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Original Article

Tsc1/Tsc2 complex: A molecular target of capsaicin for protection against testicular torsion induced injury in rats

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ABSTRACT

Objective: The detailed knowledge about protective effects of capsaicin (cap) and involved mechanisms against testicular torsion (TT) is still not available completely.
Methods: Male Wistar rats were assigned into four major cohorts: (i) sham, (ii) TT, (iii) three subgroups subjected to TT and different doses of cap (100, 500, and 1000 μg/mL), and (iv) three subgroups of healthy animals subjected to various concentrations of cap. The animals were decapitated at 24 h after reperfusion, and the evaluation of protein expression was performed by Western blotting assay. At 72 h after reperfusion, apoptotic cell death and tissue injury were evaluated by TUNEL nuclear and H&E staining, respectively.
Results: The results showed that cap administration following TT significantly increased the expression of tuberous sclerosis proteins 1 and 2 (Tsc1/Tsc2) in a dose-dependent manner ($P < 0.05$). Cap decreased cell apoptosis at highest dose. Likewise, cap contributed to the preservation of tubular morphology and decreased tissue injury at the highest tested concentration (1000 μg/mL).
Conclusion: Collectively, our findings demonstrate the validity of cap as a therapeutic agent against TT through targeting Tsc1/Tsc2 in a dose-dependent manner.

1. Introduction

In recent years, an increasing trend in the incidence of testicular torsion (TT), an ordinary urological emergency, has been observed which, consecutively, enhance the risk of male infertility (Bayne et al., 2017). The decreasing of blood flow after TT and twisting of the spermatic cord induce ischemia, and following by the production of reactive oxygen species (ROS), oxidative stress and cell injury (Boettcher et al., 2016). Although reperfusion may confer the cell survival in the site of stress, there is accumulating evidences which may trigger a pathophysiological chain including the recruitment of neutrophils, activation of inflammatory cytokines, high release of Ca^{2+} ions, and production of free radicals that, in turn, aggravating injury (Amani et al., 2017b; Çayan et al., 2014; Farahini et al., 2012; Feng et al., 2017; Habibey et al., 2010; Moghtadaei et al., 2012). Complex molecular mechanisms are responsible for modulation of the endogenous anti-oxidant pool and cellular function (Ajami et al., 2017; Amani et al., 2019; Arabian et al., 2017, 2018; Pazoki-Toroudi et al., 2011). The degree of atrophy and sperm count is dependent on time and degree of torsion (Javdan et al., 2018). Administration of pharmacological agents may give rise to cell survival by enforcing intracellular ROS equilibrium, scavenging free radicals and subsequently apoptosis inhibition by targeting various cellular signaling pathways (Ajami et al., 2013, 2011; Ghadernezhad, Khalaj, Pazoki-Toroudi, Mirmasoumi, & Ashabi, 2016).

Accumulating evidences in experimental animal models and humans have demonstrated that dietary interventions rich in the phytochemical compounds play an important role in public health through ameliorating cell death under oxidative stress related

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disorders (Hargraves, He, & Firestone, 2015; Pazoki-Toroudi et al., 2016).

Capsaicin (Cap) is a phytochemical compound, presents in chili peppers, from genus Capsicum and family Solanaceae that widely has been used in herbal medicine as a pain killer, modulator of energy expenditure, inhibitor of cytokines and antimutagenic agent (Derry, Sven-Rice, Cole, Tan, & Moore, 2013; Sun, Xiong, & Zhu, 2016). Cap can regulate many biological functions including thermal regulation, vascular physiology, cell development and growth by binding to the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor (Gaete, Lillo, Poblete, & Figueroa, 2014; Maiese, 2017; Szolcsányi, 2015).

It is known that cap can lead to cell survival against oxidative stress through free radical scavenging and apoptosis inhibition (Kursunluoglu, Kayali, & Taskiran, 2014; Pyun, Kim, Han, Hong, & Lee, 2014). Activated TRPV1 receptor modulates some biological processes such as vasomotor control (Korishettar, Nishijima, Cao, & Zhang, 2017), exercise endurance (Luo et al., 2012), energy metabolism (Luo et al., 2012), autophagy (Farfariello, Amantini, & Santoni, 2012), apoptosis (Amantini et al., 2007), and oxidative stress (Kahya, Nazıroğlu, & Övey, 2016) through modulation of different cellular signaling pathways.

The beneficial effects of TRPV1 activation on determining the cell fate under oxidative stress is achieved partially by affecting calcium signaling while it also affects other signaling pathways. Previous attempts have shown that the possible therapeutic potential of TRPV1 activation is closely associated with activated protein kinase (AMPK), protein kinase B (Akt), and mammalian TOR (mTOR), downstream of tuberous sclerosis proteins 1 and 2 (TSC1/TSC2) complex (Maiese, 2017). The TSC1/TSC2 complex has emerged as central negative regulator of mTORC1 and a master modulator of stress signals and nutrient to control a large numerous of cell functions such as protein synthesis and cell growth. Under oxidative stress, activation of TSC1/TSC2 complex confers promotion of cell survival by induction of protective autophagy in an mTORC1-dependent mechanism (Papadakis et al., 2013). Accordingly, TRPV1 receptor activation has been found to promote cell survival under oxidative stress through induction of protective autophagy (Liu et al., 2013).

The objectives of the present work were to examine the possible molecular targets of cap which attenuate ischemia/reperfusion (I/R) injury following TT.

2. Materials and methods

2.1. Chemicals

Cap, bovine serum albumin (BSA), RIPA buffer, In Situ Cell Death Detection Kit, and Immobilon®-FL Polyvinylidene Fluoride (PVDF) membrane were obtained from Sigma Company (St. Louis, MO, USA). Hamartin/TSC1 antibody (1: 1000; 4906) and phospho-Tuberin/TSC2 (Ser1387) antibody (1: 1000; 5584) were provided by Cell Signaling Technology Company (Beverly, MA, USA). Anti-beta actin antibody (1/5000; ab8227) was obtained from Abcam Company (Cambridge, UK).

2.2. Ethical guidelines and animals

Eight-week old male Wistar rats weighing 200–220 g, were obtained from Iran University of Medical Sciences, and housed four per cage to maintain social relationships. Animals were placed on a 12 h standard day/night schedule at a controlled room temperature [(25 ± 2) °C] with (60 ± 5)% humidity and free access to standard chow pellets and water ad libitum. All experimental tests and the procedures of this study were affirmed by the Institutional Animal Ethical Committee of Iran University of Medical Sciences and were conducted according to ethical guidelines for the care of laboratory animals and Principles of Laboratory Animals Care (NIH Publication No. 85–23, revised 1996).

2.3. Experimental groups

A total of 48 adult male Wistar rats were used in this study. As shown in Fig. 1, the animals were allocated into four major cohorts: (i) sham-operated control animals received all surgical steps except twisting the testis (sham; $n = 8$), (ii) in twisting the testis cohort, the animals were only subjected to twisting the testis for 30 min and removing of twisting for 24 h or 72 h (TT; $n = 8$), (iii) three cohorts of animals were subjected to twisting the testis and treated with various concentrations of cap 1 h after reperfusion (TT + 100, 500, and 1000 μg/mL cap; $n = 16$), (iv) three cohorts of healthy animals were only treated with various concentrations of cap (100, 500, and 1000 μg/mL cap; $n = 16$). In the case of treatment cohorts, non-fat dry milk solution was used as the solvent of cap. Drug was administrated 1 h after reperfusion.

2.4. Surgical procedures

Intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg) was performed to anesthetize rats. A low midline laparotomy was made to divide the gubernaculum; then the testis was dissected from its longitudinal and distal pole attachment to the epididymis. To induce ischemia, the left testis was rotated 720° clockwise and testis fixation to the scrotum was performed by a silk suture which was inserted through the tunica albuginea. Thirty minutes after the twisting, the tissue was counter-rotated back to the normal condition and reinserted into the scrotum torsion. After surgery, the animals were permitted to recover spontaneous breathing and were housed in their cages with free access to standard chow pellets and water ad libitum.

2.5. Anesthesia and tissue collection

At the end of the experimental period, rats were terminally killed under deep anesthesia and the testis was removed on ice and maintained at −80 °C prior to Western blotting assay.

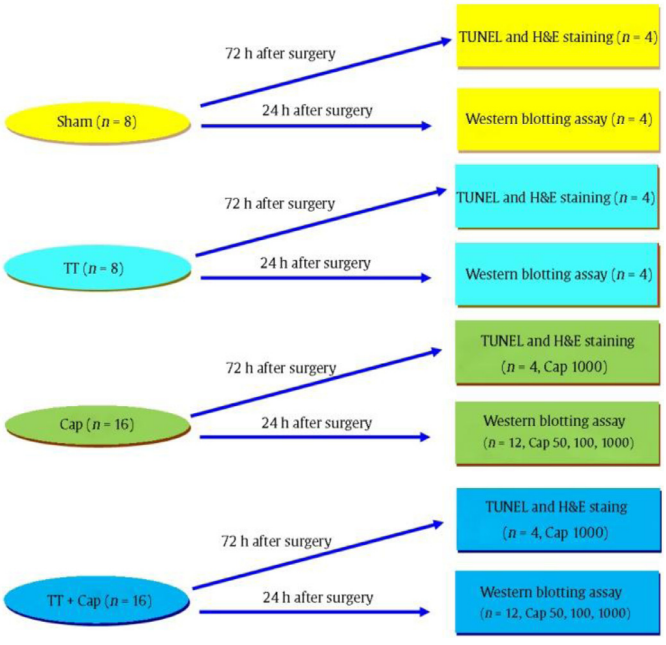

Fig. 1. Outline scheme of experimental designs.

2.6. Apoptosis assessment

Apoptotic cell death on paraffin slices of tissue was evaluated by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The amount of TUNEL-positive cells was calculated per tubule. Propidium iodide (PI) was used to stain the nuclei of cells. Five fields were investigated per slide and 100 cells were counted per field. The percentage of apoptotic cells or apoptotic index (AI) was calculated based on the following formula: AI = (number of apoptotic cells/the total number of counted cells) × 100%. In sum, the slices were deparaffinized and incubated with proteinase K solution. Then, slices were treated with 3% H_2O_2 for 10 min, and inserted in TUNEL reaction buffer for 10 min, and then immersed again in TUNEL reaction mixture at 37–40 °C for 60 min. Cell counting was performed by an experimenter blinded to experimental design.

2.7. Histological evaluations

To stain the slices by hematoxylin and eosin (H&E) method, tissues were fixed in 10% formalin for 4 d, and then embedded in paraffin. Microtome apparatus was used to prepare 4 µm-thick slices. Assessment of tissue damage was performed by an expert under light microscope.

2.8. Western blotting assay

For western blotting analysis, small pieces of tissues were homogenized in cell lysis buffer containing complete protease and phosphatase inhibitor. After centrifugation (16 400 r/min for 30 min) of lysates tissues, we determined protein concentrations by nanodrop. The samples were denatured at 90 °C for 4 min. Equal amounts of the protein (50 µg) were loaded in wells, separated by 8% SDS polyacrylamide gel electrophoresis and then transferred onto PVDF membrane (Millipore). Then, the samples were blocked with a solution containing BSA+ 0.05% Tween-20 at 4 °C for 12 h, followed by incubation with primary antibodies for 1 h and subsequently a horseradish peroxidase-conjugated secondary antibody (Anti-rabbit IgG). Blots were developed with Chemiluminescent HRP Substrate (Millipore) for 5 min and visualized by Kodak X-OMAT films.

2.9. Statistical analysis

The data are expressed as mean ± SEM. Analysis was performed using Prism Software, version 5 (CA, USA). One-way analysis of variance followed by LSD test for post hoc analysis was used for comparison of three or more groups. Values were considered statistically significant in $P < 0.05$.

3. Results

3.1. Effects of capsaicin on Tsc1 (Hamartin) expression

To obtain greater insight into cap effect on cell survival and clarify the molecular targets, we evaluated the expression of TSC1 protein 24 h after reperfusion in different groups. As depicted in Fig. 2A, no remarkable differences were found between sham, Cap (100, 500, and 1000 µg/mL), and TT cohorts. Significant ($P < 0.05$) differences were found between treated rats at the highest tested dose and other groups, giving clear evidence that cap promotes cell survival in a dose-dependent manner.

3.2. Effects of capsaicin on Tsc2 (Tuberin) expression

To prove cap promotes cell survival against following testicular I/R injury through activation of negative central modulators of mTOR, we evaluated phosphorylation of Tsc2 by Western blotting assay. As shown in Fig. 2B, phosphorylation of Tsc2 did not show a significant change in TT cohort relative to sham, cap 100, 500, and 1000 µg/mL. A significant increase in Tsc2 phosphorylation was found in animal subjected to TT and treated with 500 and 1000 µg/mL doses of cap.

3.3. Effects of capsaicin on DNA fragmentation and apoptosis cell death

To evaluate the effect of cap on DNA fragmentation and apoptotic cell death, we performed TUNEL staining. TUNEL staining sections were shown in Fig. 3. The ratio of apoptotic cells to the total cell number was denoted as AI. The AI value of TT group was significantly higher relative to sham and cap 1000 groups. Cap significantly decreased AI value at the highest tested concentration.

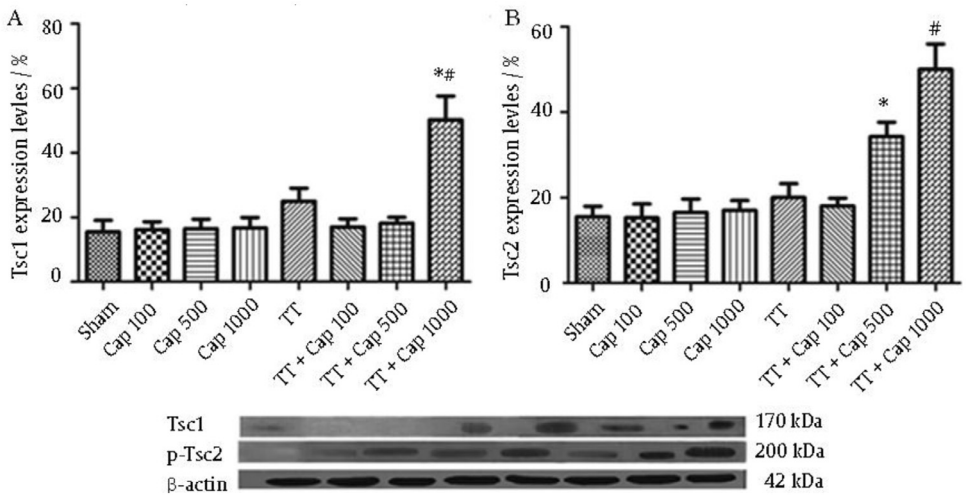

Fig. 2. Capsaicin pos-treatment increased expression of Tsc1 and phosphorylation of Tsc2 in a dose-dependent fashion. (A) Slightly increased expression of Tsc1 was observed in TT group. Cap administration at the highest dose (1000 µg/mL) significantly increased expression of TSC1, a negative central regulator of mTOR complex ($^{\#}P < 0.01$ *vs* TT group, $^{*}P < 0.001$ *vs* other groups). (B) Capsaicin administration significantly increased phosphorylation of Tsc2 after reperfusion in a dose-related manner. ($^{\#}P < 0.001$ *vs* sham, cap 100, cap 500, cap 1000, TT, and TT+ Cap 100 groups, $^{*}P < 0.05$ *vs* sham, cap 100, cap500, cap 1000, TT, and TT+ Cap 100 groups).

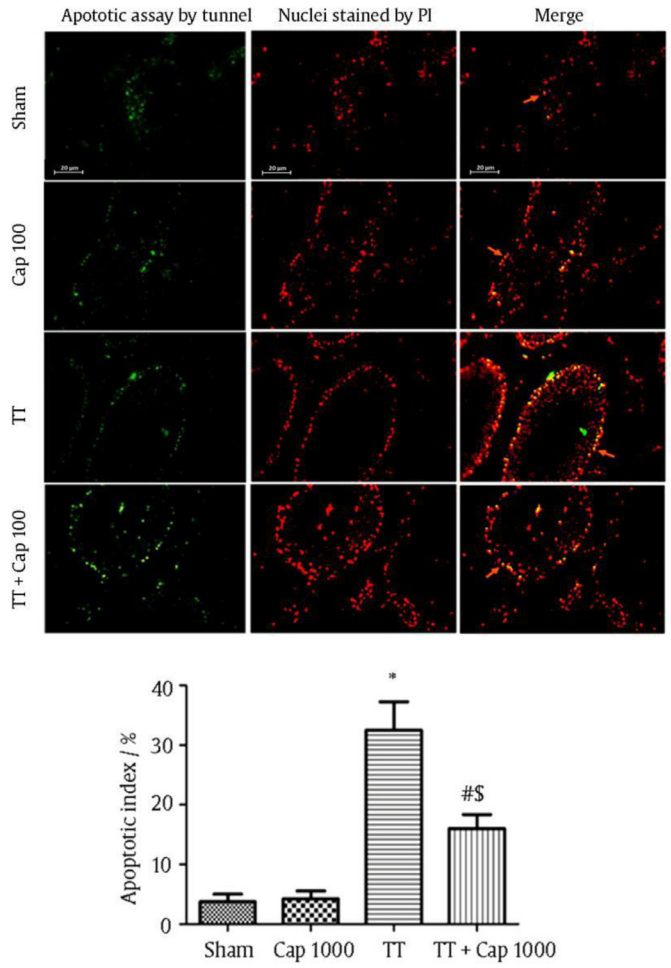

Fig. 3. TUNEL staining of testicular tissue (scale bar, 100 um). A few TUNEL-positive cells were observed in Sham and Cap 1000 group. In TT group, TUNEL-positive cells were highly observed in Sertoli and spermatogonia cells of testis ($^{*}P < 0.001$ *vs* sham and cap 1000 groups). Capsaicin post treatment significantly decreased TUNEL-positive cells ($^{\#}P < 0.01$ *vs* TT group) ($^{\$}P < 0.01$ *vs* sham and cap 1000 groups).

3.4. Effects of capsaicin on tissue injury

To determine tissue damage, H&E staining was used. Fig. 4 showed the findings of the light microscopy for different groups. Normal testicular structure was found in sham and cap 1000 groups. Severe distortion of tubules and cell damage were seen in the TT group. IP injection of cap at the highest tested concentration significantly reduced injury in testes after torsion/detorsion.

4. Discussion

In this study, we found that administration of cap 1 h after reperfusion in a rat model of testicular torsion could inhibit apoptosis cell death and tissue injury. We also sought to clarify the possible mechanisms of cap in protection against testicular torsion. Some factors including the expression of Tsc1, phosphorylation of Tsc2, tissue injury, DNA fragment and apoptosis activity have been detected 24 or 72 h after reperfusion. Cap administration markedly increased the expression of Tsc1 and Tsc2 phosphorylation in animals subjected to TT in a dose-dependent manner. Likewise, cap mitigated cell apoptosis death and abrogated tissue injury in the ipsilateral testis following TT at the highest tested concentration. High polyunsaturated fatty acid content of the sperm membrane makes it highly sensitive to oxidative stress. ROS attacks to high long chain polyunsaturated fatty acid content of the plasma membrane that may result in necrotic or apoptotic cell death (Nissen & Kreysel, 1983). On the other hand, oxidative stress might dysregulate different cellular signaling pathways which lead to generation of ROS and free radicals, imbalance in electron transport chain and consequently poor cell survival (Amani et al., 2017a; Pazoki-Toroudi et al., 2003; Pazoki-Toroudi, Ajami, & Habibey, 2010).

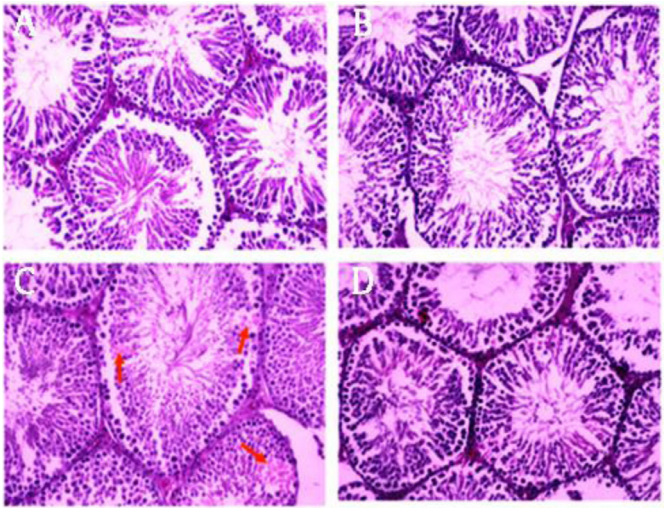

Fig. 4. Light microscope images of H&E stained sections (Magnification × 200). (A and B) Sham and Cap1000 group: normal testicular tissue was observed. (C) TT group: severe damage to testis was seen. (D) Capsaicin post treatment remarkably decreased tissue /or cell damage at the highest dose.

Among these various cellular signaling pathways, aberrant activation of Akt and mTOR trigger necrotic cell death (Liu et al., 2014). It is well documented that mTOR is strongly expressed at the site of the blood-testis barrier, microtubules and by Sertoli cell and plays a pivotal role in the preservation of testicular junctions and Sertoli cell polarity. Dysregulated activation of mTOR following deletion of Lkb1 and Tsc1/ Tsc2 complex results in large loss of sertoli and germ cells and subsequently hamper spermatogenesis (Tanwar, Kaneko-Tarui, Zhang, & Teixeira, 2012). Previous studies have reported that many new agents play a great part in the treatment of diseases through modulation of different cellular signaling pathways (Habibey & Pazoki-Toroudi, 2008; Pazoki-Toroudi et al., 2010; Zarch et al., 2009). Pharmacological studies have introduced many new agents that contribute to diagnosis and treatment of oxidative stress related diseases and other disorders (Firooz et al., 2005; Gu et al., 2018; Jazayeri et al., 2016; Song, Su, Wei, Liu, & Yin, 2017; Sun et al., 2018).

Many recent studies have shown that cap can be partly responsible for protection against IR in various tissues such as lung, kidney, and heart via activation of TRPV1 and subsequently inhibition of acute inflammatory responses and oxidative stress (Ueda, Tsuji, Hirata, Takaoka, & Matsumura, 2008; Wang, Ji, Wang, Zhao, & Xia, 2012; Zhong & Wang, 2008). Moreover, some existing researches indicated that activation of TRPV1 can promote cell survival by modulating various cellular signaling pathways (Chen, Geis, & Sommer, 2008). Maiese *et al.*, indicated that TRPV1 receptors not only affect cellular biology through regulation of calcium signaling but also are closely associated with mTOR, AMPK, and Akt that are master regulators of cellular metabolism, stem cell development, pain sensitivity, and cell survival (Maiese, 2017). Tuberins (Tsc1/Tsc2), the tumor-suppressor proteins, are negative central regulators of mTORC1 complex which play a master role in numerous biological functions (Huang & Manning, 2008). Boothby and Lee reported that Tsc deficiency is a factor in aberrant activation of mTOR that, sequentially, results in imbalance in electron transport chain, generation of ROS, and cell death (Boothby & Lee, 2011).

In this study, our results showed that cap exerts protective effects against I/R injury following TT in a dose-dependent manner by activating Tsc1. In keeping with our finding in a previous study, Buchan and coworkers reported that under oxidative stress, activation of Tsc1 gave rise to neuroprotection in an mTOR dependent mechanism. Activated Tsc1 potently inhibited mTOR complex that resulted in induction of protective autophagy and subsequently cell survival (Papadakis et al., 2013). In addition, it has been shown that the possible mechanism of TRPV1 receptor activation by cap is associated with induction of protective autophagy (Liu et al., 2013; Maiese, 2016).

As a further matter, previous studies showed that activated Tsc1 enhanced phosphorylation of Tsc2 to indirectly inhibit mTORC1 complex (Inoki, Zhu, & Guan, 2003). In present study, the phosphorylated level of Tsc2 protein was determined to prove that Tsc1 activation by cap guard against I/R injury following TT in an mTOR dependent mechanism. Our results confirmed this hypothesis and showed that cap increased Tsc2 phosphorylation in a dose-dependent manner. In agreement with these findings, a previous report showed that cap exerted protection against TT through inhibition of mTOR signaling pathway (Javdan et al., 2018).

Additionally, previous reports have shown that absence of the TSC1/TSC2 complex lead to Foxo1 inactivation, a master mediator in expression of anti-oxidant enzymes, that, in turn, increase cell death under oxidative stress (Maiese, 2014; Malik & Storey, 2011). According to these reports, a current report by Pazoki-Toroudi *et al.*, demonstrated that cap promoted cell survival in I/R injury following TT by overexpression of FoxO1 (N. Javdan, Ayatollahi, Iqbal Choudhary, Al-Hasani, & Pazoki-Toroudi).

Moreover, our results indicated that cap was mitigated apoptosis cell death in the ipsilateral testis following TT through amelioration of DNA fragmentation. In keeping with our finding, Tanyel and coworkers reported that cap remarkably decreased nuclear DNA fragment in the contralateral testis after TT (Sarioglu-Buke, Erdem, Gedikoglu, Bingol-Kologlu, & Tanyel, 2001). In present work, H&E staining showed that highest tested dose of cap was abrogated tissue damage in the ipsilateral testis following TT. According to our results, some exciting researches showed that cap displayed a great role in coping with oxidative stress under pathologic conditions (Du et al., 2010; Huang et al., 2017).

5. Conclusion

In summary, our results indicated that cap displays protection against I/R injury following TT through the mitigation of apoptosis and targeting central negative regulators of mTOR complex. It is our hope that this study will serve as a base for future attempts to expand exploratory studies about the possible mechanisms and therapeutic potential of cap to rescue spermatogenesis in men with infertility.

Conflicts of interest

All authors declare that there are no conflicts of interest.

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