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Effects of *Echinacea Purpurae* Extract on Testicular Ischemia/Reperfusion (I/R) Injury in Rat

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Abstract

BACKGROUND: Ischemia/reperfusion of tetsis is a male infertility condition which occurs because of oxidation demage. *Echinacea purpurea* extract (EPE) has antioxidant and protective effect.

OBJECTIVES: So, the main purpose of this research was to determine effects of EP extract on testicular ischemia/reperfusion (I/R) injury in rat.

METHODS: 50 adult Wistar rats were randomly allocated into five groups: group one as control, group two, 2 hour I/24 hours R period, group three, 1 hour I which after 1 hour of ischemia, rat was injected 25 mg/kg EPE and ischemia continued for an hour, then was followed by 24 hours R period. Groups 4 and 5 were similar to experiment 3, except rats were injected with 50 and 100 mg/kg of EPE, respectively. Then 24 hours later, the left testis was removed for histological and antioxidant enzyme activity including malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant status (TAS).

RESUTLS: Based on the findings, MDA concentration was significantly elevated in I/R rat ($P<0.05$) while EPE diminished MDA concentration in I/R rat ($P<0.05$). SOD and GPx activity decreased in I/R rat ($P<0.05$). Injection of the of the EPE (25, 50 and 100mg/kg) increased SOD and GPx concentrations ($P<0.05$). There was significant fluctuation on TAS in EPE treated groups in comparision to the control group ($P>0.05$). Seminiferous tubules degenerated and few spermatocytes were observed in testis tubules of the I/R rat. EPE (50 and 100mg/kg) improved testis characteristics in experimental I/R-induced rat in which normal spermatocyte in seminiferous tubules was observed.

CONCLUSIONS: These results suggested EPE has protective effect against against testicular I/R.

KEYWORDS: *Echinacea purpurae*, ischemia, rat, reperfusion,testis

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Introduction

One of the emergency conditions in male infertility is testicular torsion (Taati et al. 2016). Testicular torsion needs early identification and surgical operation to prevent further damage to the testis, subfertility and infertility (Ranade et al. 2011). It is reported ischemia/reperfusion (I/R) injury leads to germ cells loss and disruption of the seminiferous epithelium (Taati et al. 2012). The main treatment for correction of testicular torsion is surgery to detorsion spermatic cord and re-establishing testis blood circulation (Asghari et al. 2016). During the long testicular torsion oxidation damage affects testis by production of the reactive oxygen species (ROS) (Asghari et al. 2016). Testis and spermatozoa contain higher fatty acids levels which are vulnerable to the ROS (Wei et al. 2011). Excessive generation of the ROS interacts with lipids, proteins and nucleic acids which has adverse effect on cell function and damage (Yuluğ et al. 2013). Testis has high cell metabolism such that excessive ROS production weakens antioxidant capacity (Tuglu et al. 2015). Malondialdehyde (MDA) is the end product of lipid peroxidation and increased MDA level has adverse effect on sperm fertility (Ghiasi Ghalehkandi, 2014). Glutathione peroxidase (GPx) is peroxidase enzyme and protects sperm from lipid peroxidation and oxidative damage (Hsieh et al. 2006; Lee et al. 2012).

Today, there is growing interest in the application of medical plants due to their medicinal properties (Mansouri and Abdennour, 2011). Echinacea purpurea (EP) is a herbal medicine belonging to the Asteraceae (Compositae) family and contains bioactive metabolites including lipophilic, alkamides, caffeic acid and polysaccharides (Bayramoglu et al. 2011). Caffeic acid is the main bioactive component of the EP which has anti-inflammatory, antivirus, anticancer and antiandrogenic activities (Rezaie et al. 2013). Also, it is used for pain relief and wound healing (Rezaie et al. 2013). In folk medicine, it is used in in bacterial and viral infections (Barnes et al. 2005). Echinacea has antioxidant and free radical scavenging properties (Bayramoglu et al. 2011). It is reported, administration of the 50 and 100 mg/kg EP reduced MDA and amplified SOD and CAT levels on experimental renal I/R injury in the rats (Bayramoglu et al. 2011). Recently, Awaad et al. (2017) reported administration of the hinacea purpurea extract (EPE) (30 mg/kg) protects against magnetic nanoparticles intra-testicular injection-induced toxicity. Also, EPE (100 mg/kg) has protective role against Gama-irradiation on hepatic and testicular in rat (Ahmed et al. 2017). Also, the EP stimulates T-cell, lymphocytic and cy¬tokine production in Arsenic-induced hepatic toxicity (Rezaie et al. 2013). Even though the correlationwith antioxidant activity of the EP has been reported, there is no previous research on effect of the EPE on testicular IR injury in rats. So, the main purpose of this reseach was to investigate effects of the EPE on testicular ischemia/reperfusion (I/R) injury in rat.

Material & Methods

Animals

Fifty healthy mature male Wistar rats (250 ± 20 g) were obtained from Razi Vaccine and Serum Research Institute (Tehran, Iran). Rats were provided commercial chow pellets and fresh water. Animals were kept in laboratory one week prior to experiments. All

experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann 1983). Each animal was used only once and killed immediately after the experiment.

Drugs

Pure sample of the EPE (Sigma Aldrich, UK; CAS Number 90028-20-9) and assay kits of MDA, SOD and GPx (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom) were purchased. The doses for the EPE were selected based on the pilot study (un-published data) and previous report (Rezaie et al. 2013; Ahmed et al. 2017; Motamedi et al. 2017).

Experimental protocol

Intraperitoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) was used for surgical procedures under anesthesia, then experimental testicular IR was created (Koksal et al. 2013). A midline longitudinal incision was made for access to both testes. Torsion was created through twistings the left testes 720° in counter clockwise direction and preserved through fixing the testes to scrotum with a 6-0 nylon suture passing by the tunica albuginea and dartos. Two hours later, suture was removed, left testes were detorted and replaced with scrotum reperfusion continued for 24 h (Sahin et al. 2005). During the surgery, heating pad was used to keep body temperature connstant, then after surgery, the incision was closed. Group 1 was kept as control with no surgery. Group 2 was subjected to 2h I /24 h R period. Group 3 was subjected to 2 h I which after 1 h of ischemia, rat was i.p. injected with 25 mg/kg EPE and ischemia continued for an hour, then followed by 24 h R period. In group 4 rat was subjected to 2 h I which after 1 h of ischemia, rat was i.p. injected with 50 mg/kg EPE and ischemia continued for an hour, then followed by 24 h R period. In group 4 rat was subjected to 2 h I which after 1 h of ischemia, rat was i.p. injected with 100 mg/kg EPE and ischemia continued for an hour, then followed by 24 h R period. The doses for EPE were selected based on the pilot study (un-published data) and previous report (Awaad et al. 2018). After 2 h of I, the suture was removed and left testis was detorted and replaced in scrotum for 24 h of reperfusion. At the end of the study, rats were euthanized (pentobarbital 300 mg/kg, i.p.), peritoneum opened and left testis was removed. The testicle was divided into two halves by a sagittal section, one half was fixed in Bouin's solution, the second half was stored at -80 °C for the biochemical analysis (Fakouri et al. 2017). The right testis was removed as control for histological investigations.

Tissue processing

The tissue was fixed in Bouin's solution (2.5 mL 7% formaldehyde, 2.65 mL glacial acetic acid and 7.5 mL saturated picric acid), post-fixed in 70% alcohol and fixed in paraffin blocks. A 5µm tissue section was obtained, deparaffinized and stained using hematoxyline eosin. The testicular tissue was observed with standard light microscopy by a sole observer 14. A 5µm thickness tissue section was taken and stained with hematoxylin and eosin [H & E]. The testis sections were graded numerically to assess the degree of seminiferous tubule injury according to the method of Johnsen (1971) as (1) neither germ cells nor Sertoli cells present, (2) no germ cells present, (3) only spermatogonia present, (4) only a few spermatocytes present, (5) no spermatozoa or spermatids present but many spermatocytes present, (6) only a few spermatids present, (7) no spermatozoa

but many spermatids present, (8) only a few spermatozoa present, (9) many spermatozoa present but disorganized spermatogenesis and (10) complete spermatogenesis and perfect tubules.

Antioxidant activity

The tissue MDA level was determined with a maximum absorption at 532 nm (Placer et al. 1966). The GPx level was measured in absorbance of 340nm (Paglia and Valentine, 1967). The GPx activity was expressed as U/mg tissue. Tissue SOD activity was measured according to the method of Paoletti and Mocali (Paoletti and Mocali, 1990). The SOD activity was expressed as nmol/g tissue. Nicotinamide adenine dinucleotide oxidation was measured at 340nm and expressed as U/mg tissue. The total antioxidant status detecting kit was obtained on the basis of suppression in color production which was measured at 600nm and expressed as mmol/ml (Miller et al. 1993).

Statistical analysis

The parametric data was analyzed by one-way analysis of variance (ANOVA) using SPSS 24.0 and expressed as mean values ± standard error of mean (SEM). The differences between groups were analysed using Duncan Multiple Range Test. The histopathological scores were analysed by KruskaleWallis test. $P<0.05$ was considered as significant difference between groups.

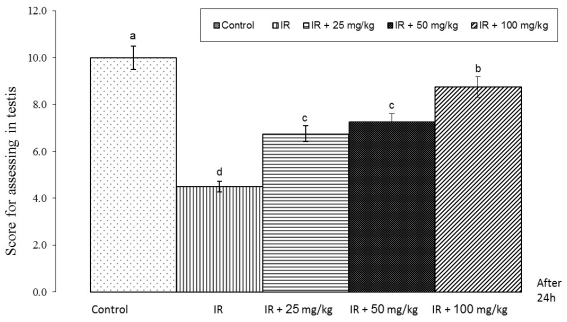

Figure 1. Histological score for assessing testis associated with seminiferous tubules injury in EPE injection followed by I/R rat. Different letters (a-d) indicate significant differences between treatments ($P<0.05$).EPE: Echinacea purpurae extract.

Results

As seen in Fig. 1, I/R group had higher testis damage compared to the other groups ($P<0.05$). The control and sham groups have the least testis damage ($P>0.05$). A dose dependent difference was detected on testis damage grade in EPE treated groups in com-

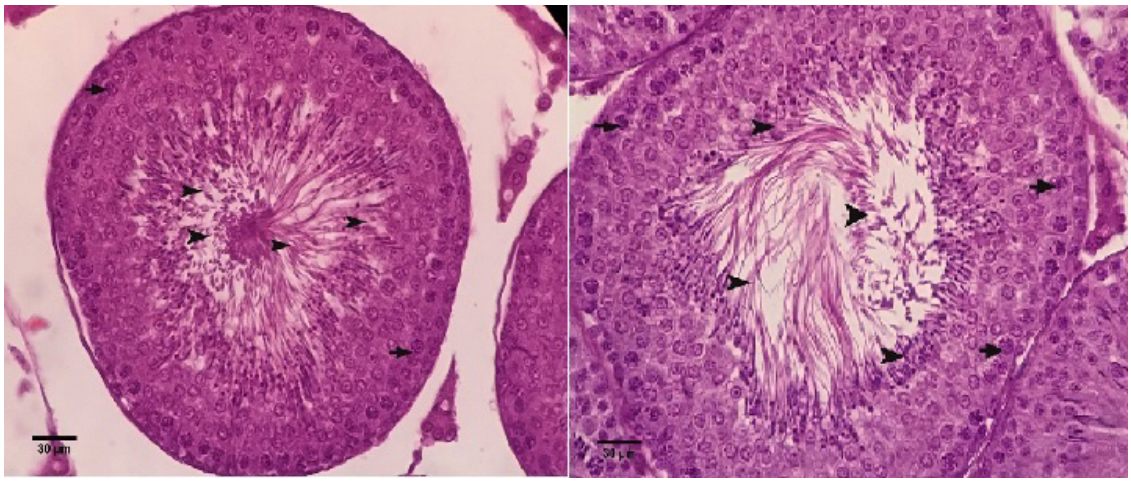

Figure 2. Testis section of left testis in control rats showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules (Left). Testis section of right testis in control rats showing normal seminiferous tubules with spermatogonia (black arrow), spermatocyte (black arrow head) and many spermatozoa (white arrow) (Right) (H&E). H & E: hematoxylin and eosin.

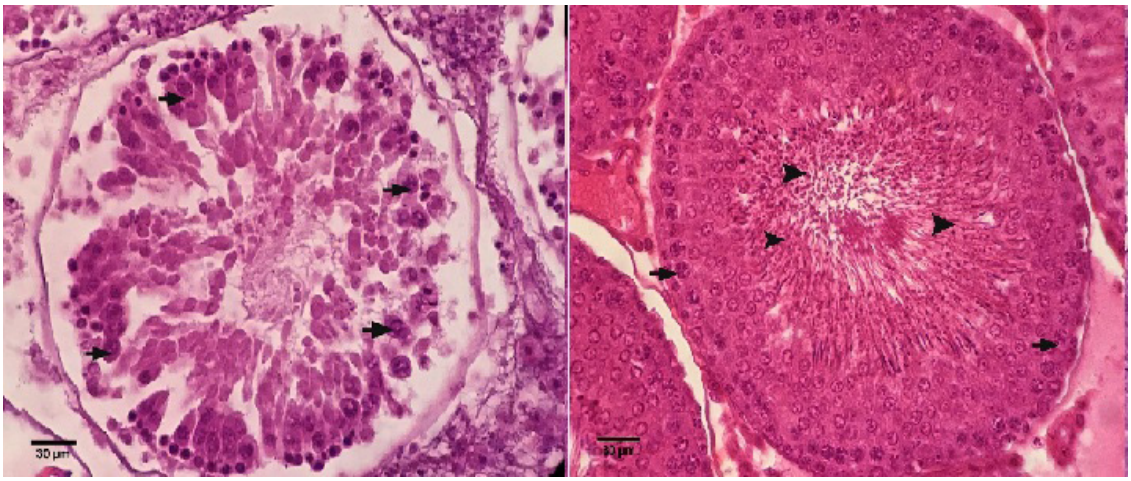

Figure 3. Testis section of left testis in I/R rats showing degenerated seminiferous tubules (arrow) and loss of spermatogenesis (H&E) (Left) and testis section of right testis in I/R rats showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules (H&E) (Right). H & E: hematoxylin and eosin.

parision with I/R group (P<0.05). No difference was observed between 25 and 50 mg/kg of the EPE (P>0.05).

Effect of various EPE on tissue MDA, SOD and GPx levels in experimental testicular I/R-induced rat is presented in Table 1. As seen, testicular MDA levels significantly increased in I/R rat (P<0.05) while i.p injection of the EPE (25, 50 and 100 mg/kg) normalized I/R-induced MDA (P<0.05). Experimental I/R significantly decreased SOD and GPx activity in comparision to control group (P<0.05). Injection of the of the EPE (25, 50 and 100 mg/kg) significantly increased SOD and GPx activity (P<0.05). No significant difference was detected on TAS in EPE treated groups compared to the control group (P>0.05).

According to the data, left and right testis section of control rats had shown normal seminiferous tubules and spermatogenesis with spermatocytes, sertoli and spermatozoa (Fig. 2).

As seen in Fig. 3, seminiferous tubules de-

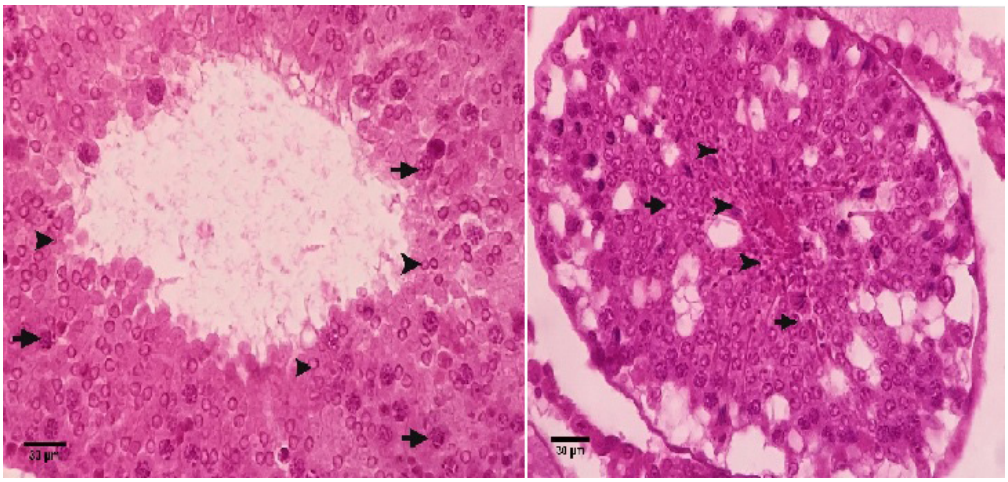

Figure 4. Testis section of left testis in the EPE (25 mg/kg) followed by I/R rats showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules (Left) and testis section of right testis in the EPE (25 mg/kg) followed by I/R rats showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules (H&E) (Right). H & E: hematoxylin and eosin. EPE: Echinacea purpurae extract.

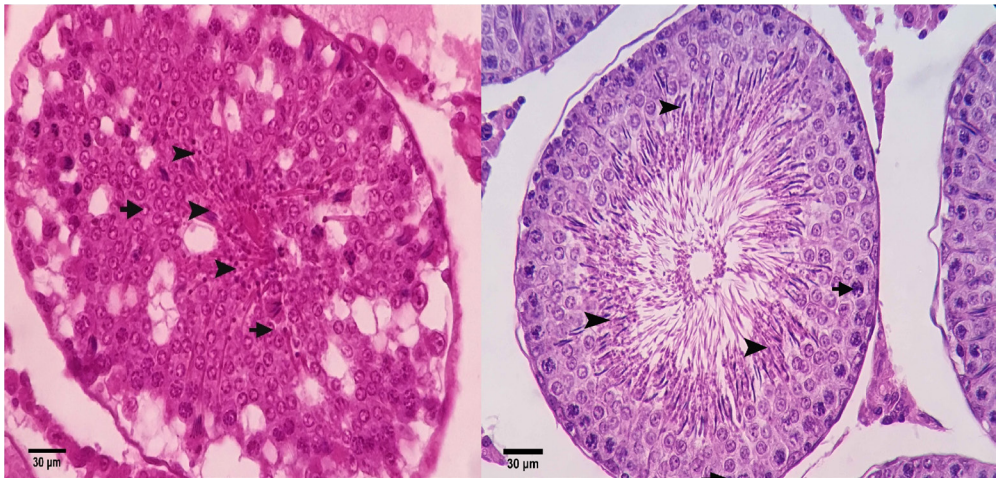

Figure 5. Testis section of left testis in the EPE (50 mg/kg) followed by I/R rats showing seminiferous tubules (Arrow) with few spermatocyte and interstitial cells (Arrow head) between tubules (Left) and testis section of right testis in the EPE (50 mg/kg) followed by I/R rats showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules (H&E). H & E: hematoxylin and eosin. EPE: Echinacea purpurae extract.

generation and loss of spermatogenesis with few spermatocytes were detected in left degenerated testis tubules in I/R injur rat. However, no significant effect was observed on right testis (Fig. 3, right).

Seminiferous tubules degenerated and loss of spermatogenesis with few spermatocytes was detected in degenerated in i.p injection of the EPE (25 mg/kg) followed by I/R injury rats (Fig. 4, left). However, no significant effect was observed on right testis (Fig. 4, right).

In this study, i.p administration of the EPE (50 mg/kg) followed by I/R improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in I/R injury rat (Fig. 5).

According to the Fig. 6, injection of the EPE (50 mg/kg) improved testis characteristics with few normal seminiferous tubules and spermatocyte in seminiferous tubules in experimental I/R-induced rat.

Table 1. Effect of different levels EPE on tissue values of Malondialdehyde, Superoxide dismutase, Glutathione peroxidase and total antioxidant status in experimental testicular I/R-induced rat

Group	**MDA (nmol/g tissue)**	**SOD (U/mg tissue)**	**GPx (U/mg tissue)**	**TAS (mmol/ml)**
Control	99.51 ±8.54 ^{d}	4.32 ± 0.25 ^{a}	4.67 ± 0.67 ^{a}	13.11 ± 1.85
I/R	176.80 ± 9.27 ^{a}	1.14 ± 0.14 ^{d}	2.22 ± 0.56 ^{d}	11.09 ± 1.47
EPE (25 mg/kg)	168.44 ± 8.33 ^{b}	1.89 ± 0.34 ^{c}	3.69 ± 0.84 ^{c}	11.45 ± 1.77
EPE (50 mg/kg)	131.12 ± 9.30 ^{b}	2.51 ± 0.27 ^{c}	3.59 ± 0.36 ^{c}	12.21 ± 1.25
EPE (100 mg/kg)	125.32 ± 8.10 ^{c}	3.26 ± 0.44 ^{b}	4.11 ± 0.29 ^{b}	12.84 ± 1.48

EPE: *Echinacea purpurae* extract, MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase, TAS: total antioxidant status, I/R: ischemia/reperfusion. Different letters (a-d) indicate significant differences between treatments ($P<0.05$).

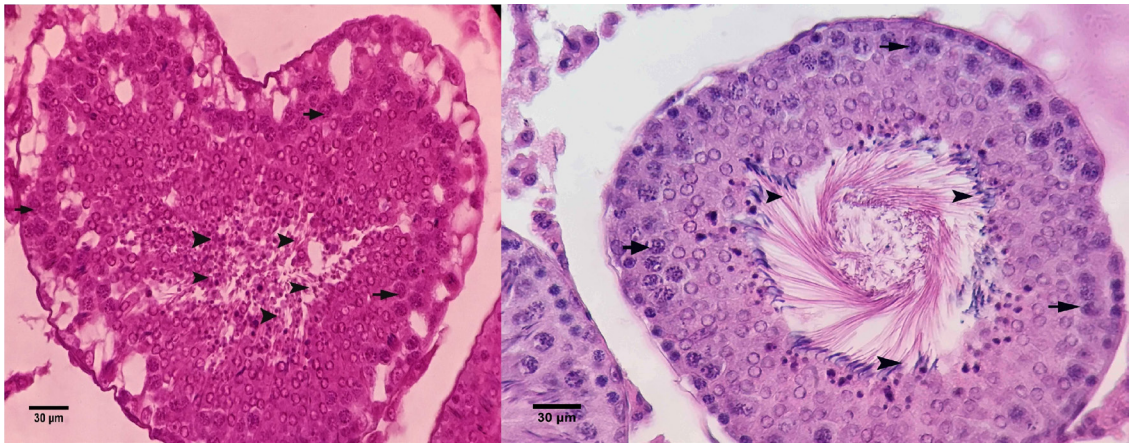

Figure 6. Testis section of left testis in the EPE (100 mg/kg) followed by I/R rats showing many normal seminiferous tubules (arrow) (H&E) (Left) with few spermatocyte (Arrow head) and testis section of right testis in the EPE (100 mg/kg) followed by I/R rats showing normal seminiferous tubules (Arrow) and interstitial cells (Arrow head) between tubules (H&E). H & E: hematoxylin and eosin. EPE: Echinacea purpurae extract.

Discussion

In the current study, untreated rats that were subjected to ischemia for 2 h followed by 24 h reperfusion and revealed testicular injury with apparent seminiferous tubular necrosis. In I/R rat seminiferous tubules degenerated and few spermatocytes were observed. The 50 and 100 mg/kg of the EPE improved testis characteristics with normal seminiferous tubules were observed in experimental I/R-induced rat.

Several medical properties were reported for the EP including antifungal, antibacterial, antiinflammatory, antioxidant and wound healing properties (Nematalla et al. 2011). Echinacea extract has protective effects on the liver against cyproterone acetate and mentioned antioxidant properties of the EP induced these effects (Nematalla et al. 2011).

It is well documented I-followed by R has adverse effects on germ cell loss and disruption of the seminiferous epithelium in the testis (Ranade et al. 2011). The ROS such as superoxide anions, singlet oxygen and hydrogen peroxide has negative adverse role in the testicular I/R injury (Kheradmand et al. 2011). In the physiologic condition, antioxidant mechanisms scavenge produced free radicals while in the oxidative stress condition, imbalance occurs between ROS and scavenge free antioxidants (Agarwal et al. 2014). Ischemia increases in intracellular hypoxanthine as a result of ATP breakdown and during R, xanthine oxidase converts hypoxanthine and superoxide radicals (Agarwal et al. 2014). The GPx and CAT are the first line of cellular defense against oxidative stress (Agarwal et al. 2014). During testicular torsion and detorsion inversly enhanced ROS indicates lipid peroxidation. Testicular cell membranes are rich in polyunsaturated fatty acids and are vulnerable to oxidative injury (Ma et al. 2018). So, oxidative stress inhibitors or increase in anti-oxidant enzymes level has beneficial effect on testicular IR injury (El-Shahat et al. 2012). In this regard, reported Caffeic Acid derivatives, and polysaccharide fractions from EP have strong antioxidative effects (Newair et al. 2017). Based on the literature, antioxidant protective effects have been reported for the EPE (Ahmed et al. 2017). In our recent study, 50 and 100 mg/

kg of the EPE improved sperm count and mobility in I/R injury rat (Motamedi et al. 2017). Oral administration of EPE (100 mg/kg for 8 weeks) before exposure to Gamma rays increased GPx, SOD and CAT in the rat liver and testes. The echinacoside and caffeic acid content of the EP are potent scavengers of free radicals which protect cell form oxidation and cellular membrane destruction (Farombi et al. 2010). Also, Bayramoglu et al. (2011) revealed EP decreased liver enzymes, inflammatory cell infiltration, necrosis in hepatic and liver. Based on the findings of the current study, MDA levels increased in I/R rat while EPE in a dose dependent manner decreased I/R-induced MDA. Experimental I/R decreased SOD and GPx activity in comparision to control group. Injection of the of the EPE (25, 50 and 100mg/kg) increased SOD and GPx activity. Under normal conditions, free radicals are produced, and their effects are counterbalanced by way of their own antioxidant mechanisms, including enzymatic and non-enzymatic antioxidant systems (Farombi et al. 2010). Intracellular glutathione is the major buffer of the cellular redox status that acts against reactive species (Ahmed et al. 2017). Despite the well documented medical properties of the Echinacea species (E. angustifolia, E. pallida, and E. purpurea) , it is reported EP has higher antioxidant activity among the other Echinacea species (Bayramoglu et al. 2011). So, because of that, in the current study we used EPE to determine its possible protective effects on experimental I/R injury in rat. Aromatic ring in Caffeic acid enhances its antioxidant efficacy against antioxidant radical scavenging (Newair et al. 2017). Caffeic acid has strong antimicrobial, anti-inflammatory, antineoplastic and antioxidant activity which decrease the oxidative damage (Arena et al. 2017). Caffeic acid (10 µmol) blocks the production of ROS and inhibits lipid peroxidation and suppresses oxidative stress (Newair et al. 2017). Caffeic acid has protective effect on spinal cord I/R injury in rabbits. In conclusion ROS elicits the apoptosis in testicular germ cells in IR injury (Arena et al. 2017). These results suggested the EPE has protective effect against against testicular I/R. Based on the literature, there was no similar report to compare results of the current paper with it. We think further researches are needed to determine direct cellular and molecular action of the EPE against I/R injury.

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Conflicts of Interest

The author declared no conflict of interest.

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اثرات عصاره *اکیناسه آ* پورپورا بر آسیب ناشی از ایسکمی رپرفیوژن در بیضه موش صحرایی

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چکیده

زمینهٔ مطالعه: ایسکمی رپرفیوژن در بیضه یکی از شرایط ناباروری در مردان است که در طی آسیب ناشی از اکسیداسیون اتفاق می افتد. عصاره گیاه اکیناسه دارای اثرات آنتی اکسیدانی و محافظتی می باشد.

هدف: مطالعه حاضر بمنظور بررسی اثرات عصاره اکیناسه اثرات عصاره بر آسیب ناشی از ایسکمی رپرفیوژن در بیضه موش صحرایی می باشد.

روش کار: در این مطالعه ۵۰ سر موش بالغ نژاد ویستار بطور تصادفی به ۵ گروه آزمایشی تقسیم شد: گروه اول کنترل، گروه دوم، ۲ ساعت اسیکمی و ۲۴ ساعت رپرفیوژن بیضه چپ، گروه سوم، ۲ ساعت ایسکمی که ۱ ساعت پس از ایجاد ایسکمی به موش ها عصاره اکیناسه (۲۵ میلی گرم/کیلوگرم) بصورت داخل صفاقی تزریق شد و سپس ۲۴ ساعت رپرفیوژن انجام شد. گروه های ۴ و ۵ مشابه آزمایش سوم بود و موش ها با سطوح ۵۰ و ۱۰۰ میلی گرم/کیلوگرم عصاره اکیناسه را دریافت کردند. پس از ۲۴ ساعت، بیضه چپ جدا و برای ارزیابی هیستولوژی و مقادیر آنزیم های سوپراکسیددسموتاز، مالون دی آلدهید، گلوتاتیون پراکسیداز مورد استفاده قرار گرفت.

نتایج: با توجه به نتایج بدست آمده، سطوح مالون دی آلدهید بطورمعنی داری در موش های دچار ایسکمی ریپرفیوژن افزایش پیدا کرد ($P<0/05$) درحالی که عصاره اکیناسه بطور وابسته به دوز موجب کاهش مالون دی آلدهید شد ($P<0/05$). ایسکمی رپرفیوژن تجربی موجب کاهش فعالیت سوپرااکسیددسموتاز و گلوتاتیون پراکسیداز در مقایسه با گروه کنترل شد ($P<0/05$). تزریق عصاره اکیناسه (۲۵، ۵۰ و ۱۰۰ میلی–گرم/کیلوگرم) بطور وابسته به دوز و معنی داری موجب افزایش فعالیت سوپرااکسیددسموتاز و گلوتاتیون پراکسیداز شد ($P<0/05$). تجویز عصاره اکیناسه تاثیر معنی داری بر مقادیر توتال آنتی اکسیدان در مقایسه با گروه کنترل نداشت ($P<0/05$). در موش های دچار ایسکمی رپرفیوژن لوله های اسپرم ساز تخریب شده و اسپرماتوسیت کمی دیده شد. عصاره اکیناسه (۵۰ و ۱۰۰ میلی گرم/کیلوگرم) موجب بهبود شاخص های بیضه به همراه توبول های سیمنی فروس و اسپرماتوسیت در مقایسه با گروه ایسکمی رپرفیوژن شد.

نتیجه‌گیری نهایی: نتایج پیشنهاد دهنده این بود که عصاره اکیناسه اثرات محافظتی در مقابل ایسکمی پرفیوژن بیضه دارد.

واژه‌های کلیدی:

اکیناسه آ پورپورا، ایسکمی رپرفیوژن، موش صحرایی

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