury in the rat testis after detorsion (Nezami et al., 2009; Jeong et al., 2010).

It is well established that 720° testicular torsion around the axis of spermatic cord for 1 h by causing an obstruction in blood flow 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 h after detorsion. In addition, germ cell-specific apoptosis significantly increases when assessed by *in situ* TUNEL technique 24 h after detorsion.

I/R injury in many aspects resembles an inflammatory response characterized by potent generators of ROS, which lead to an increase in cellular oxidative stress. Calcium influx into neutrophils during ischemia causes increased NADPH oxidase activity in the cell, which leads to release of free radicals during reperfusion (Lysiak et al., 2001; Ghasemnejad-berenji et al., 2017a, 2017b).

As mentioned above, a hallmark of tissue injury in I/R is the release of massive intracellular calcium, loss of ΔΨm, overproduction of ROS, destabilization of mitochondria membrane by facilitating mPTP opening, and swelling of the mitochondrial inner membrane (Crompton, 1999). Channel opening causes a vicious cycle to mitochondria by ATP hydrolysis, energy impaired, Ca^{+2} deregulation, further mitochondrial swelling, and mPTP opening, inevitably, a self-amplifying process, which lead to release of apoptosomes proteins from mitochondrial intermembrane and activation of cell death pathways by apoptosis (Kinnally et al., 2011). Apoptosis is executed by caspases after proteolytic cleavage and processed to their active forms and induce DNA fragmentation in nuclei, ultimately lead to subfertility or infertility *via* losing of spermatogenesis (Sakkas and Alvarez, 2010). Therefore, assembling and opening of mPTP in I/R is a critical point at which the insult becomes irreversible (Crompton et al., 2002). Based on early researches, mPTP channel forms by integration of three mitochondrial proteins named VDAC-ANT-CypD with inorganic phosphate carrier (Pic) (Crompton, 1999). Cyclophilin D (CypD), an isoform of mitochondrial cyclophilins, is located on the matrix side of inner mitochondrial membrane and has regulatory role in protein folding and/or conformational change in proteins of pore. In I/R injuries, CypD favors the opening of mPTP and CypD-depleted mitochondria show a high resistance to mPTP opening (Crompton, 1999).Several lines of evidence suggest that other proteins such the mitochondrial F1FO ATPase may participate to the formation of the pore (Bonora et al., 2013; Halestrap and Richardson, 2015). Recently, the mitochondrial F1FO ATP synthase, particularly the c subunit of the FO domain has been added to the list of permeability transition pore complex (PTPC) components and it was reported that mPTP induction is linked to F1FO ATP synthase dimers dissociation and that stabilizing F1FO ATP synthase dimers by genetic approaches inhibits PTPC opening (Morciano et al., 2017). Furthermore, it has been reported that the *c* subunit of FO is required for mPTP, mitochondrial fragmentation and cell death induced by mitochondrial Ca^{2+} overload and oxidative stress (Bonora et al., 2013).

CsA is a potent ligand for cyclophilins and its interactions may produce two curtail downstream; binding to cyclophilin A (CypA) inhibits calcineurin pathway activation and immunosuppressive effect is distinctly performed by this mechanis. On the other hand, CypD as a mPTP opening facilitator is a target for CsA, which its binding results mPTP inhibition. Upon CsA binding to CypD in mitochondrial matrix, the catalytic activities of peptidylprolyl *cis-trans* isomerase (PPIase) is blocked, consequently, the opening of pore and apoptosis death is restricted by forming a stable complex with pore components (VDAC-ANT-CypD-CsA) and cytoprotective effects in I/R injuries are mediated through blockade of CypD (Ho et al., 1996; Clarke et al., 2002; Halestrap and Richardson, 2015).

Based on previous studies on I/R, binding of CsA to CypA exerts the immunosuppressive activity through formation a complex between CsA and CypA to block calcineurin activity. Calcineurin triggers NF-κB activation by Ca^{+2}/calmodulin-dependent protein phosphatase (Ho et al., 1996). Calcium-dependent signal transduction is critical for the induction of cytokine expression by stimuli in leukocytes. The subsequent of inhibited calcineurin-catalyzed dephosphorylation is failure of cytokines to respond the production of interleukin-2 (IL-2), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α). Induction of IFN-γ and IL-2 are inhibited by CsA. This mechanism is rapidly reversible in leukocytes (Batiuk et al., 1995; Nezami et al., 2009).

CsA directly blocks pore by one of two ways; First, CsA is a reasonable size and it blocks movement of large solutes influx (Crompton, 1999). Second, CsA might induce inhibitory conformational changes in CypD conformation by preventing CypD binding to the P_iC/ANT and keeps pore in closed position (Kinnally et al., 2011). Studied have showed that under ischemia-associated oxidative damage, transcriptional activator p53, together with CypD, opens mPTP, leading to necrosis, which CsA also suppresses (Zhao et al., 2013). Recently it has been demonstrated that F1FO-ATPase *c*-ring in mPTP constitution is required for mitochondrial pore transition, mitochondrial fragmentation and cell death induced by mitochondrial Ca^{2+} overload and oxidative stress. CsA could prevent mitochondria *c*-ring detaching from F1 and subsequent pore opening (Alavian et al., 2014). Beside these evidences as anti-ischemic mechanisms of CsA, cytoprotective effect of CsA *via* mPTP inhibition has been demonstrated in some models of ischemia followed by reperfusion. Neuroprotective potency of CsA in acute brain injury and diffuse axonal injury models is related to mPTP inhibition by binding to CypD (Sullivan et al., 2000). In myocardial I/R injuries, CsA could preserve mitochondrial morphology independent of calcineurin

inhibition (Leshnower et al., 2008). A study by Chen and colleague in 2002 suggests that CsA could protect the oxidative stress-induced cardiomyocytes apoptosis not only by preventing the loss ΔΨm in mitochondria, but also through ROS generation. They also showed that pre-treating cells with CsA exerts pre-conditioning like effect. On the other hand, a research in 2015 showed that blocking the mPTP with CsA can restore cardioprotection of ischemic post-conditioning in hypercholesterolemic rat heart (Wu et al., 2015). Furthermore, mitochondrial ROS rise can modulate Ca^{2+} dynamics and augment Ca^{2+} surge. The reciprocal interactions between Ca^{2+} induced ROS increase and ROS modulated Ca^{2+} upsurge may cause a feedforward, self-amplified loop creating cellular damage far beyond direct Ca^{2+} induced damage (Kinnally et al., 2011). CsA causes delayed mPTP induction and significant resistance to calcium overload-induced injury. Based on the results of our study, the effects of increasing the antioxidant enzymes activity like SOD, CAT, GTx, and decreasing caspase activity, indicating cellular apoptosis, is in line with previous researches (Chen et al., 2002; Nezami et al., 2009).

Histological and immunohistochemical assays showed CsA could reduce the amount of tissue damage by reducing cell apoptosis, which is consistent with former results. Administration of CsA in heart failure was associated with a greater post-ischemic recovery and smaller infarction size by mPTP closing (Sharov et al., 2007; Leshnower et al., 2008; Nezami et al., 2009; Wu et al., 2015).

Despite satisfying effects, well-known side effects such as fever, vomiting, hepato-, and nephrotoxicity must be considered as restrictions on clinical practice (Amaike et al., 1996). Furthermore, testicular T/D may induce hormonal disturbances on testosterone in long-term (Turner et al., 2005) and the effects of CsA on venous testosterone concentration remains to be elucidated.

10. Conclusions

Based on our appraisal, therapeutic efficacy in T/D injury with CsA is in line with the previous studies in I/R conditions. The results of this research suggest that treatment with CsA, a potent agent in closing of mPTP, could normalize the antioxidant enzymes, inhibit oxidative stress-induced cell apoptosis, augment sperm criteria and provides evidences as a potential pharmaceutical adjunct for treatment and prevention of I/R-induced testicular injury in post-conditioning mood for increasing fertility outcome.

On the other hand, anti-inflammatory property of CsA *via* Ca^{+2}/calmodulin-dependent pathway, reduces the production of cytokines by neutrophils precluding may have a secondary role in reducing T/D injury. According to our results, adjunctive therapy in torsion insults with CsA provides significant rescue of testicular function and collapse at multiplex points along the molecular pathway to germ cell apoptosis.

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