Theriogenology 144 (2020) 98–106

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Investigating the sperm parameters, oxidative stress and histopathological effects of salvia miltiorrhiza hydroalcoholic extract in the prevention of testicular ischemia reperfusion damage in rats

Farshid Davoodi [a], Shayan Taheri [a], Abbas Raisi [a, *], Asghar Rajabzadeh [b, c], Hassan Ahmadvand [b, d], Mohammad Hassan Hablolvarid [e], Amir Zakian [a]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran
[b] Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran
[c] Department of Anatomical Sciences, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran
[d] Department of Biochemistry, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran
[e] Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran

ARTICLE INFO

Article history:
Received 1 November 2019
Received in revised form
22 December 2019
Accepted 1 January 2020
Available online 3 January 2020

Keywords:
Testicular torsion detorsion
Salvia miltiorrhiza
Sperm parameters
Oxidative stress
Antioxidant
Ischemia reperfusion

ABSTRACT

Aims: One of the most common urologic emergencies is spermatic cord torsion, which can damage testicular tissue and reduce fertility. Salvia miltiorrhiza (SM) hydroalcoholic extract possess high antioxidant properties, and its efficacy in ischemia-reperfusion (I/R) injury prevention has been demonstrated in cardiac, renal, and liver tissues. Therefore, the purpose of this study was to assess the protective mechanism of SM extract on testicular I/R damage.
Main methods: 18 mature male Wistar albino rats were randomly divided into 3 groups; with six rats in each group: Group 1 (Sham) was sham-operated. Group 2 (T-D): torsion was performed, and after 2 hours (h) detorsion was done. Group 3 (SM): (200 mg kg^{-1}) SM was intraperitoneally injected thirty minutes before detorsion. Then testicular and epididymal weight and size alterations, sperm parameters (motility, livability, concentration, and morphology), both plasma and testicular tissue levels of malondialdehyde (MDA), catalase (CAT), glutathione peroxidase (GPX), and total antioxidant capacity (TAC) were evaluated. Also, histopathological changes included mean seminiferous tubular diameter (MSTD), testicular capsule thickness (TCT), mean testicular biopsy scoring (MTBS), and germinal epithelial cell thickness (GECT) were examined.
Results: Testicular I/R significantly reduced sperm motility, viability, and normality, while SM extract administration remarkably increased sperm motility, and normality ($P < 0.05$). Induction of testicular T-D caused a significant increment in the level of MDA and notable decline in the levels of GPX, CAT, and TAC both in plasma and testis tissue, whereas administration of SM extract significantly decreased MDA level and increased GPX, CAT, and TAC levels in plasma and testicular tissue ($P < 0.05$). Histopathological parameters including MSTD, GECT, MTBS, and TCT were significantly lower in the T-D group, while pretreatment with SM extract remarkably increased MSTD, GECT, and MTBS amounts ($P < 0.05$).
Conclusion: Since the SM extract increased the activity of antioxidant enzymes, improved sperm parameters and reduced the damage to testicular tissue, therefore, its use as a potent antioxidant in reducing testicular I/R damage is suggested.

© 2020 Elsevier Inc. All rights reserved.

1. Introduction

Spermatic cord torsion is one of the common urological emergencies that can damage testes, the main reproductive organ, and cause infertility [1]. There are two timeframes for testicular torsion, the first occurring in the first year of life and before puberty, and the second, more prevalently occurring in the puberty [2]. Subfertility

* Corresponding author.
E-mail addresses: E-mail address: raisi.a@lu.ac.ir (A. Raisi).dr_abbas_raisi@yahoo.com (A. Raisi).

https://doi.org/10.1016/j.theriogenology.2020.01.002
0093-691X/© 2020 Elsevier Inc. All rights reserved.

and infertility are inevitable, if the testicular torsion is not diagnosed quickly and surgically corrected [3]. Time elapsed and the intensity of torsion are two crucial factors in the success of surgery in maintaining fertility [4]. Spermatic cord torsion leads to blood flow disruption and ischemia and following by detorsion; reperfusion, generates reactive oxygen species (ROS) [5]. ROS comprise four main groups: radical molecules, non-radical molecules, oxygen derivatives and nitrogen derivatives that are produced during the different biological activities of the body [6]. Oxidative stress is a condition that ROS is over generated in the body organs and the antioxidant defense is unable to scavenge and neutralize the extra ROS so the balance between ROS production and elimination is disturbed [6]. Natural enzymatic (superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidants (zinc, vitamin C, vitamin E, melatonin and cytochrome C) in the body effectively protect the testes from injury [7]. Oxidative stress damages all cellular components such as nucleic acids, proteins, lipids, and carbohydrates and the injury rate depends on the amount of the ROS produced and exposure time [8]. Testicular tissue, especially spermatozoa cells, possess high amount of unsaturated fatty acids and spermatogenesis in the germinal layer is performed at high speed, therefore, it consumes a lot of oxygen and consequently very sensitive to oxidative damage [8]. As demonstrated in previous studies, oxidative damage and increased lipid peroxide content lead to decreased sperm motility as well as DNA damage in the genome of spermatozoa and germ cells and induces apoptosis of testicular tissue [2,9,10].

Medicinal herbs are a group of herbs that are known in many countries for the treatment of diseases and have certain compounds that help improve human health. According to statistical analysis, approximately 80% of people in developing countries and about one-third of people in the United States use herbs to treat diseases [11]. The exact details of the properties and mechanisms of many herbs in traditional medicine are still unclear to patients and physicians [12].

The genus Salvia belongs to Lamiaceae family known throughout the world as a medicinal plant, and it is used as a flavoring agent and in fragrance compounds [13]. The genus Salivia has about 900 species, of which 58 species are grown in Iran, and about 17 are endemic to Iran [13]. Salvia miltiorrhiza (SM) is a potent antioxidant with a high ability to scavenger free radicals comprising diphenyl-2-picrylhydrazyl, hydroxyl, and superoxide anion radicals [14]. The well-known properties of salvia miltiorrhiza are antioxidant, antimicrobial, anti-inflammatory, anti-spasmodic, reduce myocardial infarction and aggregation of platelets [15]. Also, in China, Russia, and Korea, there is a dripping herbal pill known as Daneshan to cure cardiovascular disease, comprising atherosclerosis, coronary artery disease, vasculitis, and cerebral infarction, that one of the main components of which is Salvia miltiorrhiza [14]. Previous studies have shown that the beneficial properties of SM extract in ischemic injury comprised of vasodilatation, increased blood flow, and reduced free radicals [16]. To our knowledge, the effect of SM extract on ischemia-reperfusion (I/R) has been investigated in several organs, including the heart, kidney, and liver, but no studies have been accomplished on the SM extract influences on testicular I/R [17–20]. So this study was conducted to assess the mechanism that salvia miltiorrhiza protects rat testis in I/R injury induced by torsion/detorsion.

2. Material and methods

2.1. Hydroalcoholic extract preparation

Salvia miltiorrhiza leaves were obtained from the Barij Essence research farm, Kashan, Iran. One sample of the Salvia leaves were placed at Barij Essence research center, department of agriculture, under herbarium number 186-1. To prepare SM extract, 500 g of the plant was dried at room temperature (25 °C) for ten days. The dried leaves were then thoroughly powdered and dissolved in 1 L of 96% ethanol in a container and left at room temperature for two days (The container was shaken once a day for 15 min). Then the extract was filtered off, and the extract solvent was removed. Thereafter, the filtered solution was extracted by a vacuum evaporation method using a rotary evaporator (IKA RV 05 Rotary Evaporator, Iran). Approximately 100 g extract with high viscosity was obtained and placed in the oven for 24 hours (h) for further concentration. At the end, the resulted powder was dissolved in distilled water to reach the desired concentration and utilized in the experiment.

Salvia miltiorrhiza liquid extract analysis: Also, prior to removal of the solvent from the extract, the salvia liquid extract analysis in the central laboratory of the Barij Essence Pharmaceutical Co was performed (Code: FCL64-03). The results of this analysis indicate that the Specific Gravity was 0.974 (g ml^{-1}) by USP38-⟨841⟩ reference method, pH was 5.83 by ISIRI1487-(2-2-5) reference method, the Dry residue was 12.83 (%W/W) by BP2015 reference method, the Refractive Index was 1.3764 (nD) by ISIRI2274-6 reference method, and the Rosmarinic acid amount was 14.28 (mg ml^{-1}) by Barij Essence reference method.

Ferric reducing-antioxidant power (FRAP) analysis: The FRAP method was used to test the total antioxidant power of the hydroalcoholic extract of Salvia miltiorrhiza [21]. This method measures the reduction ability of the extract to convert Fe^{3+} to Fe^{2+}. Resulted iron ions (Fe^{2+}) in acidic pH and the presence of tripyridyl-s-triazine (TPTZ, Fluka, Buchs, Switzerland) as a reagent forms Fe-TPTZ complex which is blue and its intensity was measured by spectrophotometer (JENWAY 6715 UV/Vis Spectrophotometer, Staffordshire, UK) at a wavelength of 593 nm. This is a nonspecific reaction, meaning that any molecule that can reduce the ferric ion (Fe^{3+}) participates in this reaction. The standard used in this analysis was ferrous sulfate (FeSO4) with concentrations of 25–1000 μmol/l. The FRAP of the SM extract was 24.73×10^2 μmol FeSO4 equivalents per liter of sample.

2.2. Animals and groups

Ethical approval was obtained from the Animal Ethics Committee (LU.ACRA.2018.14) at the Veterinary Faculty of Lorestan University. The experiments were done using eighteen sexually mature male Wistar albino rats with an average weight of 250–300 g (10–12 weeks old). Standard conditions in terms of temperature (23 ± 1 °C), humidity (about 65%), and photoperiod 12-h were provided for the rats at the animal house. Pellet diet and water were provided as much as rats need. Prior to experimentation, the animals remained in laboratory conditions for one week to get accustomed to the conditions, and were randomly divided into 3 groups; with six rats in each group:

1 Group 1 (sham): In this group, the left testis was drawn out, and then returned to its ordinary scrotal position.
2 Group 2 (T-D): Torsion was performed, and after 2 h detorsion was done.
3 Group 3 (SM): Testicular torsion/detorsion was induced, and thirty minutes before testicular detorsion rats received Salvia miltiorrhiza (200 mg kg-1) intraperitoneally [22–24].

2.3. Surgical procedure

The surgical procedure of this study was conducted in sterile conditions. Anesthetics include the combination of ketamine 10%

(80 mg kg-1, Alfasan, Woerden, The Netherlands) and xylazine 2% (10 mg kg-1, Alfasan, Woerden, The Netherlands) were administered by intraperitoneal injection. After shaving the scrotal area and disinfection by a 10% povidone-iodine solution, a vertical incision in the skin of the middle part of the scrotum was made, and the left testicle was drawn out and rotated 720° clockwise. Then three simple interrupted stitches were applied to fix the testis in the torsion position (5/0 silk non-absorbable, SUPA, Iran). After 2 h, testicular detorsion was performed, and a simple running suture technique was carried out in the closure of the scrotal skin (4/0 nylon non-absorbable, SUPA, Iran). Two hours later, thiopental sodium (250 mg kg-1, i.p. Exipental, Exir, Iran) was injected to euthanize rats, and 3 ml blood samples were obtained (left ventricle), and they experienced an orchiectomy surgery. The caudal part of the epididymis was separated to examine the sperm parameters, and with a longitudinal incision in the middle of the testis, the testis was divided into equal parts. One part was kept in a 10% buffered formalin solution, and the second part was frozen at −80 °C (Ultra-low temp freezer, JAL TAJHIZ, Iran) in order to determine the biochemical parameters. The blood samples were poured into the EDTA containing tubes (K2 EDTA CBC, FARTEST, Isfahan, Iran) to prevent coagulation, Then, 10 min centrifugation (Hettich ROTOFIX 32 A, Germany) at 3000 rpm was performed to separate the plasma, and the plasma was placed in a microtube and frozen at −80 °C.

2.4. Macroscopic measurements

After orchiectomy, the testes and the epididymis were examined for appearance and testicular and epididymal size and weight were measured in centimeter and gram (Sartorius BP61S, Göttingen, Germany) in all experimental groups, respectively (Fig. 1).

2.5. Evaluation of sperm parameters

Sperm collection: Varisli et al. 's method was used to collect mature sperm stored in the epididymis [25]. Petri dishes were filled with 5 ml of RPMI 1640 medium (INOCLON, Karaj, Iran), and the caudal part of the epididymis was separated and inserted in the petri dishes. Then different cuts were made to allow spermatozoa to exit the epididymis more easily, and petri dish containing epididymis was incubated in the incubator at 37 °C for 15 min. Sperm parameters were then measured according to the methods proposed by the World Health Organization (WHO) in 2010 [26].

Sperm motility: Diluted sperm samples in 5 ml RPMI medium were utilized to evaluate sperm motility. A phase-contrast microscope (Olympus IMT-2, Japan) was used to observe the samples at 400× magnification. According to the WHO method, about two hundred spermatozoa in each sample were examined for different types of sperm motions, including progressive (PR), non-progressive (NP), and Immotile (IM) [26].

Sperm viability: The second parameter after sperm motility that should be evaluated quickly is the viability of the sperms. Based on WHO sperm analysis methods, there are three methods to assess sperm vitality, including the eosin-nigrosin staining method, eosin staining method, and hypo-osmotic swelling method. In this study, we used the eosin staining method as follows: 100 ml sodium chloride 0.9% solution was prepared, and 0.5 g eosin Y (Merck, Germany) was dissolved in it. Then 5 μL of 0.5% eosin Y solution was mixed with 5 μL of the sperm sample, and a thin smear was placed on the slide and incubated at 37 °C for 30 s. Subsequently, the light microscope (CX21, Olympus, Japan) was used to determine the viability of spermatozoa. In each slide, approximately 200 spermatozoa were evaluated for viability to minimize test error. In this staining method, live sperm's heads are stained in white or faint pink, and dead sperm's heads are stained in red or dark pink [26] (Fig. 2).

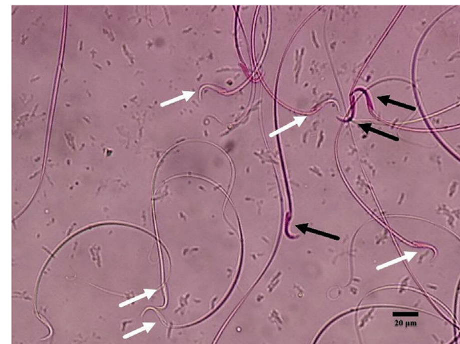

Fig. 2. Eosin stained slide for evaluation of sperm vitality. Black arrows show dead spermatozoa with red or dark pink heads and white arrows show live spermatozoa with white and or pale pink heads. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

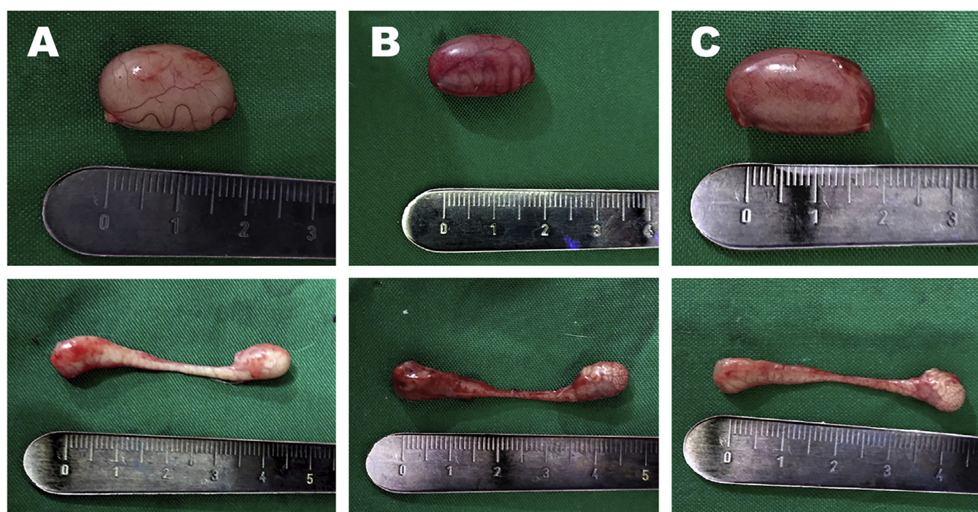

Fig. 1. Macroscopic evaluation of testis and epididymis sizes. Column **A** shows the sham group, Column **B** shows the T-D group, and Column **C** shows the SM treatment group.

Sperm concentration: To count and determine sperm concentration, sperm motilities must be stopped (in order to ease counting) therefore, spermatozoa should be fixed using a fixative solution. The fixative solution was made from 0.5 g sodium bicarbonate (NaHCO3) and 1 ml of 35% formalin solution dissolved in 100 ml of purified water. Then 50 μL of the sample was mixed with 200 μL of the fixative solution to achieve a 1:5 dilution rate, and a 10 μL aliquot of the diluted sample was loaded to Neubauer hemocytometer (Brand, Germany). Then, according to the WHO method, the sperm concentration as sperm/ml was calculated by counting 200 spermatozoa with the phase-contrast microscope at 400× magnification [26].

Sperm morphology: Sperm morphology was determined following these steps: First, an aliquot of sperm suspension (10 μL) was located on a slide, and a smear was prepared. The slide was air-dried and then fixed with 70% ethanol and finally stained with hematoxylin Papanicolaou's solution. (Padtan Teb, Iran). About 200 spermatozoa were examined for normal and abnormalities. Abnormal sperms were classified into categories of short tail, coiled tail, bent tail, cytoplasmic droplet, elongated head, and detached head with a very short tail (Fig. 3).

2.6. Biochemical assays

Preparing testis tissue for enzyme analysis: Testis tissue specimens stored in −80 °C, were defrosted and separated into minor pieces and placed in phosphate buffer solution (PH = 7.4). The samples were then homogenized with homogenizer (Cole-Parmer LabGEN 700 Homogenizer, U.S) and centrifuged at 3000 g for ten minutes to separate the supernatants. We used the supernatants to ascertain oxidative stress.

Oxidative stress biomarkers assessment: In order to determine total protein concentrations, malondialdehyde (MDA), catalase (CAT), glutathione peroxidase (GPx), and total antioxidant capacity (TAC) levels, the colorimetrical commercial biochemical kits (Asan, Khorramabad, Iran) were used.

2.7. Histopathologic evaluations

In this study, two types of tissue staining methods, including hematoxylin and eosin (H&E) (Fig. 4 a, b), and Masson's trichrome (Fig. 4 c, d), were performed. H&E staining protocols were carried out as follows: tissue samples were removed from the fixative (10% neutral buffered formalin) and embedded in paraffin wax for preparation of tissue blocks. Subsequently, thin sections (4 μm thickness) were made from all parts of the testis and after paraffin removal, staining was performed. The pathology slides were appraised by a skilled pathologist, unknowing the previous steps of the experiment. Microscope slides were observed using a light microscope (CX21, Olympus, Japan) with different magnifications of 100×, 200×, and 400×. Histopathological changes and testis tissue necrosis were examined. A Microscope Eyepiece Reticle was used to investigate germinal epithelial cell thickness (GECT) in two cases according to micron thickness and the number of cell layers, mean seminiferous tubular diameter (MSTD), and mean testicular biopsy scoring (MTBS) according to Johnson's method (Table 1) [27]. Masson's trichrome staining method was performed to measure testicular capsule thickness (TCT).

2.8. Analytical approach

The statistical analysis was carried out by MedCalc software (Ver. 14.8.1, Ostend, Belgium) and Analyse-it software (Ver. 4.80.8, Leeds, UK) in order to examine the effects of different treatments on sperm parameters, oxidative damage markers, and histopathological alterations with final significance declared at a P-value ≤0.05. Descriptive statistics of each variable were calculated for sham, T-D and SM groups. Distributions of data in each experimental groups were evaluated relying on one-sample Kolmogorov-Smirnov test, then data with statistically normal distribution were shown as mean ± standard deviation, and data with non-normal distribution were reported as median and interquartile range. Groups with normal distribution data were evaluated using one-way ANOVA with Tukey-Kramer's post hoc, and non-normal distributed data were compared using the non-parametric Kruskal-Wallis test.

3. Results

3.1. Testicular, epididymal weight and size measurements

Testis and epididymis weight, as well as size (Fig. 1), are presented in Table 2. There was no significant difference between groups (p > 0.05).

3.2. Sperm parameters

The results of the sperm motility parameter are shown in Fig. 5(a). In the T-D group, the PR motility is significantly lower than the sham group, and the NPR, and IM motilities were significantly

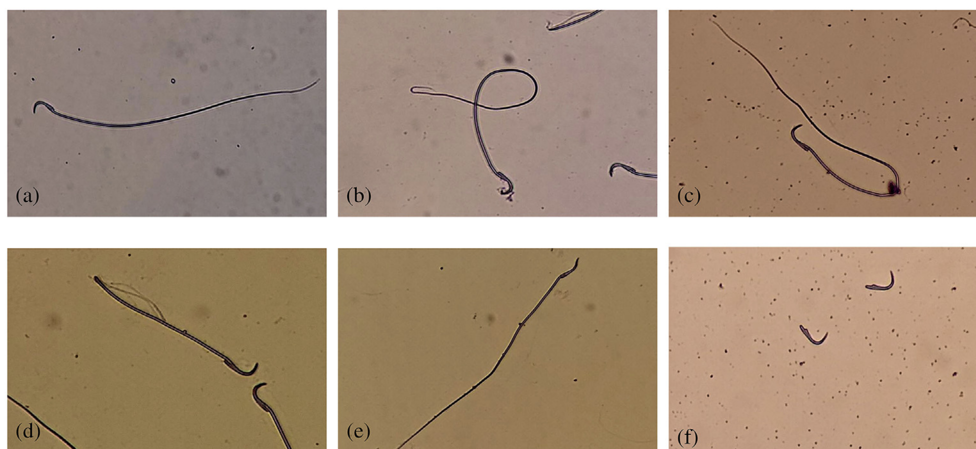

Fig. 3. Hematoxylin Papanicolaou's solution stained spermatozoa with normal and abnormal morphology. (400x) **(a)** normal spermatozoa; **(b)** coiled tail; **(c)** bent tail; **(d)** short tail; **(e)** abnormal (elongated) head; **(f)** free head with a very short tail.

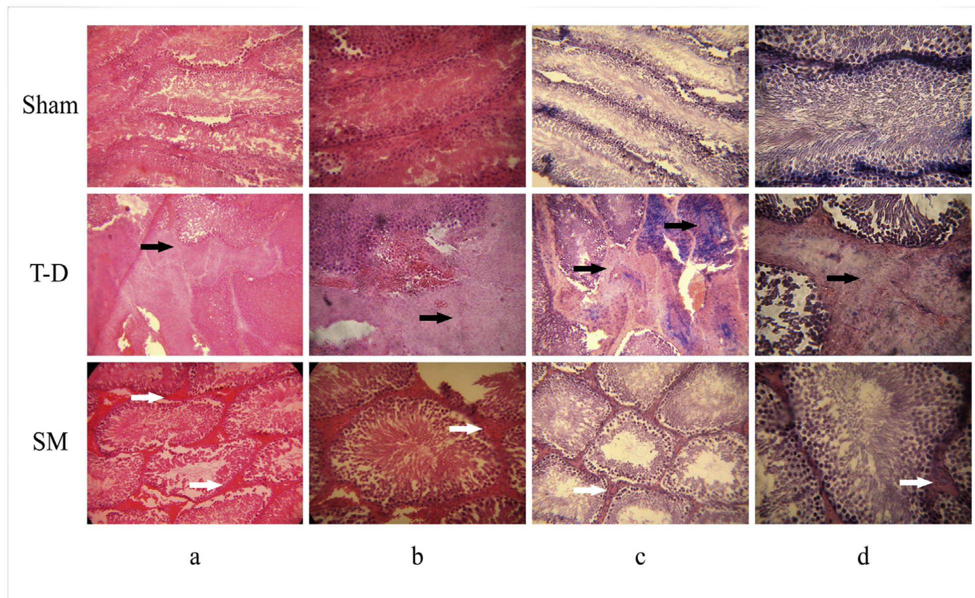

Fig. 4. Hematoxylin and eosin (H&E) and Masson's trichrome stained slides of seminiferous tubules for histopathological evaluation. Column **a** shows H&E with 200X magnification, column **b** shows H&E with 400X magnification, column **c** shows Masson's trichrome with 200X magnification, and column **d** shows Masson's trichrome with 400X magnification. Black arrows indicate extensive coagulation necrosis in the T-D group. White arrows indicate interstitial haemorrhage in the SM group.

Table 1
Mean testicular biopsy scoring (MTBS) according to Johnson's method.

Score	Spermatogenesis level
10	Full spermatogenesis along with the presence of numerous spermatozoa
9	Slightly impaired spermatogenesis, many late spermatids, disorganized epithelium
8	Less than five spermatozoa per tubule, few late spermatids
7	Absence of spermatozoa and late spermatids, many early spermatids
6	Absence of spermatozoa and late spermatids, few early spermatids
5	Absence of spermatozoa and spermatids, many spermatocytes
4	Absence of spermatozoa and spermatids, few spermatocytes
3	Spermatogonia only
2	Absence of germ cells, Sertoli cells only
1	Absence of seminiferous epithelial cells

higher than the sham group ($p < 0.05$). The SM administration could significantly increase the PR motility compared to the T-D group and reduced the NPR motility in comparison with the T-D group ($p < 0.05$). It can be seen from the data in Fig. 5(b) that sperm viability was significantly lower in the T-D and SM groups compared with the sham group, but no significant difference was observed between the T-D and SM groups ($p > 0.05$)(Fig. 2). The results obtained from the preliminary analysis of sperm concentration are presented in Fig. 5(c). It is apparent from this figure that sperm concentration in the SM group is significantly higher than the T-D and sham groups ($p < 0.05$). Table 3 presents the summary statistics for sperm morphology parameters. In the sham group the normal morphology percentage of spermatozoa was significantly greater than the T-D group ($p < 0.05$). SM administration could substantially increase the normal morphology percentage compared with the T-D group ($p < 0.05$). In the other morphological sperm parameters, there was a significant difference just for the coiled tail percentage among the T-D group and sham group ($p < 0.05$).

3.3. Oxidative stress parameters

The results of the testicular tissue and plasma oxidative stress biomarkers evaluation are set out in Table 4. A significant increment in the MDA levels of the testicular tissue and plasma in the T-D group were observed compared to the sham group ($p < 0.05$), and both testicular tissue and plasma levels of the MDA in the SM group were significantly lower than the T-D group ($p < 0.05$). As it can be observed in Table 4, testicular torsion detorsion significantly reduced both testicular tissue and plasma levels of the GPX, CAT and TAC in comparison with the sham group ($p < 0.05$), also SM pretreatment notably increased all the mentioned parameters ($p < 0.05$) except for the testis tissue CAT that its change was not notable ($p > 0.05$).

3.4. Histopathological parameters

The histopathological parameters are illustrated in Table 5. Experimental testicular torsion detorsion (T-D group) significantly decreased all histopathological parameters including MSTD, GECT, GECT based on cell layer, MTBS, and TCT in comparison with the sham group ($p < 0.05$). The MSTD, GECT, GECT based on cell layer, and MTBS values were significantly increased in the SM group than the T-D group ($p < 0.05$). The difference between the SM group and the T-D group for the TCT parameter was not notable ($p > 0.05$).

Table 2
Macroscopic evaluation of testicular and epididymal weight and size. All values are displayed as Mean ± SD.

Groups	Testis weight (g)	Testis size (cm)	Epididymis weight (g)	Epididymis Size (cm)
Sham	1.48 ± 0.10	1.72 ± 0.16	0.72 ± 0.17	4.12 ± 0.13
T-D	1.39 ± 0.08	1.68 ± 0.08	0.66 ± 0.16	4.24 ± 0.23
SM	1.44 ± 0.09	1.74 ± 0.11	0.70 ± 0.18	4.28 ± 0.25

[a] $p < 0.05$ compared with the sham group.
[b] $p < 0.05$ compared with the T-D group.

4. Discussion

Spermatic cord torsion is classified as pediatric urological emergencies, which can lead to infertility if not diagnosed quickly, and no treatment is performed [28]. The only way to treat testicular torsion after diagnosis is to have immediate surgery for reperfusion in ischemic tissue. Many previous studies have shown that reperfusion is a factor in further damage to testicular tissue [29]. Testicular tissue is a tissue very sensitive to free radicals because it is highly active in cell division and spermatogenesis is constantly ongoing, so it consumes a great deal of oxygen and is extremely vulnerable to oxygen depletion [8]. All living cells of the body, including spermatozoa and testicular tissue cells, produce ROS during their normal metabolism, that the abnormal increase in the concentration of these ROS causes severe damage to these cells [30]. Therefore, it has been shown that the original factor in male infertility is oxidative damage, as measured by the level of ROS in seminal fluid in infertile men [10,31]. Testicular tissue, semen, and spermatozoa contain antioxidant enzymes including MDA, SOD, CAT, and GPX, which neutralize free radicals, but due to I/R injury, excessive production of ROS causes the enzyme to be insufficient to scavenger all free radicals [32]. There are several studies investigating the effects of potent antioxidants (phytochemicals, vitamins, and enzymes) in preventing testicular torsion/detorsion damage in rats. Effects of melatonin on sperm parameters after testicular torsion/detorsion were evaluated and it was concluded that melatonin significantly reduced sperm abnormalities [33]. In another study, the effects of lycopene on testicular torsion were examined and showed that lycopene remarkably increased sperm motility and reduced abnormal sperm count [34]. In an investigation, the effects of ghrelin on sperm parameters in testicular I/R were examined and it was concluded that ghrelin significantly increased sperm motility and concentration [35]. Azizollahi et al., 2009 in a study on effects of vitamin C in rats, after testicular torsion induction, found that vitamin C treated group notably increased the spermatogenesis rate and seminiferous diameter when compared to the I/R group [36]. Obzel et al., 2012 reported that α-Lipoic Acid administration after experimental testicular torsion enhanced the MSTD and Johnsen's score than control group [37]. However, most powerful antioxidants are found in plants. Plenty of herbal extracts with high antioxidant properties have been used in previous studies to prevent I/R injury in testis [38–43].

One of the herbs used in folk medicine as a choice for cardiovascular ischemia is the Salvia miltiorrhiza [44–46]. Previous studies have shown that SM has vasodilatory properties and increases blood flow, including in the coronary artery, and reduces damage in ischemic complications by neutralizing free radicals [16]. Studies on SM have proven that the plant contains active compounds such as terpenoids, flavonoids, and phenolic acids [47,48]. It has also been shown in other studies that rosmarinic acid is the main antioxidant agent found in the SM [49]. Many flavonoids have been studied so far, but the structure of rosmarinic acid is very different from all of them. Extensive research into the antioxidant capacity of rosmarinic acid shows three times higher

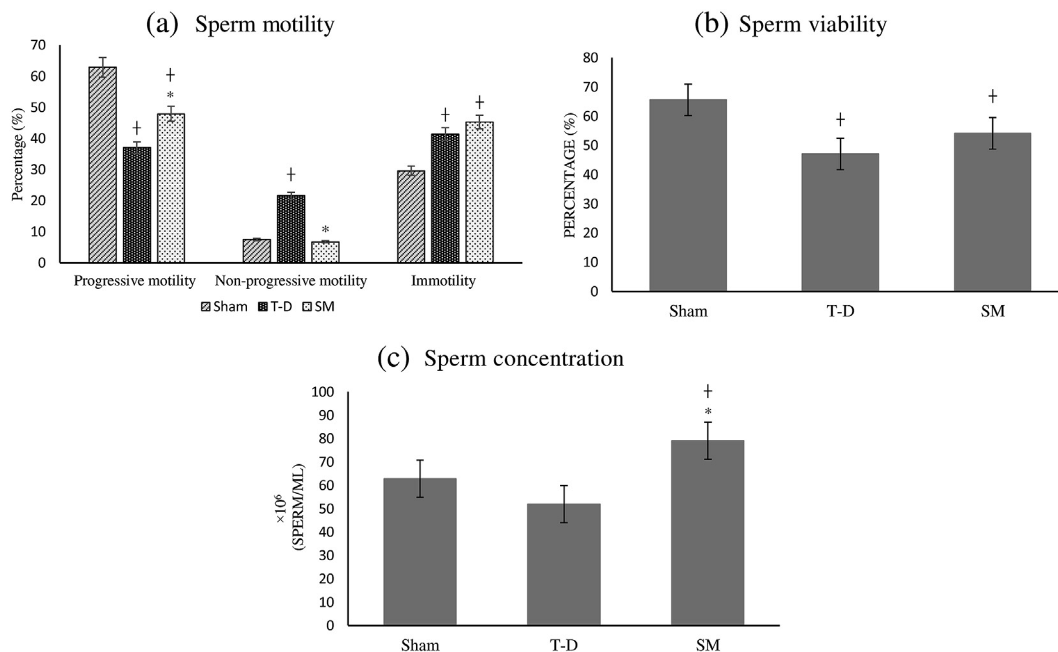

Fig. 5. **(a)** The comparison of sperm motility percentage, **(b)** the comparison of sperm viability percentage, and **(c)** the comparison of sperm concentration in different experimental groups of study. The symbol of † means the significant difference with sham group ($p < 0.05$). The asterisk * shows the significant difference with the T-D group ($p < 0.05$).

Table 3
Morphology of cauda epididymal spermatozoa. Data with normal distribution are displayed as Mean ± SD; Non-normal distribution data are expressed as median and interquartile range.

Groups	Normal morphology (%)	Bent tail (%)	Coiled tail (%)	Distal cytoplasmic droplet (%)	Short tail (%)	Abnormal head (%)
Sham	82.33 ± 3.44	14.48 ± 2.42	2.35(1.20,3.00)	0.21 ± 0.53	0 ± 0.00	1.01 ± 1.29
T-D	71.41 ± 3.15[a]	19.37 ± 5.28	8.06(3.98,14.24)[a]	0.83 ± 1.35	0.39 ± 0.61	0 ± 0.00
SM	80.92 ± 4.52[b]	14.06 ± 3.78	5.00(2.70,5.96)	0.20 ± 0.48	0.16 ± 0.40	0.16 ± 0.40

[a] $p < 0.05$ compared with the sham group.
[b] $p < 0.05$ compared with the T-D group.

Table 4
Malondialdehyde (MDA), glutathione peroxidase (GPX), catalase (CAT), and total antioxidant capacity (TAC) activities in testis tissue and plasma.

Groups	Testis tissue				Plasma			
	MDA (μmol/mg-pr)	GPX (Unit/mg-pr)	CAT (Unit/mg-pr)	TAC (nmol Trolox equivalent/mg-protein)	MDA (μmol/mg-pr)	GPX (Unit/mg-pr)	CAT (Unit/mg-pr)	TAC (nmol Trolox equivalent/mg-protein)
Sham	0.24(0.17,0.33)	20.62 ± 1.92	0.41(0.34,0.51)	0.43 ± 0.08	0.15(0.15,0.16)	10.16 ± 0.04	4.53(3.47,7.72)	1.21 ± 0.19
T-D	0.98(0.93,1.01)[a]	8.24 ± 2.57[a]	0.13(0.10,0.18)[a]	0.15 ± 0.04[a]	0.52(0.48,0.59)[a]	8.07 ± 0.51[a]	0.44(0.33,0.64)[a]	0.45 ± 0.08[a]
SM	0.21(0.19,0.32)[b]	19.8 ± 2.99[b]	0.33(0.21,0.34)	0.50 ± 0.03[b]	0.29(0.18,0.40)[a,b]	11.67 ± 0.55[a,b]	5.73(4.94,6.13)[b]	1.44 ± 0.12[b]

Data with normal distribution are displayed as Mean ± SD; Non-normal distribution data are expressed as median and interquartile range.
[a] $p < 0.05$ compared with the sham group.
[b] $p < 0.05$ compared with the T-D group.

antioxidant activity than torolox, prevention of xanthine oxidase activity, and scavenging excessive free radicals. It has also been demonstrated that rosmarinic acid is able to reduce molybdenum blue (VI) to (V), which reduces the free radicals produced by metals [50]. In addition to the antioxidant properties, protection against HIV type 1, anticarcinogenic, liver protection, and anti-hepatitis, preventing coagulation and the formation of blood clots and anti-inflammatory are also other properties of rosmarinic acid [50]. As mentioned above, in our study, the SM extract was comprising 14.28 (mg ml^{-1}) rosmarinic acid.

ROS generation is biphasic in testicular I/R. The onset of phase I is associated with tissue reperfusion and lasts for short hours and is also associated with oxidative stress, but damage to cells in this phase may be repaired. Phase II occurs when oxidative damage persists for several days, and damage to the testicular tissue in this phase is irreversible [1,40,51]. The results reported in this study were determined for the first phase and for a short period of reperfusion.

Our macroscopic results included testicular and epididymal weight, and size did not have any significant difference between the T-D group and the sham group, and these results were different from previous findings that reported a significant reduction in testicular weight and size following I/R [52,53]. A possible explanation for this might be that our results were indicated in a short reperfusion time and only in the first phase of ROS generation.

As the amount of ROS in the tissue increases, plasma membrane lipids are damaged and peroxidated, and the intracellular components are destroyed, and the marker for lipid peroxidation is MDA. Also, in the male reproductive system, GPX is one of the major enzymes involved in reducing free radicals [54]. In an investigation, the oxidative stress and histopathological effects of SM extract pretreatment in the prevention of renal I/R damage in rats have been evaluated and indicated that SM prescription considerably reduced renal MDA levels and increased the levels of GSH, SOD, CAT, and GPX [20]. In another study of the oxidative stress inhibition impacts of Salvia militiorihiza mixed with Radix Puerariae on vascular damage in diabetic rats, it has been concluded that the extract could ameliorate the oxidative stress by a significant reduction in the level of MDA and increment in the serum levels of the SOD and CAT [24]. In the present study, in the T-D group the plasma and testicular tissue level of MDA significantly increased and levels of GPX, CAT, and TAC reduced considerably, and the SM pretreatment could significantly reduce the MDA level and elevate the levels of GPX, CAT, and TAC both in the plasma and testis tissue compared to the T-D group which is in keeping with previous observational studies [20,24,29,54].

Previously, it was thought that ischemia/reperfusion-induced cell damage were only caused by necrosis. It has now been shown that changes in ion exchange in the plasma membrane and the loss of cell membrane integrity cause secretion of toxic molecules and inflammatory reactions that both damage and cause cell death [55]. This is the mechanism that can damage cells, including spermatozoa, in I/R injury. In 2015, Shen et al. published a paper in which they described that application of the SM polysaccharides in freezing and thawing of boar sperms prevented oxidative damage to spermatozoa and increased the percentage of motile sperms [56]. Jasem et al. (2010) carried out an investigation on salvia hypoleuca (one of the species of salvia) effects on rat spermatogenesis and found that the extract could significantly increase the motility of spermatozoa, epididymal sperm concentration, and

Table 5
Histopathological evaluations. MSTD, mean seminiferous tubular diameter; GECT, germinal epithelial cell thickness; MTBS, mean testicular biopsy scoring; TCT, testicular capsule thickness. All values are displayed as Mean ± SD.

Groups	MSTD (μm)	GECT (μm)	GECT (Cell Layer)	MTBS (Score)	TCT (μm)
Sham	294.83 ± 22.01	78.16 ± 7.83	8.66 ± 0.51	9.33 ± 0.51	37.66 ± 4.71
T-D	195.16 ± 26.97[a]	32.66 ± 5.34[a]	4.00 ± 0.63[a]	6.66 ± 0.51[a]	28.16 ± 4.53[a]
SM	272.00 ± 10.71[b]	61.00 ± 6.38[a,b]	6.5 ± 0.83[a,b]	8.50 ± 0.54[a,b]	33.00 ± 3.03

[a] $p < 0.05$ compared with the sham group.
[b] $p < 0.05$ compared with the T-D group.

testicular weights [57]. The beneficial impacts of rosmarinic acid in sex hormones and testicular apoptosis induced by the electromagnetic field in male rats were evaluated by Khaki et al. (2012), and an increment in the testosterone level of the serum and positive effects on histology of the testis was observed in the rosmarinic acid treatment group [58]. In accordance with previous studies, our results proved that I/R could damage the epididymal stored spermatozoa, and the SM extract pretreatment could markedly increase progressive motile spermatozoa, normal morphological spermatozoa, sperm concentration, and significant reduction in non-progressive motile spermatozoa was observed when compared to the T-D group.

5. Conclusion

In this investigation, the aim was to assess the protective effects of Salvia miltiorrhiza hydroalcoholic extract pretreatment in preventing the ischemia/reperfusion damage caused by torsion detorsion of testis. The findings of the present study suggest that Salvia miltiorrhiza is a potent antioxidant that can reduce oxidative stress and improve sperm parameters as well as reduce the rate of testicular tissue injury. One limitation of this study was that the precise timing of the administration of the extract and the appropriate dose to achieve the best effect of the extract were not exactly known, and the dose and time of injection in our study were based on previous documentations. Further research is required to completely understand the suitable dose and administration time to achieve the best treatment efficiency.

Declaration of competing interest

Authors declare no conflict of interest.

CRediT authorship contribution statement

Farshid Davoodi: Conceptualization, Methodology, Investigation, Writing - original draft. **Shayan Taheri:** Conceptualization, Methodology, Investigation. **Abbas Raisi:** Project administration, Writing - original draft, Writing - review & editing. **Asghar Rajabzadeh:** Writing - original draft, Writing - review & editing. **Hassan Ahmadvand:** Visualization, Resources. **Mohammad Hassan Hablolvarid:** Methodology, Validation. **Amir Zakian:** Data curation, Formal analysis, Writing - review & editing.

Acknowledgment

This paper has been extracted from a DVM thesis of Farshid Davoodi and Shayan Taheri carried out in Lorestan University and the authors want to appreciate the vice-chancellor of research of Lorestan University, Razi Herbal Medicines Research Center of Lorestan Medical University, Khorramabad, Iran, and Razi Vaccine and Serum Research Institute, Karaj, Iran.

Funding: This work was supported by the Lorestan University grant number: [LU-9211501009-2020]

References

[1] Abdel-Gaber SA, Mohammed RK, Refaie MM. Mechanism mediating the protective effect of diacerein in ischemia-reperfusion-induced testicular injury in rats. Life Sci 2018;209:57–62.
[2] Wilhelm Filho D, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia–reperfusion injury. Mol Asp Med 2004;25:199–210.
[3] Bozlu M, Acar D, Cayan S, Aktas S, Tunckiran A. Protective effect of trapidil on long-term histologic damage in a rat model of testicular ischemia-reperfusion injury. World J Urol 2009;27:117.
[4] Gökçe A, Oktar S, Koc A, Gonenci R, Yalcinkaya F, Yonden Z, et al. Protective effect of thymoquinone in experimental testicular torsion. Urol Int 2010;85:461–5.
[5] Bilommi R, Nawas BA, Kusmayadi DD, Diposarosa R, Chairul A, Hernowo BS. The effects of glutathione on malondialdehyde expression and seminiferous tubule damage in experimental testicular torsion–detorsion in Wistar rats. J Pediatr Urol 2013;9:1059–63.
[6] Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology 2005;43:963–74.
[7] Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longevity 2008;1:15–24.
[8] Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol 2008;59:2–11.
[9] Sawyer DE, Mercer BG, Wiklendt AM, Aitken RJ. Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutat Res Fundam Mol Mech Mutagen 2003;529:21–34.
[10] Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. Mol Cell Endocrinol 2006;250:66–9.
[11] Mikaili P, Shayegh J, Asghari MH, Sarahroodi S, Sharifi M. Currently used traditional phytomedicines with hot nature in Iran. Biol Res 2011;2:56–68.
[12] O'hara M, Kiefer D, Farrell K, Kemper K. A review of 12 commonly used medicinal herbs. Arch Fam Med 1998;7:523–36.
[13] Abad ANA, Nouri MHK, Tavakkoli F. Effect of Salvia officinalis hydroalcoholic extract on vincristine-induced neuropathy in mice. Chin J Nat Med 2011;9:354–8.
[14] Zhao G-R, Xiang Z-J, Ye T-X, Yuan Y-J, Guo Z-X. Antioxidant activities of Salvia miltiorrhiza and Panax notoginseng. Food Chem 2006;99:767–74.
[15] Gordon M, Weng X. Antioxidant properties of extracts from tanshen (Salvia miltiorrhiza Bunge). Food Chem 1992;44:119–22.
[16] Anversa P, Olivetti G, Meggs L, Sonnenblick E, Capasso J. Cardiac anatomy and ventricular loading after myocardial infarction. MonogrAm Heart Assoc 1993;87. VII. 22-VII. 7.
[17] Sun J, Huang SH, Tan BK-H, Whiteman M, Zhu YC, Wu YJ, et al. Effects of purified herbal extract of Salvia miltiorrhiza on ischemic rat myocardium after acute myocardial infarction. Life Sci 2005;76:2849–60.
[18] Jia Y, Huang F, Zhang S, Leung S-w. Is danshen (Salvia miltiorrhiza) dripping pill more effective than isosorbide dinitrate in treating angina pectoris? A systematic review of randomized controlled trials. Int J Cardiol 2012;157:330–40.
[19] Nan JX, Park EJ, Kang HC, Park PH, Kim JY, Sohn DH. Anti-fibrotic effects of a hot-water extract from Salvia miltiorrhiza roots on liver fibrosis induced by biliary obstruction in rats. J Pharm Pharmacol 2001;53:197–204.
[20] Chen G, Fu Y, Wu X. Protective effect of Salvia miltiorrhiza extract against renal ischemia-reperfusion-induced injury in rats. Molecules 2012;17:1191–202.
[21] Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70–6.
[22] Wang X, Lee WY, Zhou X, Or PM, Yeung JH. A pharmacodynamic–pharmacokinetic (PD–PK) study on the effects of Danshen (Salvia miltiorrhiza) on midazolam, a model CYP3A probe substrate, in the rat. Phytomedicine 2010;17:876–83.
[23] Wang X, Yeung JH. Effects of the aqueous extract from Salvia miltiorrhiza Bunge on caffeine pharmacokinetics and liver microsomal CYP1A2 activity in humans and rats. J Pharm Pharmacol 2010;62:1077–83.
[24] Zhao W, Yuan Y, Zhao H, Han Y, Chen X. Aqueous extract of Salvia miltiorrhiza Bunge-Radix Puerariae herb pair ameliorates diabetic vascular injury by inhibiting oxidative stress in streptozotocin-induced diabetic rats. Food Chem Toxicol 2019;129:97–107.
[25] Varisli O, Uguz C, Agca C, Agca Y. Various physical stress factors on rat sperm motility, integrity of acrosome, and plasma membrane. J Androl 2009;30:75–86.
[26] World Health Organization DoRHaR. WHO laboratory manual for the examination and processing of human semen. fifth ed. Geneva: Switzerland: World Health Organization; 2010. ed. Switzerland, 2010.
[27] Johnsen SG. Testicular biopsy score count–a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormone Res Paediatr 1970;1:2–25.
[28] Lee J-W, Kim J-I, Lee Y-A, Lee D-H, Song C-S, Cho YJ, et al. Inhaled hydrogen gas therapy for prevention of testicular ischemia/reperfusion injury in rats. J Pediatr Surg 2012;47:736–42.
[29] Ameli M, Hashemi MS, Moghimian M, Shokoohi M. Protective effect of tadalafil and verapamil on testicular function and oxidative stress after torsion/detorsion in adult male rat. Andrologia 2018;50:e13068.
[30] Dokmeci D, Inan M, Basaran UN, Yalcin O, Aydogdu N, Turan FN, et al. Protective effect of L-carnitine on testicular ischaemia–reperfusion injury in rats. Cell Biochem Funct: Cellular biochemistry and its modulation by active agents or disease 2007;25:611–8.
[31] Allamaneni SS, Agarwal A, Nallella KP, Sharma RK, Thomas Jr AJ, Sikka SC. Characterization of oxidative stress status by evaluation of reactive oxygen species levels in whole semen and isolated spermatozoa. Fertil Steril 2005;83:800–3.
[32] Dogan C, Halici Z, Topcu A, Cadirci E, Karakus E, Bayir Y, et al. Effects of amlodipine on ischaemia/reperfusion injury in the rat testis. Andrologia 2016;48:441–52.
[33] Kurcer Z, Hekimoglu A, Aral F, Baba F, Sahna E. Effect of melatonin on epididymal sperm quality after testicular ischemia/reperfusion in rats. Fertil

Steril 2010;93:1545–9.

[34] Hekimoglu A, Kurcer Z, Aral F, Baba F, Sahna E, Atessahin A. Lycopene, an antioxidant carotenoid, attenuates testicular injury caused by ischemia/reperfusion in rats. Tohoku J Exp Med 2009;218:141–7.

[35] Taati M, Moghadasi M, Dezfoulian O, Asadian P, Kheradmand A, Abbasi M, et al. The effect of ghrelin pretreatment on epididymal sperm quality and tissue antioxidant enzyme activities after testicular ischemia/reperfusion in rats. J Physiol Biochem 2012;68:91–7.

[36] Azizollahi S, Babaei H, Derakhshanfar A, Oloumi M. Effects of co-administration of dopamine and vitamin C on ischaemia-reperfusion injury after experimental testicular torsion-detorsion in rats. Andrologia 2011;43:100–5.

[37] Ozbal S, Ergur BU, Erbil G, Tekmen I, Bagrıyanık A, Cavdar Z. The effects of α-lipoic acid against testicular ischemia-reperfusion injury in rats. Sci World J 2012;2012.

[38] Aktas C, Erboga M, Fidanol Erboga Z, Bozdemir Donmez Y, Topcu B, Gurel A. Protective effects of Urtica dioica L. on experimental testicular ischaemia reperfusion injury in rats. Andrologia 2017;49:e12636.

[39] Bayatli F, Akkuş D, Kilic E, Saraymen R, Sönmez MF. The protective effects of grape seed extract on MDA, AOPP, apoptosis and eNOS expression in testicular torsion: an experimental study. World J Urol 2013;31:615–22.

[40] Kim Y-H, Kim G-H, Shin J-H, Kim K-S, Lim J-S. Effect of Korean red ginseng on testicular tissue injury after torsion and detorsion. Korean J Urol 2010;51:794–9.

[41] Shokoohi M, Shoorei H, Soltani M, Abtahi-Eivari SH, Salimnejad R, Moghimian M. Protective effects of the hydroalcoholic extract of Fumaria parviflora on testicular injury induced by torsion/detorsion in adult rats. Andrologia 2018;50:e13047.

[42] Uyeturk U, Terzi EH, Gucuk A, Kemahli E, Ozturk H, Tosun M. Prevention of torsion-induced testicular injury by Rhodiola rosea. Urology 2013;82:254. e1-. e6.

[43] Dursun R, Zengin Y, Gündüz E, İçer M, Durgun HM, Dağgulli M, et al. The protective effect of goji berry extract in ischemic reperfusion in testis torsion. Int J Clin Exp Med 2015;8:2727.

[44] Ji X, Tan BK-H, Zhu YC, Linz W, Zhu YZ. Comparison of cardioprotective effects using ramipril and DanShen for the treatment of acute myocardial infarction in rats. Life Sci 2003;73:1413–26.

[45] Ji X. Effects of Salvia miltiorrhiza in ischemic myocardium in experimental rats. Perspective. In: Tan B, Bay BH, Zhu YZ, editors. Novel compounds from natural products in new millenniums. London: World Scientific Press; 2004. p. 183–95.

[46] Zhu Y, Huang S, Tan B, Sun J, Whiteman M, Zhu Y-C. Antioxidants in Chinese herbal medicines: a biochemical perspective. Nat Prod Rep 2004;21:478–89.

[47] Bisio A, Romussi G, Ciarallo G, De Tommasi N. Flavonoide und triterpenoide aus Salvia blepharophylla Brandegee ex Epling. Pharmazie 1997;52:330–1.

[48] Gökdil G, Topcu G, Sönmez U, Ulubelen A. Terpenoids and flavonoids from Salvia cyanescens. Phytochemistry 1997;46:799–800.

[49] Lu Y, Foo LY. Antioxidant activities of polyphenols from sage (Salvia officinalis). Food Chem 2001;75:197–202.

[50] Petersen M, Simmonds MS. Rosmarinic acid. Phytochemistry 2003;62:121–5.

[51] Cutrìn JC, Boveris A, Zingaro B, Corvetti G, Poli G. In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment. Hepatology 2000;31:622–32.

[52] Dejban P, Rahimi N, Takzare N, Jahansouz M, Dehpour AR. Protective effects of sumatriptan on ischaemia/reperfusion injury following torsion/detorsion in ipsilateral and contralateral testes of rat. Andrologia 2019:e13358.

[53] Mogilner JG, Lurie M, Coran AG, Nativ O, Shiloni E, Sukhotnik I. Effect of diclofenac on germ cell apoptosis following testicular ischemia-reperfusion injury in a rat. Pediatr Surg Int 2006;22:99–105.

[54] Asghari A, Akbari G, Meghdadi A, Mortazavi P. Protective effect of metformin on testicular ischemia/reperfusion injury in rats. Acta Cir Bras 2016;31:411–6.

[55] Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Ischemia/reperfusion. Comprehensive Physiology 2016;7:113–70.

[56] Shen T, Jiang Z-L, Liu H, Li Q-W. Effect of Salvia miltiorrhiza polysaccharides on boar spermatozoa during freezing–thawing. Anim Reprod Sci 2015;159:25–30.

[57] Jasem E, Nasim J, Gholamreza M, Naser S, Nader M, Maryam SL, et al. Evaluation of the effects of Salvia hypoleuca on the cAMP-responsive element modulator (CREM) gene expression and spermatogenesis in rat. Middle East Fertil Soc J 2010;15:274–7.

[58] Khaki A, Imani S, Golzar FS. Effects of rosmarinic acid on male sex hormones (testosterone-FSH-LH) and testis tissue apoptosis after exposure to electromagnetic field (EMF) in rats. Afr J Pharm Pharmacol 2012;6:248–52.