Protection against ischemia/reperfusion-induced renal injury by co-treatment with erythropoietin and sodium selenite

LU LIU[1*], CHAO LIU[1*], LAN HOU[1*], JUAN LV[2], FANG WU[1], XUEFEI YANG[2], SHUTING REN[3], WENJUN JI[2], MENG WANG[2] and LINA CHEN[2]

[1]Department of Clinical Medicine, College of Clinical Medicine; [2]Department of Pharmacology; [3]Department of Pathology, College of Basic Medicine, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi 710061, P.R. China

Received December 9, 2014; Accepted September 9, 2015

DOI: 10.3892/mmr.2015.4426

Abstract. Ischemia/reperfusion injury (IRI) has lzong been an area of concern and focus of investigations. Erythropoietin (EPO) exhibits multiple protective effects, and selenium is an antioxidant trace element in the body, however, there have been no reports concerning the effects of EPO combined with sodium selenite on IRI. In the present study, a mouse model of renal IRI (RIRI) was pre-treated with EPO and sodium selenite to determine the most appropriate combination ratio of the two for further investigation. The results revealed that EPO and sodium selenite had synergistic protective effects in RIRI. EPO was identified as the predominant treatment component, with sodium selenite serving as an adjuvant, and combination treatment was markedly more effective, compared with treatment with either drug alone. The optimal ratio of treatment was 10:1 (10 IU EPO: 1 μg sodium selenite). The results indicated that RIRI markedly induced renal injury, as evidenced by elevated levels of blood urea nitrogen (BUN), as well as higher pathological scores, based on hematoxylin and eosin staining. Pre-treatment with EPO and sodium selenite significantly decreased serum expression levels of BUN and malonaldehyde, and increased the expression levels of superoxide dismutase, glutathione peroxidase and nitric oxide (NO), compared with the model group. Furthermore, co-treatment with EPO and sodium selenite upregulated the protein expression levels of phosphatidylinositol-3 kinase (PI3K) in renal tissue samples. Together, the results suggested that co-administration of EPO and sodium selenite effectively ameliorates IRI-induced renal injury by reducing oxidative stress and activating the PI3K/NO signaling pathway.

Correspondence to: Dr Lina Chen, Department of Pharmacology, College of Basic Medicine, Xi'an Jiaotong University Health Science Center, 76 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: chenlin@mail.xjtu.edu.cn; 394127769@qq.com

*Contributed equally

Abbreviations: RIRI, renal ischemic/reperfusion injury; ROS, reactive oxygen species; EPO, erythropoietin; EPOR, erythropoietin receptor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol-3 kinase; Se, sodium selenite; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; BUN, blood urea nitrogen; Cr, creatinine; eNOS, endothelial nitric oxide synthase; NO, nitric oxide

Key words: erythropoietin, sodium selenite, renal ischemia/reperfusion injury, phosphatidylinositol-3 kinase signaling pathway

Introduction

Ischemia/reperfusion injury (IRI) is commonly observed in clinical settings following stroke, trauma surgery and organ transplantation (1). Renal IRI (RIRI), which is commonly observed following hypovolemic shock, renal surgical procedures, renal transplantation and acute renal arterial occlusion, is a major cause of acute renal failure, and is a complex pathophysiological process leading to high rates of morbidity and mortality (2-4). However, no effective therapy is currently available, with the exception of supportive treatment. Therefore, the identification of effective therapeutic intervention strategies for RIRI is required. The pathophysiological mechanism underlying RIRI is complex, and involves the release of reactive oxygen species (ROS) (5), generation of pro-inflammatory mediators (6), calcium overload and activation of the apoptotic genes (7). A previous study demonstrated that ROS are important in the pathophysiology of RIRI (8).

Erythropoietin (EPO) is a hematopoietic hormone produced by the kidney and the fetal liver in response to hypoxia (9). Previous studies have revealed that EPO has numerous protective effects, including anti-oxidative (10,11), anti-inflammatory (12) and anti-apoptotic effects (13,14). EPO can exert these protective effects against IRI in the brain (15), liver, heart and intestine (16,17). Furthermore, it has been reported that renal expression levels of EPO are decreased following RIRI (18). EPO exerts cytoprotective effects by modulating a variety of signal transduction pathways, which involve mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) and phosphatidylinositol 3-kinase (PI3K)/Akt (19,20). It has been reported that PI3K/Akt signaling pathway activation exerts protective effects on IRI, as activated Akt elevates

the expression of endothelial nitric oxide synthase (eNOS) and the production of nitric oxide (NO) in endothelial cells (21). However, the protective mechanisms underlying the effects of EPO on RIRI remain to be fully elucidated.

Selenium is an essential trace element, which scavenges free radicals (22), and its anti-oxidative function is associated with selenoproteins, including glutathione peroxidase (GSH-Px) and thioredoxin reductase (23). Selenium deficiency has been demonstrated to be closely associated with various diseases, including cancer, coronary artery disease and osteoarthritis (24-26). Previous studies have reported that selenium supplementation has a number of beneficial effects in IRI in different tissues, including the brain, heart, liver and kidney (27-30).

To the best of our knowledge, no studies have been performed on the effects of combined treatment with EPO and sodium selenite in IRI. Therefore, the aim of the present study was to investigate the synergistic effects of EPO and sodium selenite in RIRI. In addition, the mechanisms underlying the action of EPO and sodium selenite on RIRI were investigated.

Materials and methods

Determination of optimal treatment components

Animals, grouping and treatment. A total of 60 male Kun-Ming mice, weighing 18-22 g (provided by the Experimental Animal Center of Xi'an Jiaotong University, Xi'an, China) were used in the present study, in accordance with the recommended guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research. The present study was approved by the Ethics Committee at Xian Jiaotong University (Xian, China). The mice were randomly divided into six groups: In groups 1-6, drug dosage was determined based on the Uniform Design's Table of U6 (6^6) (31). EPO was obtained from Shengyang Sunshine Pharmaceutical Co., Ltd. (Shengyang, China). Sodium selenite was purchased from Shanghai Tiancifu Bioengineering Co., Ltd. (Shanghai, China). Following treatment for 3 days with the concentrations indicated in Table I, an acute RIRI model was established in the mice. The dosage ranges of EPO and sodium selenite were based on previous reports (11-13,19-23). Briefly, following anesthesia with 10% chloral hydrate, the abdomens of the mice were opened through ventrimeson, and the renal pedicle was exposed by incision. The right kidney was removed and the left kidney pedicle was clamped for 1 h. For reperfusion, the clamp was removed, and blood flow was allowed to return to the renal tissues. Following reperfusion for 4 h, blood samples were collected through the venous plexus behind the eyeball, and the mice were sacrificed with 10% chloral hydrate. The samples were placed into eppendorf tubes and centrifuged at 1,368 x g for 10 min at room temperature, from which the plasma was collected and stored at -80˚C until further analysis.

Treatment with EPO and sodium selenite in RIPI

Animals, grouping and treatment. Following treatment with the optimal dosage ratio of EPO and sodium selenite, 70 male Sprague-Dawley rats, weighing 180-220 g (obtained from the Experimental Animal Center of Xi'an Jiaotong University) were used to undertake factorial analysis. The rats were used in accordance with recommended guidelines on the care and use of laboratory animals issued by the Chinese Council on Animal Research. The study was approved by the Ethics Committee at Xian Jiaotong University. The Kun-Ming mice and Sprague-Dawley rats were fed with standard laboratory diets *ad libitum*, and were maintained at a temperature of 20-25˚C and a humidity of 45-55% with illumination for 12 h in a specific pathogen-free environment.

The rats were randomly divided into seven groups (n=10/group): Sham group, intraperitoneal (i.p) injection with normal saline; a model group, i.p injection with normal saline; EPO group, i.p injection with 1,500 U/kg/day EPO; Se group (i.p) injection with 150 μg/kg/day sodium selenite); EPO + Se (L) group, i.p injection with 750 U/kg/day EPO and 75 μg/kg/day sodium selenite); EPO + Se (M) group, i.p injection with 1,500 U/kg/day EPO and 150 μg/kg/day sodium selenite); EPO + Se (H) group, intraperitoneal (i.p) injection with 3,000 U/kg/day EPO and 300 μg/kg/day sodium selenite. Following three days of treatment, an acute RIRI model was established. Briefly, following anesthesia with 10% chloral hydrate, the renal pedicle of the rats was exposed by incision. The right kidney was removed and the left kidney pedicle was clamped for 1 h. The clamp was subsequently removed and blood flow was permitted to return to the renal tissues for reperfusion. Following 4 h reperfusion, blood samples were collected through the abdominal aorta under anesthesia. The blood samples were placed into eppendorf tubes and centrifuged at 1,368 x g for 10 min at room temperature, and the plasma was collected and stored at -80˚C until further analysis. The rats were sacrificed by cervical dislocation, and the left kidney was separated into two specimens, one of which was immediately stored in eppendorf tubes and frozen in liquid nitrogen; whereas the other half was fixed in 10% formalin for hematoxylin and eosin (H&E) staining and immunohistochemical examination.

Analysis of serum and renal biochemical indices. The serum levels of BUN were measured via urease methods using commercial kits (cat. no. C013-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The levels of serum and renal superoxide dismutase (SOD), malonic dialdehyde (MDA) and renal glutathione peroxidase (GSH-Px) were measured using a Total Superoxide Dismutase Assay kit (cat. no. A001-1; hydroxylamine method), Malondialdehyde Assay kit (cat. no. A003-1; TBA method) and Glutathione Peroxidase Assay kit (cat. no. A005; colorimetric method), according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute). Renal expression levels of nitric oxide (NO) were determined indirectly as the concentration of nitrite from nitrates, using an NO assay kit (cat. no. A012; Nanjing Jiancheng Bioengineering Institute).

Histological changes in the kidney. Renal tissue samples were embedded in paraffin (Xi'an Zaolutang Pharmaceutical Co, Ltd., Xi'an, China), sectioned at 5 μm and stained with H&E (Xi'an Laibo Bio-technology Co, Ltd., Xi'an, China). Renal tubule damage was scored by calculating the percentage of tubules in the corticomedullary junction, which exhibited cell necrosis, loss of the brush border, cast formation and tubular dilatation. The scoring of renal damage was performed in a double-blinded manner, as follows: Score 0, no damage; score 1, 10%; score 2, 11-25%; score 3, 26-45%; score 4, 46-75%;

score 5, >76%. Images of the representative fields were captured using an Olympus DP71 digital camera (Olympus Corporation, Tokyo, Japan).

Immunohistochemical detection in the renal tissue samples. Immunohistochemistry was performed to examine the expression levels of PI3K in the renal tissue samples. The tissue sections were deparaffinized and rehydrated, followed by immersion in citrate buffer (0.01 M at pH 6.0) and heating for 10 min (Gree Co., Guangzhou, China) in a microwave oven for antigen retrieval. Following cooling to room temperature, the tissue sections were rinsed with distilled water twice and PBS twice, and then incubated with 3% H_2O_2 solution to block endogenous enzymes. The tissue sections were subsequently incubated with normal goat serum to block non-specific binding following rinsing with phosphate-buffered saline. Subsequently, the tissue sections were incubated overnight at 4˚C with monoclonal mouse anti-human PI3K primary antibody (cat. no. ab86714; Abcam, Cambridge, USA; 1:200), followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 30 mins. Substrate-chromogen DAB reagent (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) was added for coloration, following which the tissue specimens were dehydrated, counterstained with xylene, and mounted under glass cover slips. Brown colored sites were observed at a final magnification of x400 under a microscope by a pathologist, and images were captured using an Olympus DP71 digital camera (Olympus Corporation, Tokyo, Japan).

Statistical analysis. The data are presented as the mean ± standard error of the mean. Multiple comparisons were performed using analysis of variance with SPSS v13.0 software (SPSS, Inc., Chicago, IL, USA). Weighted modification analyses were performed using DAS 1.0 software (Shanghai University of Traditional Chinese Medicine, Shanghai, China). d represents the standardized dose and b represents the coefficient of standardized dose; the value of b (d) reflects the relative importance of the component. The higher the value of b (d), the more significant the dose-dependency. The component exhibiting the highest b value was deemed the predominant drug. b (d1d2) represents the interaction between the two drugs, with higher values representing increased synergy. Figures were generated using Prism 5.0 software (GraphPad Software Inc, La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Determination of optimal treatment components

Effects of EPO and sodium selenite on serum expression levels of BUN in RIRI mice. To evaluate the protective effects of EPO and sodium selenite on RIRI in mice, the expression levels of BUN were quantified. The expression levels of BUN were significantly reduced following injection with EPO and sodium selenite prior to RIRI. As shown in Table I, the serum expression levels of BUN decreased between 16.29±1.71 mmol/l (group 3) and 9.09±2.35 mmol/l (group 5).

Optimization formulation analysis. Further analysis demonstrated that the optimal proportion of compounds in the preparation was 10:1 (10 IU EPO: 1 μg sodium selenite), which was calculated using DAS 1.0 software (24). The standardized dose (d), the coefficient of the standardized dose (b), the value of b (d) reflected the relative importance of the components of the prescription (Table. II). The higher the value of b (d), the higher the significance of the dose-dependency. The component with the maximum b (d) value was the predominant drug, whereas, b (d1d2) indicated the interaction between the two drugs, of which a higher value indicated increased synergy. EPO and sodium selenite exhibited synergistically protective effects on RIRI. EPO was identified as the predominant drug and sodium selenite served as the adjuvant. The theoretical optimization formulation was determined as 4,000 IU/kg EPO and 400 μg/kg sodium selenite.

Effects of treatment with EPI and sodium selenite in RIPI. EPO combined with sodium selenite decreases serum expression levels of BUN in rats with RIRI. As shown in Fig. 1, the

Table I. Effects of EPO and sodium selenite on serum expression levels of BUN in mice with renal ischemia/reperfusion injury (n=10 for each group).

Group	EPO (IU/kg)	Se (μg/kg)	BUN (mmol/l)	1/BUN
1	125	50	15.13±1.48	0.067±0.006
2	250	400	14.16±3.13	0.074±0.018
3	500	25	16.29±1.71	0.062±0.007
4	1,000	200	10.74±1.65	0.098±0.017
5	2,000	12.5	9.09±2.35	0.117±0.031
6	4,000	100	9.76±1.66	0.112±0.020

Results are expressed as the mean ± standard error of the mean. EPO, erythropoietin; Se, sodium selenite; BUN, blood urea nitrogen.

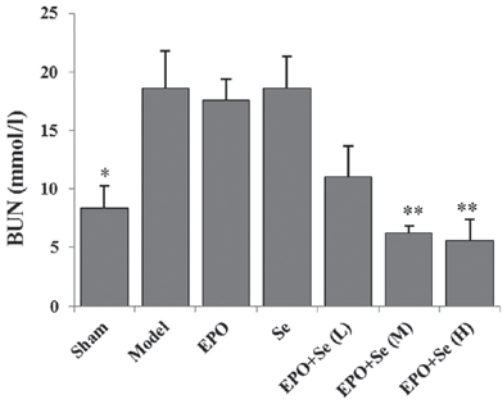

Figure 1. Effects of treatment with EPO combined with sodium selenite on serum the expression levels of BUN in rats with RIRI. Drugs were intraperitoneally injected prior to RIRI. Sham, no RIRI, normal saline; model, normal saline; EPO, 1,500 U/kg/day EPO; Se, 150 μg/kg/day Se; EPO + Se (L), 750 U/kg/day EPO+75 μg/kg/day Se; EPO + Se (M), 1,500 U/kg/day EPO+150 μg/kg/day Se; EPO + Se (H), 3,000 U/kg/day EPO+300 μg/kg/day Se. Data are presented as the mean ± standard error of the mean. $^{*}P<0.05$ and $^{**}P<0.01$, vs. model group. RIRI, renal ischemia/reperfusion injury; BUN, blood urea nitrogen; EPO, erythropoietin; Se, sodium selenite.

Table II. Effects of each component in combination in its interaction with 1/BUN.

Component	Standardized dose	b (d1)	b (d1d2)	P-value	Optimized dose	Annotation
EPO	d1	2.085	-	0.013	4,000.0 IU/kg	Dmax of EPO
Se	d2	0.314	-	0.042	400.0 μg/kg	Dmax of Se
EPO+Se	d1d2	-	0.698	0.041	400.0	Synergistic

A higher b (d) value indicates a more significant the dose-dependent association. b (d1d2) represents the interaction between the two drugs, and a high b (d1d2) value indicates increased synergy. Data analysis revealed that the optimal ratio of EPO to sodium selenite in the treatment preparation was 10:1 (10 IU EPO: 1 μg Se), determined using DAS 1.0 software. EPO, erythropoietin; Se, sodium selenite; d, standardized dose; b, coefficient of standardized dose. b (d) reflects the relative importance of the component; Dmax, sufficient drug quantity.

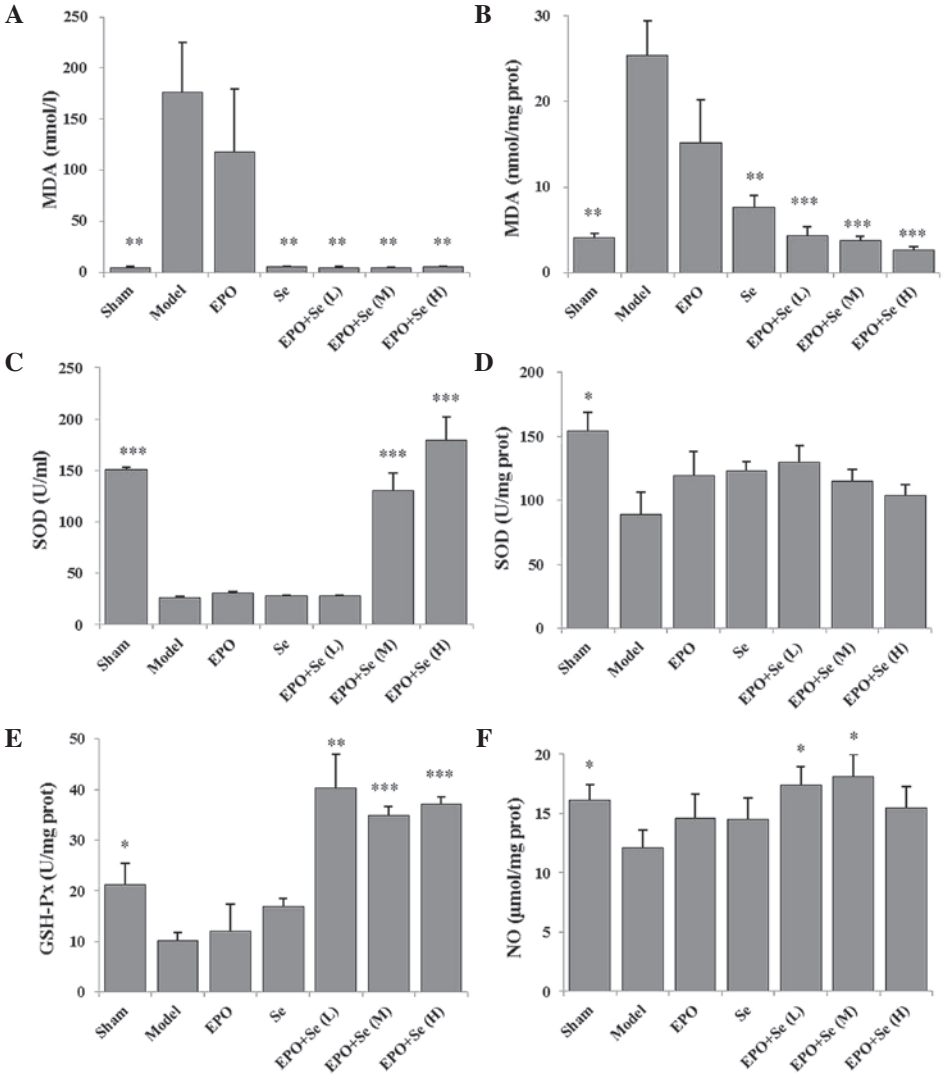

Figure 2. Effects of treatment with EPO and Se on the expression levels of MDA, SOD, GSH-Px and NO in rats with renal ischemia/reperfusion injury. (A) Serum expression levels of MDA. (B) Tissue expression levels of MDA. (C) Serum SOD activity. (D) Tissue SOD activity. (E) Tissue GSH-Px activity. (F) Tissue expression levels of NO. Data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001, vs. model group. EPO, erythropoietin; Se, sodium selenite; MDA, malondialdehyde; SOD, superoxide dismutase; GPH-Px, glutathione peroxidase; NO, nitric oxide.

serum expression levels of BUN in the RIRI model group were significantly higher, compared with the sham group (P<0.05), suggesting marked glomerular dysfunction. Compared with the model group, intraperitoneal administration of medium (1,500 U/kg/day EPO, 150 μg/kg/day Se); and high (3,000 U/kg/day EPO, 300 μg/kg/day Se) doses of EPO and sodium selenite combined resulted in a significant reduction in serum expression levels of BUN (P<0.01), which occurred in a dose-dependent manner. However, no significant effects on the expression levels of BUN were observed following treatment with EPO or sodium selenite alone, compared with the model group.

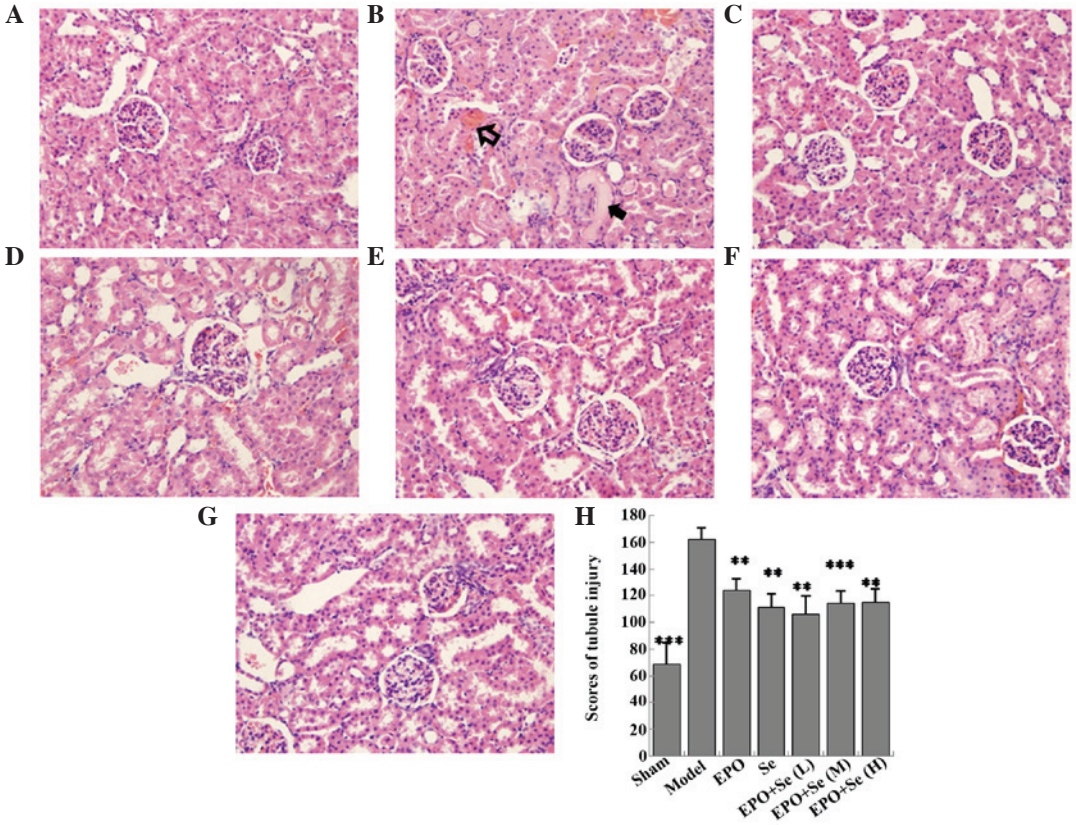

Figure 3. Effects of treatment with EPO and sodium selenite on renal histological sections of renal tissues from rats with renal ischemia/reperfusion injury. (A) Sham group; (B) model group; (C) EPO group; (D) Se group; (E) EPO + Se (L) group; (F) EPO + Se (M) group; (G) EPO + Se (H) group; (H) renal tubular lesion scores (magnification, x400). The solid arrow indicates the protein cast; the hollow arrow indicates the congestion of the blood vessel. Data are expressed as the mean ± standard error of the mean. **P<0.01 and ***P<0.001, vs. model group. EPO, erythropoietin; Se, sodium selenite.

EPO combined with sodium selenite improves serum biochemical parameters of rats with RIRI. As shown in Fig. 2A and B, the serum and tissue expression levels of MDA in the model group were significantly higher, compared with the sham group (P<0.01). Pre-treatment with EPO and sodium selenite significantly reduced the increased expression levels observed in the model group to varying degrees. Furthermore, the expression levels of MDA decreased in the sodium selenite-treated group, compared with the model group (P<0.01). Antioxidant enzyme activity levels were also measured, including those of SOD and GSH-Px (Fig. 2C-E). Tissue activities of SOD and GSH-Px in the model group were significantly lower than those in the sham group (P<0.01). Pre-treatment with EPO and sodium selenite significantly increased tissue activities of GSH-Px. However, the serum expression levels of SOD in the treated groups were not significantly different from those in the model group (P>0.05). As shown in Fig. 2F the tissue levels of NO were significantly lower in the model group, compared with the sham group (P<0.05). However, pre-treatment with EPO and sodium selenite increased tissue levels of NO, compared with the model group (P<0.05).

EPO and sodium selenite ameliorate histological alterations in rats with RIRI. Histological alterations were observed in the renal tissue samples of the rats in each group (Fig. 3). Histological analysis of the H&E-stained renal sections in the sham group revealed an integrated architectural structure of healthy glomerular and tubular cells without necrosis (Fig. 3A). However, the renal sections of the model group showed dilation of the Bowman's capsule, degeneration of the tubular epithelium, necrosis of the tubular epithelium, tubular dilation, protein cast, interstitial inflammatory cell infiltration and congestion of the blood vessels (Fig. 3B). The degree of renal tissue damage was significantly ameliorated in the rats pretreated with EPO and sodium selenite (Fig. 3C-G). The tubule injury scores (Fig. 3H) also showed that EPO and sodium selenite reduced tubule injury in renal ischemia reperfusion (P<0.01).

Immunohistochemical analysis of the renal tissue samples and evaluation of the expression of PI3K. In order to further investigate the mechanisms underlying the effects of EPO and sodium selenite on RIRI, the protein expression levels of PI3K were quantified using immunohistochemistry. Positive expression of PI3K was indicated by brown staining following treatment with DAB (Fig. 4). Image-Pro Plus 6.0 was used to analyze the immunohistochemical images, which revealed that pre-treatment with EPO and sodium selenite significantly increased the expression levels of PI3K in the renal tissue samples, compared with the model group (P<0.01; Fig. 4). No differences in expression levels were observed following treatment with EPO alone, sodium selenite alone or treatment with EPO and sodium selenite combined (P>0.05). The results suggested that EPO and sodium selenite upregulated the expression levels of PI3K in the renal tissues of the RIRI rats.

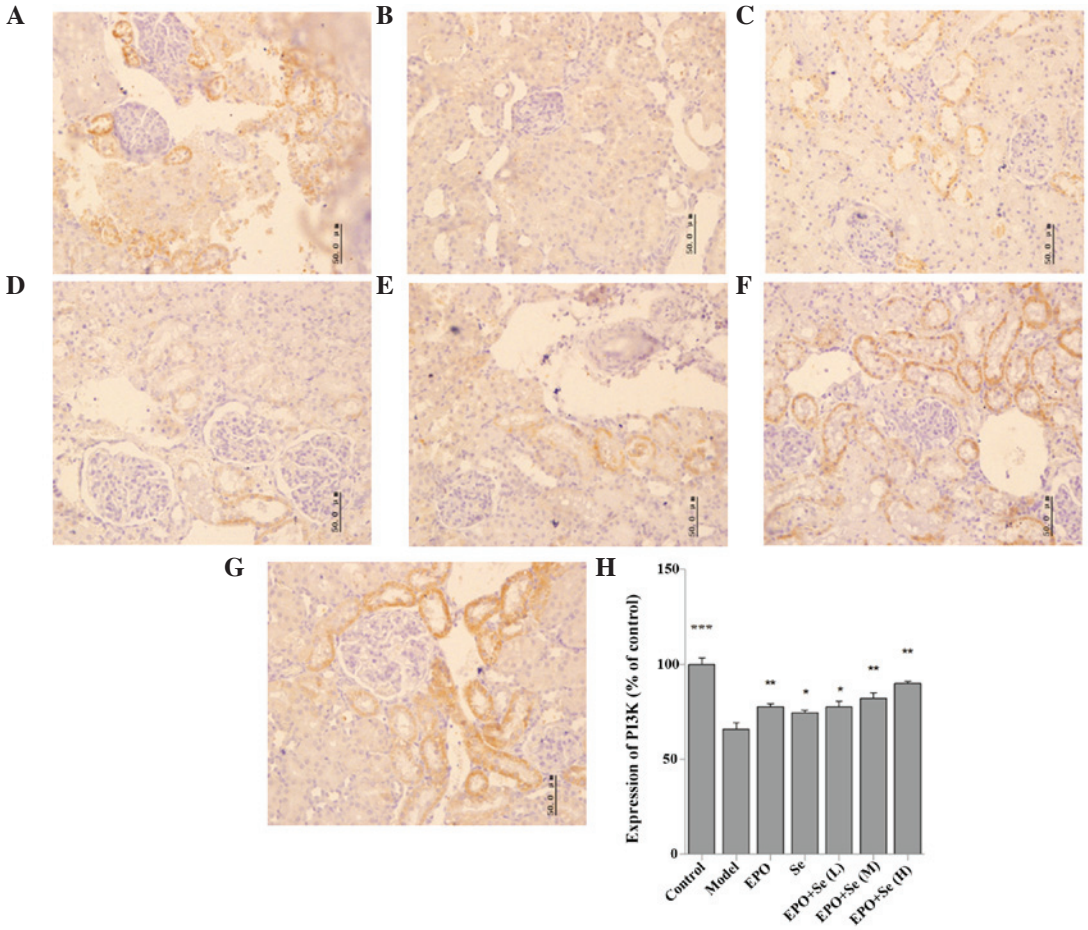

Figure 4. Effects of treatment with EPO and sodium selenite on the expression levels of PI3K in renal tissue samples of rats with renal ischemia/reperfusion injury. (A) Sham group; (B) model group; (C) EPO group; (D) Se group; (E) EPO + Se (L) group; (F) EPO + Se (M) group; (G) EPO + Se (H) group. (Magnification, x400); (H) Expression levels of PI3K. Data are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001, vs. model group. EPO, erythropoietin; Se, sodium selenite; PI3K, phosphatidylinositol-3 kinase.

Discussion

RIRI is often associated with rates of high morbidity and mortality in acute kidney injury (32). However, the pathophysiology of RIRI is complex and undefined, and there remains no effective treatment, requiring future investigations into drug combinations.

Weighted modification design was first developed by Zheng and Sun (31) using U6 (6^6), uniform design and 6x6 optimization latin square design theory, and the laws of compound drug dose-effect association to establish the multiple factors of multilevel data analysis to develop an efficient, simple drug formula method. It enables compound drug formula optimization, precise ratio proportioning, and can reduce the number of experimental group required (31). In the present study, weighted modification design was used for combined EPO and sodium selenite for the treatment of RIRI. In pre-renal injury, the expression levels of BUN increase disproportionately to those of creatinine due to the enhanced proximal tubular reabsorption, which follows the enhanced transport of sodium and water. Acute RIRI is a form of pre-renal injury. The results of the present study demonstrated that co-treatment with EPO and sodium selenite induced coordinated protection against RIRI, based on the expression levels of BUN. EPO was the predominant effective drug, and sodium selenite served as an adjuvant. The optimal ratio of treatment preparation was 10:1 (10 IU EPO: 1 μg sodium selenite).

IRI leads to the generation of ROS, which are critical in pathological processes and indirectly leads to lipid peroxidation (33). The superoxide radical is considered a 'primary' ROS, and is produced by the xanthine oxidase enzyme in the early stages of ischemia. The superoxide radical is depleted through a dismutation reaction, which is catalyzed by SOD to produce the less reactive H_2O_2, which is further converted to H_2O and O_2 by catalase and GSH-Px enzymes, preventing the formation of highly reactive hydroxyl radicals (34,35). The results of the present study demonstrated that pre-treatment with EPO and sodium selenite significantly increased the expression levels of serum SOD and renal SOD, as well as the activity of GSH-Px. MDA is a final product of lipid peroxidation, and is generally accepted as a sensitive marker of the rate of lipid peroxidation (34). In the present study, the serum and renal expression levels of MDA were significantly higher in the model group, compared with the groups pre-treated with EPO and sodium selenite, which exhibited significantly reduced MDA content and protected the tissue against further injury.

Concordant with these results, Hussein *et al* (36) demonstrated that treatment with EPO reduced the expression levels of renal MDA and increased the activity of SOD and

concentration of GSH, thereby improving renal function in IRI. EPO may exert its antioxidative effects directly by upregulating the activity of hemoglobin oxidase-1 (37). In addition, EPO may exert beneficial effects on ischemic preconditioning indirectly by inhibiting the activity of induced nitric oxide synthase (38), increasing the concentration of iron and increasing the number of immature red blood cells in the circulation to reduce cellular oxidative stress, as red blood cells contain high levels of anti-oxidative enzymes (39). Bozkurt *et al* (40) demonstrated that pre-treatment with selenium significantly decreases tissue expression levels of MDA and increases the activities of SOD and GSH-Px in tissues, which prevents tissue oxidative damage induced IRI in rat ovaries. The results of the present study suggested that treatment with EPO and sodium selenite significantly reduced IRI-induced oxidative stress via antioxidative properties.

Evidence has indicated that EPO specifically prevents the destruction of living cells surrounding areas of injury by signaling through a non-hematopoietic receptor (41). The expression of EPO receptor is also present in the kidney, liver, brain and heart (41-44). Following the binding of EPO to its receptors, phosphorylation of Janus kinase 2 occurs, which subsequently activated multiple signaling cascades that recruit PI3K, MAPK and NF-κB (45). Activation of the PI3K/Akt signaling pathway can regulate cell apoptosis, survival and the proliferation of downstream factors. Akt phosphorylates and inhibits Bas, resulting in B-cell lymphoma (Bcl) dissociation. Akt also activates inhibitor of κB kinase α, which leads to NF-κB activation, and induces the expression of anti-apoptotic genes, including Bcl-2, resulting in inhibition of apoptosis (41,45). Previous studies have reported that activation of the PI3K/Akt signaling pathway is also essential for EPO-mediated organ protection in renal and heart IRI (46,47). A previous study also demonstrated that activation of the apoptosis signal regulating kinase 1 (ASK1)/c-jun N-terminal kinase signaling cascade in cerebral IRI, which is closely associated with oxidative stress, can be suppressed by selenite through activation of the PI3K/Akt signaling pathway in rat hippocampi (48). Yoon *et al* (49) demonstrated that selenite suppresses H_2O_2-induced apoptosis, through inhibition of ASK1 and stimulation of PI3K/Akt activities (49). The results of the present study demonstrated that pre-treatment with EPO and sodium selenite increased the protein expression levels of PI3K to varying degrees, suggesting that activation of the PI3K/Akt signaling pathway during reperfusion is important in the renal protective effects of EPO and sodium selenite.

Further investigations demonstrated that activation of the PI3K/Akt signaling pathway elevated the expression levels of eNOS, thus increasing NO production. NO promotes vasorelaxation and reduces apoptosis by decreasing oxidative stress via the inhibition of NADPH oxidase (50). NO is also reported to attenuate vasoconstriction, reduce apoptosis and protect organs from IRI (51-53). In a previous study, Teng *et al* (54) demonstrated that cardioprotection effects induced by EPO were due to the stimulation of coronary artery endothelial cells, upregulated eNOS activity and enhanced NO production. In the present study, pre-treatment with EPO and sodium selenite increased the tissue expression levels of NO.

In conclusion, the data of the present study suggested that EPO and sodium selenite prevented much of the renal damage from IRI. These beneficial effects were closely associated with a decrease in oxidative stress and an increase in the production of NO via stimulation of the PI3K/NO signaling pathway. To the best of our knowledge, the present study is the first to report that EPO and sodium selenite exhibit synergistic protection on RIRI, and has important potential implications in the development of novel strategies to prevent and treat renal disease.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant. nos. 81471031 and 30901808), the Technology and Innovation Project of the Shaanxi Province (grant. no. 2011KTCL03-20) and the National College Students Innovation Training Project (grant. no. 201210698117).

References

1. Xia D, Shen K, Zhong W and Pan H: Administration of minocycline ameliorates damage in a renal ischemia/reperfusion injury model. Clin Invest Med 34: E55-E63, 2011.
2. Li YW, Zhang Y, Zhang L, Li X, Yu JB, Zhang HT, Tan BB, Jiang LH, Wang YX, Liang Y, *et al*: Protective effect of tea polyphenols on renal ischemia/reperfusion injury via suppressing the activation of TLR4/NF-κB p65 signal pathway. Gene 542: 46-51, 2014.
3. Salvadori M, Rosso G and Bertoni E: Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment. World J Transplant 5: 52-67, 2015.
4. Kiliç K, Hanci V, Selek S, Sözmen M, Kiliç N, Citil M, Yurtlu DA and Yurtlu BS: The effects of dexmedetomidine on mesenteric arterial occlusion-associated gut ischemia and reperfusion-induced gut and kidney injury in rabbits. J Surg Res 178: 223-232, 2012.
5. Cong G, Cui L, Zang M and Hao L: Attenuation of renal ischemia/reperfusion injury by a polysaccharide from the roots of Dipsacus asperoides. Int J Biol Macromol 56: 14-19, 2013.
6. Wang F, Yu G, Liu SY, Li JB, Wang JF, Bo LL, Qian LR, Sun XJ and Deng XM: Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats. J Surg Res 167: e339-e344, 2011.
7. He X, Xu X, Fan M, Chen X, Sun X, Luo G, Chen L, Mu Q, Feng Y, Mao Q and Chao Z: Preconditioning with hyperbaric oxygen induces tolerance against renal ischemia/reperfusion injury via increased expression of heme oxygenase-1. J Surg Res 170: e271-e277, 2011.
8. Qiao X, Li RS, Li H, Zhu GZ, Huang XG, Shao S and Bai B: Intermedin protects against renal ischemia/reperfusion injury by inhibition of soxidative stress. Am J Physiol Renal Physiol 304: F112-F119, 2013.
9. Fisher JW: Erythropoietin: Physiology and pharmacology update. Exp Biol Med (Maywood) 228: 1-14, 2003.
10. Lippi G, Franchini M and Banfi G: Biochemistry and physiology of anabolic androgenic steroids doping. Mini Rev Med Chem 11: 362-373, 2011.
11. Dimitrijevic ZM, Cvetkovic TP, Djordjevic VM, Pavlovic DD, Stefanovic NZ, Stojanovic IR, Paunovic GJ and Velickovic-Radovanovic RM: How the duration period of erythropoietin treatment influences the oxidative status of hemodialysis patients. Int J Med Sci 9: 808-815, 2012.
12. Nairz M, Sonnweber T, Schroll A, Theurl I and Weiss G: The pleiotropic effects of erythropoietin in infection and inflammation. Microbes Infect 14: 238-246, 2012.
13. Stoyanoff TR, Todaro JS, Aguirre MV, Zimmermann MC and Brandan NC: Amelioration of lipopolysaccharide-induced acute kidney injury by erythropoietin: Involvement of mitochondria-regulated apoptosis. Toxicology 318: 13-21, 2014.
14. Chen S, Li J, Peng H, Zhou J and Fang H: Administration of erythropoietin exerts protective effects against glucocorticoid-induced osteonecrosis of the femoral head in rats. Int J Mol Med 33: 840-848, 2014.
15. Pellegrini L, Bennis Y, Velly L, Grandvuillemin I, Pisano P, Bruder N and Guillet B: Erythropoietin protects newborn rat against sevoflurane-induced neurotoxicity. Paediatr Anaesth 24: 749-759, 2014.

16. Gul M, Cömert M, Çakmak GK, Kertis G, Ugurbas E and Oner MO: Effect of erythropoietin on liver regeneration in an experimental model of partial hepatectomy. Int J Surg 11: 59-63, 2013.
17. Yu Y, Shiou SR, Guo Y, Lu L, Westerhoff M, Sun J, Petrof EO and Claud EC: Erythropoietin protects epithelial cells from excessive autophagy and apoptosis in experimental neonatal necrotizing enterocolitis. PLoS One 8: e69620, 2013.
18. Zhang J, Zou YR, Zhong X, Deng HD, Pu L, Peng K and Wang L: Erythropoietin pretreatment ameliorates renal ischaemia-reperfusion injury by activating PI3K/Akt signalling. Nephrology (Carlton) 20: 266-272, 2015.
19. Kwon MS, Kim MH, Kim SH, Park KD, Yoo SH, Oh IU, Pak S and Seo YJ: Erythropoietin exerts cell protective effect by activating PI3K/Akt and MAPK pathways in C6 Cells. Neurol Res 36: 215-223, 2014.
20. Li XJ, Zhang GX, Sun N, Sun Y, Yang LZ and Du YJ: Protective effects of erythropoietin on endotoxin-related organ injury in rats. J Huazhong Univ Sci Technolog Med Sci 33: 680-686, 2013.
21. Yao L, Lu P, Li Y, Yang L, Feng H, Huang Y, Zhang D, Chen J and Zhu D: Osthole relaxes pulmonary arteries through endothelial phosphatidylinositol 3-kinase/Akt-eNOS-NO signaling pathway in rats. Eur J Pharmacol 699: 23-32, 2013.
22. Ošt'ádalová I: Biological effects of selenium compounds with a particular attention to the ontogenetic development. Physiol Res 61 (Suppl 1): S19-S34, 2012.
23. Guo F, Monsefi N, Moritz A and Beiras-Fernandez A: Selenium and cardiovascular surgery: An overview. Curr Drug Saf 7: 321-327, 2012.
24. Turan B, Saini HK, Zhang M, Prajapati D, Elimban V and Dhalla NS: Selenium improves cardiac function by attenuating the activation of NF-kappaB due to ischemia/reperfusion injury. Antioxid Redox Signal 7: 1388-1397, 2005.
25. Karaman S, Mansuroğlu B, Kizilbey K, Derman S and Hazar AB: Selenium status in blood, urine, and hair samples of newly diagnosed pediatric cancer patients. Turk J Med Sci 45: 329-334, 2015.
26. Shi XW, Guo X, Ren FL, Li J and Wu XM: The effect of short tandem repeat loci and low selenium levels on endemic osteoarthritis in China. J Bone Joint Surg Am 92: 72-80, 2010.
27. Mehta SL, Kumari S, Mendelev N and Li PA: Selenium preserves mitochondrial function, stimulates mitochondrial biogenesis, and reduces infarct volume after focal cerebral ischemia. BMC Neurosc 13: 79, 2012.
28. Treska V, Kuntscher V, Hasman D, Neprasová P, Kobr J, Racek J, Trefil L and Hes O: Importance of selenium for the influence of ischemia-reperfusion syndrome after kidney transplantation from a non-heart beating donor in a pig model. Transplant Proc 34: 3057-3059, 2002.
29. Lin TT, Wang BM, Li XY, Pan Y, Wang W, Mu Y, Liu JQ, Shen JC and Luo GM: An insight into the protection of rat liver against ischemia/reperfusion injury by 2-selenium-bridged beta-cyclodextrin. Hepatol Res 39: 1125-1136, 2009.
30. Ostadalova I, Vobecky M, Chvojkova Z, Mikova D, Hampl V, Wilhelm J and Ostadal B: Selenium protects the immature rat heart against ischemia/reperfusion injury. Mol Cell Biochem 300: 259-267, 2007.
31. Zheng QS and Sun RY: Quantitative design of drug compatibility by weighted modification method. Zhongguo Yao Li Xue Bao 20: 1043-1051, 1999.
32. Shih YC, Lee PY, Cheng H, Tsai CH, Ma H and Tarng DC: Adipose-derived stem cells exhibit antioxidative and anti-apoptotic properties to rescue ischemic acute kidney injury in rats. Plast Reconstr Surg 132: 940e-951e, 2013.
33. Wang HB, Li YX, Hao YJ, Wang TF, Lei Z, Wu Y, Zhao QP, Ang H, Ma L, Liu J, *et al*: Neuroprotective effects of LBP on brain ischemic reperfusion neurodegeneration. Eur Rev Med Pharmacol Sci 17: 2760-2765, 2013.
34. Kim J, Jang HS and Park KM: Reactive oxygen species generated by renal ischemia and reperfusion trigger protection against subsequent renal ischemia and reperfusion injury in mice. Am J Physiol Renal Physiol 298: F158-F166, 2010.
35. Guo C, Tong L, Xi M, Yang H, Dong H and Wen A: Neuroprotective effect of calycosin on cerebral ischemia and reperfusion injury in rats. J Ethnopharmacol 144: 768-774, 2012.
36. Hussein A, Shokeir AA, Sarhan ME, El-Menabawy FR, Abd-Elmoneim HA, El-Nashar EM and Barakat NM: Effects of combined erythropoietin and epidermal growth factor on renal ischaemia/reperfusion injury: A randomized experimental controlled study. BJU Int 107: 323-328, 2011.
37. Luo YH, Li ZD, Liu LX and Dong GH: Pretreatment with erythropoietin reduces hepatic ischemia/reperfusion injury. Hepatobiliary Pancreat Dis Int 8: 294-299, 2009.
38. Yang FL, Subeq YM, Chiu YH, Lee RP, Lee CJ and Hsu BG: Recombinant human erythropoietin reduces rhabdomyolysis-induced acute renal failure in rats. Injury 43: 367-373, 2012.
39. Liu N, Tian J, Wang W, Cheng J, Hu D and Zhang J: Effect and mechanism of erythropoietin on mesenchymal stem cell proliferation in vitro under the acute kidney injury microenvironment. Exp Biol Med (Maywood) 236: 1093-1099, 2011.
40. Bozkurt S, Arikan DC, Kurutas EB, Sayar H, Okumus M, Coskun A and Bakan V: Selenium has a protective effect on ischemia/reperfusion injury in a rat ovary model: Biochemical and histopathologic evaluation. J Pediatr Surg 47: 1735-1741, 2012.
41. Brines M and Cerami A: Emerging biological roles for erythropoietin in the nervous system. Nat Rev Neurosci 6: 484-494, 2005.
42. Lei DM, Piao SG, Jin YS, Jin H, Cui ZH, Jin HF, Jin JZ, Zheng HL, Li JJ, Jiang YJ, Yang CW and Li C: Expression of erythropoietin and its receptor in kidneys from normal and cyclosporine-treated rats. Transplant Proc 46: 521-528, 2014.
43. Yang XF, He Y, Li HY, Liu X, Chen H, Liu JB, Ji WJ, Wang B and Chen LN: Hepatoprotective effects of erythropoietin on D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure in mice. Mol Med Rep 10: 555-559, 2014.
44. Sanchis-Gomar F, Garcia-Gimenez JL, Pareja-Galeano H, Romagnoli M, Perez-Quilis C and Lippi G: Erythropoietin and the heart: physiological effects and the therapeutic perspective. Int J Cardiol 171: 116-125, 2014.
45. Rusai K, Prókai A, Szebeni B, Fekete A, Treszl A, Vannay A, Müller V, Reusz G, Heemann U, Lutz J, *et al*: Role of serum and glucocorticoid-regulated kinase-1 in the protective effects of erythropoietin during renal ischemia/reperfusion injury. Biochem Pharmacol 79: 1173-1181, 2010.
46. Yang C, Zhao T, Lin M, Zhao Z, Hu L, Jia Y, Xue Y, Xu M, Tang Q, Yang B, *et al*: Helix B surface peptide administered after insult of ischemia reperfusion improved renal function, structure and apoptosis through beta common receptor/erythropoietin receptor and PI3K/Akt pathway in a murine model. Exp Biol Med (Maywood) 238: 111-119, 2013.
47. Xu X, Cao Z, Cao B, Li J, Guo L, Que L, Ha T, Chen Q, Li C and Li Y: Carbamylated erythropoietin protects the myocardium from acute ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. Surgery 146: 506-514, 2009.
48. Wang Q, Zhang QG, Wu DN, Yin XH and Zhang GY: Neuroprotection of selenite against ischemic brain injury through negatively regulating early activation of ASK1/JNK cascade via activation of PI3K/AKT pathway. Acta Pharmacol Sin 28: 19-27, 2007.
49. Yoon SO, Kim MM, Park SJ, Kim D, Chung J and Chung AS: Selenite suppresses hydrogen peroxide-induced cell apoptosis through inhibition of ASK1/JNK and activation of PI3-K/Akt pathways. FASEB J 16: 111-113, 2002.
50. Mastromarino V, Volpe M, Musumeci MB, Autore C and Conti E: Erythropoietin and the heart: Facts and perspectives. Clin Sci (Lond) 120: 51-63, 2011.
51. Gliemann L, Nyberg M and Hellsten Y: Nitric oxide and reactive oxygen species in limb vascular function: What is the effect of physical activity? Free Radic Res 8: 71-83, 2014.
52. Dziodzio T, Biebl M and Pratschke J: Impact of brain death on ischemia/reperfusion injury in liver transplantation. Curr Opin Organ Transplant 19: 108-114, 2014.
53. Zhang W, Han Y, Meng G, Bai W, Xie L, Lu H, Shao Y, Wei L, Pan S, Zhou S, *et al*: Direct Renin inhibition with aliskiren protects against myocardial ischemia/reperfusion injury by activating nitric oxide synthase signaling in spontaneously hypertensive rats. J Am Heart Assoc 3: e000606, 2014.
54. Teng R, Calvert JW, Sibmooh N, Piknova B, Suzuki N, Sun J, Martinez K, Yamamoto M, Schechter AN, Lefer DJ and Noguchi CT: Acute erythropoietin cardioprotection is mediated by endothelial response. Basic Res Cardiol 106: 343-354, 2011.