

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

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Abstract. Ischemia/reperfusion injury (IRI) has long 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 μ g 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.

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- κ B (NF- κ B) 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

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Abbreviations: RIRI, renal ischemic/reperfusion injury; ROS, reactive oxygen species; EPO, erythropoietin; EPOR, erythropoietin receptor; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; 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

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 (6⁶) (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 ventrimidline, 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 RIRI

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% H₂O₂ 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* (*d*₁*d*₂) 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

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.

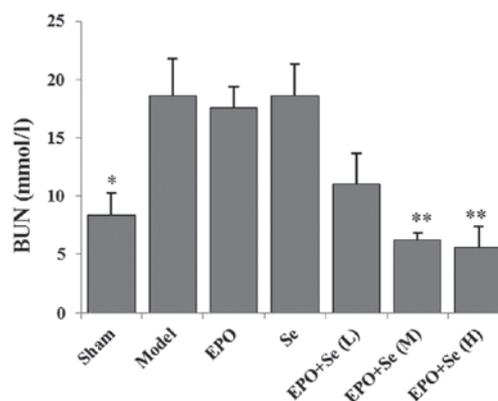


Figure 1. Effects of treatment with EPO combined with sodium selenite on serum the expression levels of BUN in rats with RIRI. Drugs were intraperitoneally injected prior to RIRI. Sham, no RIRI, normal saline; model, normal saline; EPO, 1,500 U/kg/day EPO; Se, 150 μg/kg/day Se; EPO + Se (L), 750 U/kg/day 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.

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* (*d*₁*d*₂) 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 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 $\mu\text{g}/\text{kg}$	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.

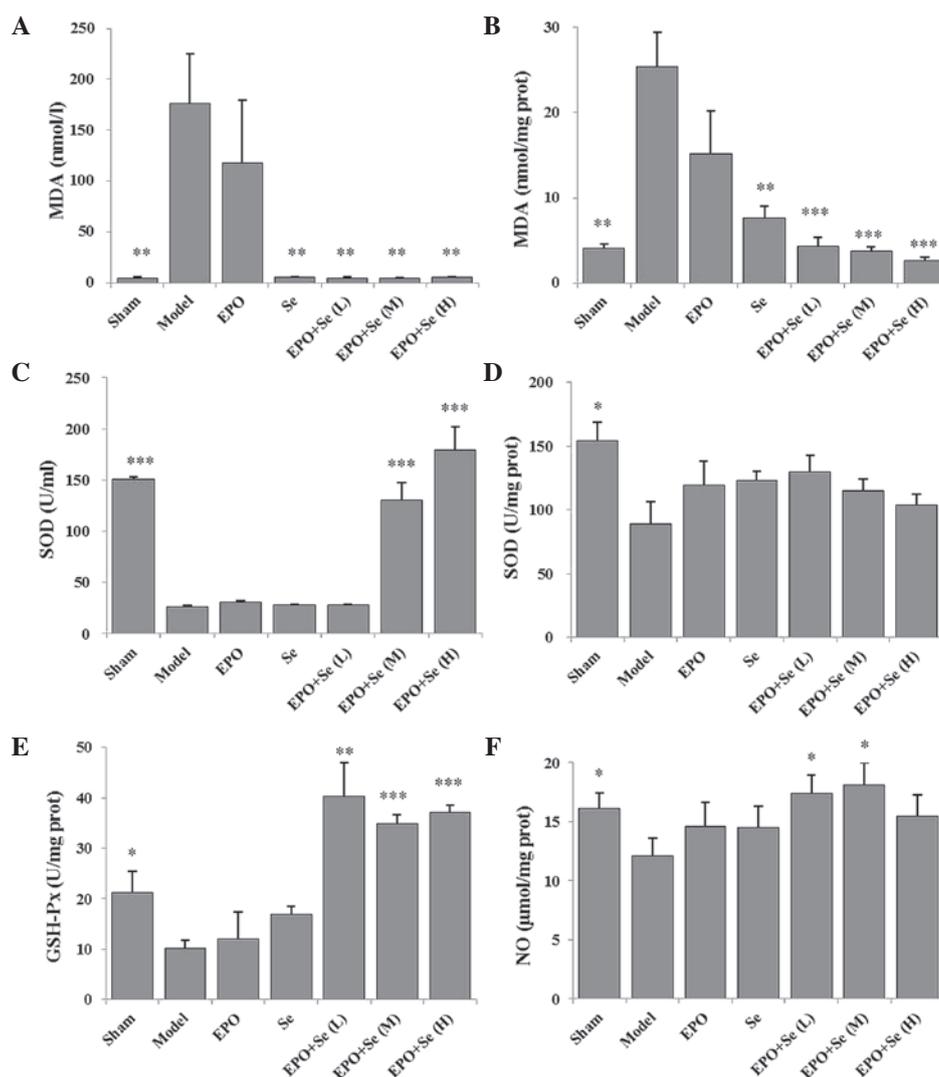


Figure 2. Effects of treatment with EPO and Se on the expression levels of MDA, SOD, GSH-Px and NO in rats with renal ischemia/reperfusion injury. (A) Serum expression levels of MDA. (B) Tissue expression levels of MDA. (C) Serum SOD activity. (D) Tissue SOD activity. (E) Tissue GSH-Px activity. (F) Tissue expression levels of NO. Data are expressed as the mean \pm 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; GSH-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 $\mu\text{g}/\text{kg}/\text{day}$ Se); and high (3,000 U/kg/day EPO, 300 $\mu\text{g}/\text{kg}/\text{day}$ 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.

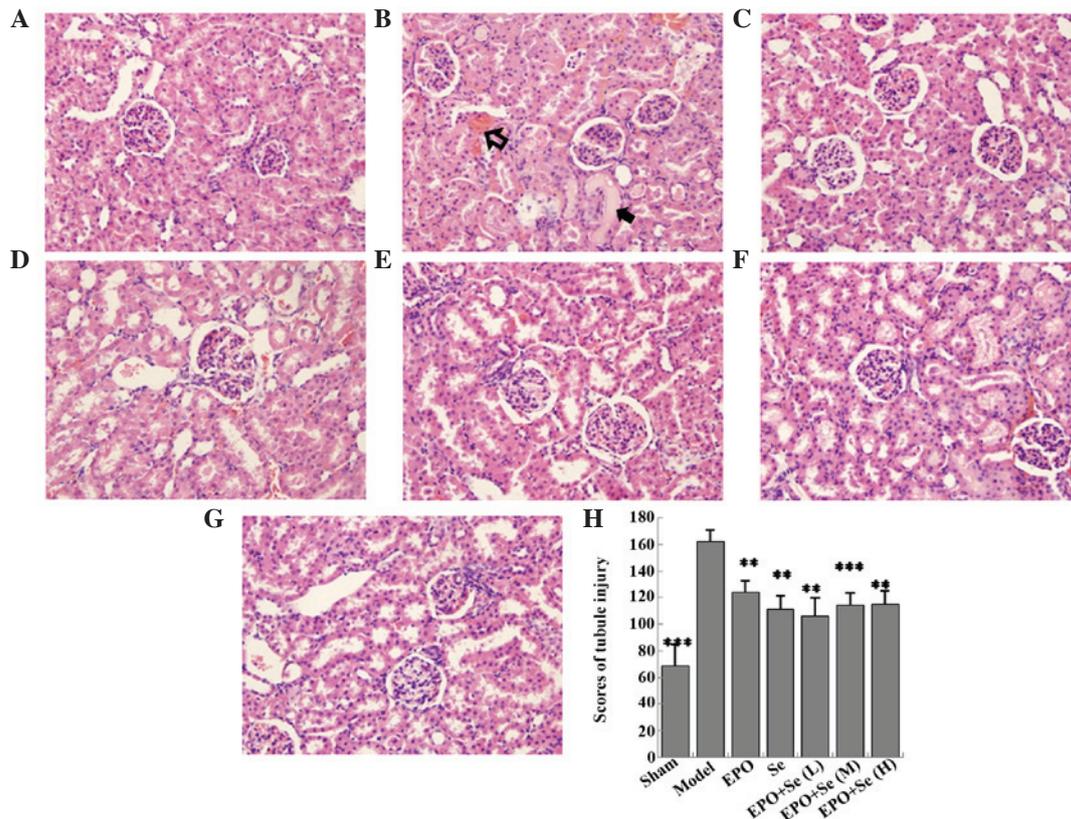


Figure 3. Effects of treatment with EPO and sodium selenite on renal histological sections of renal tissues from rats with renal ischemia/reperfusion injury. (A) Sham group; (B) model group; (C) EPO group; (D) Se group; (E) EPO + Se (L) group; (F) EPO + Se (M) group; (G) EPO + Se (H) group; (H) renal 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 \pm 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.

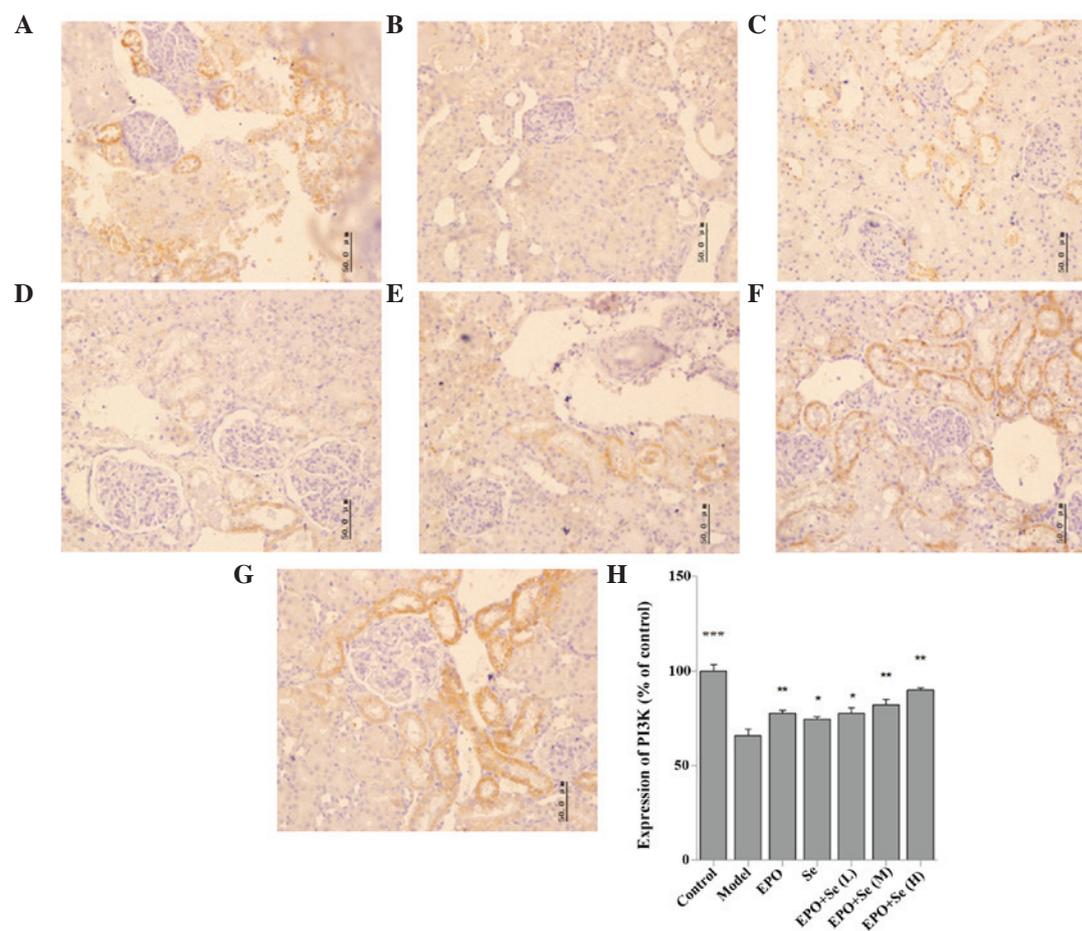


Figure 4. Effects of treatment with EPO and sodium selenite on the expression levels of PI3K in renal tissue samples of rats with renal ischemia/reperfusion injury. (A) Sham group; (B) model group; (C) EPO group; (D) Se group; (E) EPO + Se (L) group; (F) EPO + Se (M) group; (G) EPO + Se (H) group. (Magnification, x400); (H) Expression levels of PI3K. Data are expressed as the mean \pm 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 (6⁶), 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 optimization 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 μ g 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 H₂O₂, which is further converted to H₂O and O₂ 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- κ B (45). Activation of the PI3K/Akt signaling pathway can regulate cell apoptosis, survival and the proliferation of downstream factors. Akt phosphorylates and inhibits Bcl, resulting in B-cell lymphoma (Bcl) dissociation. Akt also activates inhibitor of κ B kinase α , which leads to NF- κ B 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 H₂O₂-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.

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