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Reduction of Acute Lung Injury by Administration of Spironolactone After Intestinal Ischemia and Reperfusion in Rats

Abstract

Purpose: Multiple organ failure, including acute lung injury, is a common complication of intestinal ischemia and reperfusion (I/R) injury and contributes to its high mortality rate. Activated polymorphonuclear neutrophils and reactive oxygen species contribute to the lung injury caused by intestinal I/R. Mineralokortikoid receptor antagonist spironolactone has a protective effect against I/R injury in animal models of retina, kidney, heart, and brain. The aim of the present study is to investigate the effect of aldosteron receptor blocker spironolactone on lung injury induced by intestinal I/R.

Methods: Wistar albino rats were divided into four groups: (1) sham control; (2) intestinal I/R (30 min of ischemia by superior mesenteric artery occlusion followed by 3 h of reperfusion); (3) spironolactone pretreatment (20 mg/kg) + I/R; and, (4) spironolactone pretreatment without I/R. Spironolactone was given orally 3 days prior to intestinal I/R. A marker for lipid peroxidation (malondialdehyde; MDA), an indicator of oxidation state (reduced glutathione; GSH), an index of polymorphonuclear neutrophil sequestration (myeloperoxidase; MPO), inducible nitric oxide synthase (iNOS) immunoreactivity, and the histopathology of the lung tissue were analyzed.

Results: Spironolactone pretreatment markedly reduced intestinal I/R-induced lung injury as indicated by histology and MDA and MPO levels. Moreover, the pretreatment decreased the iNOS immunoreactivity.

Conclusion: The present study strongly suggests that spironolactone pretreatment decreased neutrophil infiltration, iNOS induction, oxidative stress, and histopathological injury in an experimental model of intestinal I/R induced-lung injury of rats.

Manuscript submitted 23rd July, 2015

Manuscript accepted 7th January, 2016

Clin Invest Med 2016; 39 (1): E15-E24.

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Intestinal ischemia and reperfusion (I/R) leads to the production of numerous mediators, including reactive oxygen species (ROS), pro-inflammatory cytokines and nitric oxide (NO). Multiple enzymes that may harm distant organs are also activated [1,2]. Interactions between polymorphonuclear neutrophils and endothelial cells are instrumental in inducing secondary, inflammatory injury to distant organs including lung, heart, kidney and liver and this inflammatory injury is the leading cause of death in critically ill patients [2]. Intestinal I/R is, thus, a common feature of critical care and represents a useful and clinically relevant area of study of the pathophysiological aspects of indirect acute lung injury (ALI) [3].

The lung is a vulnerable organ, and lung injury following intestinal I/R has been well-characterized as an acute inflammation, with sequestration of leukocytes and their enzymatic products in lung tissue, leading to increased microvascular permeability and causing perivascular and interstitial edema [1,4]. It is thought that damage to the intestinal mucosal barrier following I/R causes dislocation of bacteria or endogenous endotoxins, leading to systemic inflammatory reactions. I/R involves the release of a large number of inflammatory mediators, including cytokines, arachidonic acid metabolites and NO [5]. Activated neutrophils, ROS, complement activation and endotoxemia also play a critical role in the development of lung injury [6]. The neutrophils and their enzymatic products are sequestered in lung tissue, which causes increased microvascular permeability, perivascular and interstitial edema, and ultimately leads to pulmonary edema [5]. ALI is characterized by the development of hypoxemia, damage to the alveolar-capillary membrane barrier, pulmonary edema and resultant respiratory failure [7]; therefore, it is important to reduce the immune response in order to avoid multi-organ injury. Several protective approaches for I/R injury have been investigated by targeting such individual mediators or mechanisms as antioxidants, anti-leukocyte factors and anticomplements [8-12].

Oxidative stress has been strongly implicated in the development of ALI after intestinal I/R [13,14]. In addition, the induction of inducible nitric oxide synthase (iNOS) was suggested in the pathogenesis of intestinal I/R-induced lung injury. The mechanisms of the cytotoxic actions of excessive NO production are not fully understood. It has been suggested that the superoxide ions react with NO to produce peroxynitrite, which then causes accentuated lipid peroxidation and protein and deoxyribonucleic acid (DNA) modifications, resulting in cellular damage. Taken together,

these results suggest that the iNOS-NO-peroxynitrite-dependent pathway may be one of the mechanisms of I/R-induced lung injury [15]. During intestinal I/R, the level of NO has been shown to be altered. Furthermore, NO induces the formation of vermiculite mucosa in the intestinal tract. NO is postulated to be a cytotoxic factor, similar to free radicals, which plays a role in pathophysiological processes, including various forms of inflammation and circulatory shock [16].

Mineralocorticoid receptor (MR) activation is a contributing factor in the pathophysiology of a wide range of diseases. Elevated levels of aldosterone, which is a physiological activator of MR, are known to induce hypertension, alter inflammation and fibrosis and exacerbate cardiovascular diseases [17,18]. MR antagonists have successfully been used as clinical interventions for the treatment of hypertension, heart failure and post-myocardial infarction remodelling [17,19]. Prophylactic MR antagonism with spironolactone (Sp) in rats has been shown to completely prevent the ischemic injuries of kidney, heart and brain [15,19-22]. Several studies have tried to elucidate the underlying mechanism of MR antagonist-induced cardioprotection [23]. Limitation of IR-induced apoptosis plays a pivotal role in the cardioprotective effect of these drug [24]. Several reports also indicate that Sp can reduce oxidative stress and inflammatory response [25]. Our previous study demonstrated that Sp, a aldosterone receptor antagonist, significantly decreases intestinal ischemic damage [26]; however, the effect of Sp on intestinal I/R-induced lung injury is unknown.

The aim of the present study was to investigate the effect of Sp, a aldosterone receptor blocker, on the lung tissue injury following intestinal I/R, focusing particularly on the effects of oxidative damage, neutrophil infiltration and iNOS expression.

Methods

Animals

Adult male Wistar albino rats weighing approximately 300-350 g were housed in cages at room temperature (25 ± 2 °C; 45-55 % relative humidity) with a 12 h light-12 h dark cycle. They were allowed free access to water and commercial chow before the surgery. All procedures involved in animals were approved by the Ethics Committee (Animal Investigation Committee) of Bulent Ecevit University Medical School. Maximum effort was made to ensure humane treatment of the animals. Each animal was fasted overnight the day before surgery, but had access to water *ad libitum*.

Experimental Design

Thirty-two male Wistar albino rats were randomly divided into four groups, each consisting of eight animals: (1) sham-operated control group; (2) intestinal I/R group, animals subjected to 30 min ischemia and 3 h reperfusion; (3) Sp+intestinal I/R group, animals treated with 20 mg/kg of Sp for 3 days before I/R was performed; and, (4) Sp+sham group, animals treated with 20 mg/kg of Sp for 3 days in the absence of intestinal I/R. Each pill of Sp was carefully crushed followed by appropriate weighing of the powder for each animal. The powder of Sp was then mixed with peanut butter and given orally at a dose of 20 mg/kg to the animals daily. The three-day treatment started 3 days prior surgery. All animals showed a remarkable adjustment for eating the newly introduced food in the training period. Animals in sham-operated control group and I/R group were given only the peanut butter. The dosage of Sp was the most commonly used one in rat models of experiments [21].

Intestinal Ischemia/Reperfusion

Rats were anesthetized with sodium thiopental (90 mg/kg, i.p.) and then a laparotomy through a midline incision into the peritoneal cavity was performed. After the small bowel was exteriorized gently to the left on to moist gauze, animals were subjected to 30 min of ischemia by occlusion of the superior mesenteric artery, using a microvascular clamp. Intestinal ischemia was confirmed by observing loss of pulsation of the mesenteric artery and its branches as well as paleness of the jejunum and ileum. Afterwards the intestines were returned to the abdomen, which was then closed with two small clamps. At the end of 30 min of ischemia, the clamp was gently removed to allow reperfusion of the blood flow, which was confirmed by observing the pulsation of the artery and its branches on the intestine.

Duration of the reperfusion period was 3 hours. Body temperature was maintained at 37 °C by a heating lamp during I/R. Sham control animals underwent the same procedure but without vascular occlusion. The rats were sacrificed at the end of the intestinal reperfusion. The thoraco-abdominal cavity was opened and the hilus of the right lung was clamped and resected. The upper lobe of the right lung was placed in 10% formaldehyde and subjected to routine processing for histopathologic examination and immunohistochemical staining. The middle lobe of the right lung was stored at -80 °C for later analysis of the tissue myeloperoxidase (MPO), malondialdehyde (MDA) and reduced glutathione (GSH) levels.

Detection of Myeloperoxidase Activity in Lung Tissue

MPO activity was measured to determine the degree of neutrophil accumulation in the tissue samples according to a method described by Bradley *et al.* [27].

Detection of MDA and GSH Levels in Lung Tissue

MDA, which reflect lipid peroxidation level, was measured in the lung tissue samples based on a method described by Casini *et al.* [28]. Briefly, by using a motor-driven pestle, tissue samples were homogenized in ice-cold trichloroacetic acid (TCA) by adding 10 ml of 10% TCA per g of tissue. After centrifugation, 750 µl supernatant was added to an equal volume of 0.67% thiobarbituric acid and heated to 100°C for 15 min. The absorbance of the samples was then measured spectrophotometrically at 535 nm. To measure GSH levels, 2 ml of 0.3 M Na₂HPO₄ solution was added to the 0.5 ml of supernatant obtained by using the same homogenization procedure as described above. A 0.2 ml solution of dithiobisnitrobenzoate was added into the mixture, and the absorbance at 412 nm was measured immediately after vortexing.

Histopathological Examination

For morphological evaluation of the lung specimens, each sample was fixed with 10% formaldehyde immediately after collection. Specimens were embedded in paraffin, cut with a cryostat at 4-5 µm thickness, deparaffinized and stained with hematoxylin and eosin (H&E) for histological evaluation in a single-blinded fashion. Histopathological examination of the lung tissue was based on a staging method described by Pirat *et al.* [29-31]. A light microscope was used for the evaluation of the lung tissue injury. The severity of the tissue damage was semi-quantitatively assessed. Scores were given as absent (0), minimal change (1), mild change (2), moderate change (3) and severe change (4), for each criteria. The injury criteria used for the scoring system are as follows: 1) interstitial edema; 2) hemorrhage; 3) airway epithelial-cell damage; 4) hyaline membrane formation; 5) neutrophil infiltration; 6) alveolar damage/disruption; and, 7) total lung injury score. The microscopic score of each tissue was calculated as the sum of the scores given to each criteria.

Immunohistochemical analysis

Immunohistochemical evaluation for iNOS (rabbit polyclonal antibody, RB-9242-R7, ready to-use for immunohistology, LabVision, Fremont, CA, USA) was performed using a

combination of the streptavidin-biotin-peroxidase method with the microwave antigen retrieval on formalin-fixed paraffin-embedded tissues. Following the deparaffinization process, the sections were treated with 10% hydrogen peroxidase in filtered water to block endogenous peroxidase activity. To retrieve the antigen, the slides were boiled with 10 mM of citrate buffer (pH 7) for 10 min. Preincubating with Ultra V block (Lab Vision) for 20 min, the sections were then incubated with the primary antibody for 1 hour at room temperature followed sequentially by biotinylated goat antipolyvalen (Lab Vision) for 20 min and streptavidin peroxidase complex (Lab Vision) for 20 min. DAB (3,3'-diaminobenzidine tetrahydrochloride) (Lab Vision) was used as the chromogen. The section was then counterstained with hematoxylin, rinsed and mounted. The sections were evaluated in a blinded fashion by the same investigator. Immunoreactivity was scored using a semiquantitative scale for intensity of staining as follows: 0 (negative, no staining); 1+ (minimally positive); 2+ (weakly positive); 3+ (moderately positive); and, 4+ (strongly positive).

Statistical analysis

Statistical analyses were carried out using the SPSS software (v. 20.0 for Windows, SPSS, Chicago, IL, USA). Continuous variables were presented as mean $\pm$ SD. The normality of distribution for continuous variables were detected through Shapiro-Wilk test. A nonparametric Levene's test was used to verify the homogeneity of variances in each study group. Group comparisons were performed by one-way analysis of variance (Kruskal-Wallis) followed, in case of significance, by the Mann-Whitney U test. Categorical variables are presented as median (min-max). Group comparisons were performed by median test and, in the case of significance, by chi-square test. P values less than 0.05 were deemed to be statistically significant.

Results

Biochemical findings

MDA Levels:

MDA levels in lung tissue samples are documented on Table 1. Average MDA content of lung samples from sham-operated group was 20.38 ± 1.34 nmol/g tissue, whereas that from I/R group was 27.18 ± 5.23 nmol/g (Figure 1A). There was significant difference between these two groups ($p=0.002$). Administration of Sp before the induction of ischemia significantly reduced the elevated MDA content: mean value

of the Sp+I/R group (20.57 ± 1.62 nmol/g tissue) was significantly different from that of the I/R group ($p = 0.002$). Average MDA content of lung samples from Sp+sham group was 21.17 ± 2.39 nmol/g tissue. On the other hand, average MDA contents of tissues from sham control, Sp+I/R group and Sp+sham group were not statistically different from each other.

GSH Levels:

As demonstrated on Table 1 and Figure 1B, the amount of GSH measured in the tissues subjected to I/R group (1.36 ± 0.44 μ mol/g tissue) was significantly decreased when compared with that measured in the tissues from sham-operated group (3.61 ± 1.42 μ mol/g tissue) ($p<0.001$). Mean GSH content of the Sp+I/R group was 2.95 ± 0.48 μ mol/g tissue, which was significantly different from that measured in I/R group ($p<0.001$). GSH contents measured in samples from sham-operated control group and Sp+sham group (3.86 ± 0.71 μ mol/g tissue) were statistically indistinguishable ($p=0.294$). Sp+sham group was significantly different from that measured in I/R group ($p<0.001$).

MPO activity:

As presented in Table 1, MPO enzyme activities in the lung samples from animals subjected to sham operation, I/R, Sp+I/R, and Sp+sham group were averaged 0.16 ± 0.10 , 3.49 ± 1.04 , 2.44 ± 0.41 and 0.20 ± 0.10 U/mg tissue, respectively (Figure 1C). The enzyme activity in sham control group was significantly different from both I/R ($p<0.001$) and Sp+I/R ($p<0.001$) groups. In the Sp+sham group, average MPO activity was not statistically different from that in sham-operated control group ($p = 0.292$). In terms of average MPO enzyme activities, Sp+sham group was significantly different from both I/R ($p<0.001$) and Sp+I/R ($p<0.001$) groups.

Histopathological findings

Analyzing eight sections per group, microscopic findings in the lung specimens revealed a minimal injury of the lung parenchyma in the sham group (Figure 2A), but severe lung injury in the I/R group (Figure 2B). Similar minimal lung parenchyma injury was observed both in Sp+sham group and sham group (Figure 2D), but significant improvement in lung injury was observed in Sp+I/R group (Figure 2C). Table 2 shows the scores given to each group and statistical comparison of the groups.

TABLE 1. Comparison of the biochemical markers of ischemia-related tissue injury

	Sham (n:8) Mean $\pm$ SD	I/R (n:8) Mean $\pm$ SD	Sp+I/R (n:8) Mean $\pm$ SD	Sp+sham (n:8) Mean $\pm$ SD	p	p1	p2	p3	p4	p5	p6
Malondialdehyde (nmol/g tissue)	20.38 $\pm$ 1.34	27.18 $\pm$ 5.23	20.57 $\pm$ 1.62	21.17 $\pm$ 2.39	0.02	0.002	0.527	0.115	0.002	0.009	0.344
Glutathione (μ mol/g tissue)	3.61 $\pm$ 1.42	1.36 $\pm$ 0.44	2.95 $\pm$ 0.48	3.86 $\pm$ 0.71	0.001	0.001	0.345	0.294	0.001	0.001	0.006
Myeloperoxidase (U/mg tissue)	0.16 $\pm$ 0.10	3.49 $\pm$ 1.04	2.44 $\pm$ 0.41	0.20 $\pm$ 0.10	0.001	0.001	0.001	0.292	0.036	0.001	0.001

I/R; Ischemia-Reperfusion

Sp; Spironolactone

Values are mean $\pm$ SD

p Kruskal-Wallis analysis of variance p value

p1 Comparison of Sham group and I/R group (Mann Whitney U test)

p2 Comparison of Sham group and Sp+I/R group (Mann Whitney U test)

p3 Comparison of Sham group and Sp+sham group (Mann Whitney U test)

p4 Comparison of I/R group and Sp+I/R group (Mann Whitney U test)

p5 Comparison of I/R group and Sp+sham group (Mann Whitney U test)

p6 Comparison of Sp+I/R group and Sp+sham group (Mann Whitney U test)

TABLE 2. Comparison of the histopathological (H&E) and immunohistochemical staining scores (0-4)

	Sham (n:8) Median, min-max	I/R (n:8) Median, min-max	Sp+I/R (n:8) Median, min-max	Sp+sham (n:8) Median, min-max	p	p1	p2	p3	p4	p5	p6
Interstitial edema	1.5 (1-2)	2.0 (2-2)	2.0 (2-2)	1.0 (1-1)	†	†	†	†	†	†	†
Hemorrhage	1.5 (1-2)	3.0 (3-3)	2.0 (2-2)	2.0 (1-2)	<0.001	<0.001	0.038	†	<0.001	<0.001	†
Epithelial injury	1.0 (1-1)	2.0 (2-2)	2.0 (2-2)	1.0 (1-1)	<0.001	<0.001	<0.001	†	†	<0.001	<0.001
Hyalin membrane formation	1.0 (0-1)	2.0 (2-2)	2.0 (2-2)	1.0 (1-1)	<0.001	<0.001	<0.001	†	†	<0.001	<0.001
Neutrophil infiltration	2.0 (1-2)	4.0 (4-4)	3.0 (3-3)	2.0 (2-2)	<0.001	<0.001	<0.001	†	<0.001	<0.001	<0.001
Alveolar damage/ disruption	2.0 (2-2)	4.0 (4-4)	2.0 (2-2)	2.0 (2-2)	<0.001	<0.001	†	†	<0.001	<0.001	†
Total injury score	8.0 (7-10)	17.0 (17-17)	13.0 (13-13)	9.0 (8-9)	<0.001	<0.001	<0.001	0.097	<0.001	<0.001	<0.001
iNOS	2.0 (1-2)	3.5 (3-4)	2.5 (2-3)	2.0 (2-3)	<0.001	<0.001	0.013	0.120	0.004	<0.001	0.097

I/R; Ischemia-Reperfusion

Sp; Spironolactone

iNOS; inducible nitric oxide synthase

H&E; hematoxylin and eosin

† All values are equal to or less than median

p Median test

p1 Comparison of Sham group and I/R group (Chi-square test)

p2 Comparison of Sham group and Sp+I/R group (Chi-square test)

p3 Comparison of Sham group and Sp+sham group (Chi-square test)

p4 Comparison of I/R group and Sp+I/R group (Chi-square test)

p5 Comparison of I/R group and Sp+sham group (Chi-square test)

p6 Comparison of Sp+I/R group and Sp+sham group (Chi-square test)

FIGURE 1: Effect of spironolactone (Sp) on lipid peroxidation, antioxidant status and neutrophil accumulation in lung tissue during acute lung injury induced by intestinal I/R. Average MDA (A), GSH (B), and MPO activity (C) of lung samples collected from sham-operated control (group 1), I/R (group 2), Sp+I/R (group 3) and Sp+sham (group 4).

Total lung injury scores of the groups were demonstrated on Table 2. There was no significant difference between Sp+sham group and sham-operated control group ($p = 0.097$) with respect to the average total lung injury scores; however, a significant difference was observed in comparison with the other groups ($p < 0.001$).

Histopathological parameters such as hemorrhage, neutrophil infiltration, and alveolar damage/disruption were significantly different in the I/R and Sp+I/R groups ($p < 0.001$). Epithelial injury, hyalin membrane formation, and neutrophil infiltration were significantly different in the sham control and Sp+I/R groups ($p < 0.001$). No statistically meaningful difference was observed in histopathological evaluation between sham control and Sp+sham groups or in the scores of interstitial edema.

Immunohistochemical finding

The images of lung tissue stained immunohistochemically with iNOS are depicted in Figure 3. The scores for staining intensities of the groups were given in Table 2. The most prominent staining for iNOS expression was detected in I/R group and pretreatment with Sp decreased the intensity of the staining. With respect to the iNOS scores, there was a significant difference between the I/R and Sp+I/R groups ($p < 0.004$). The immunoreaction with iNOS was virtually identical for sham control and Sp+sham groups ($p = 0.120$).

Discussion

In the present study, pretreatment with Sp was shown to prevent the rats from intestinal I/R-induced lung injury as evidenced by the attenuation of pathological and morphological changes as well as the decreases in oxidative stress and neutrophil accumulation in the tissue. A solid body of evidence has demonstrated that intestinal I/R rapidly activates local, remote, and systemic inflammatory responses, which contribute substantially to the injury of the intestine and of the distant organs including the lung, heart, kidney and liver, leading finally to multiple organ failure and death [3].

In the present study, intestinal I/R has been shown to cause significant lung inflammation, as indicated by increased iNOS production and by neutrophil infiltration into the lung tissue. Sp markedly alleviates this lung injury in the rat experimental model; pretreatment substantially reduced the histological damage, significantly decreased the MPO activity and MDA content and, remarkably, increased the GSH content to approximately normal levels. Previous studies have provided evidence that oxidative stress is the primary

participant in the pathogenesis of lung injury induced by intestinal I/R. Intestinal reperfusion allows ROS to “escape” from the microvasculature of the gut into the systemic circulation and to generate a cascade of various biological events including lung injury [14].

The mechanisms of remote organ injury induced by intestinal I/R are very complex. I/R injury can be initiated by the production of ROS, which seem to be responsible for the generation of the chemotactic activity in neutrophils. Accumulation of neutrophils in the lung is a prominent

feature of lung injury after intestinal I/R [3]. The lung is particularly vulnerable to the overproduced ROS and excessive levels of free radicals may lead to lipid peroxidation and damage to cell and mitochondrial membranes, which would eventually cause cell apoptosis and necrosis. MDA, a direct product of lipid peroxidation, reflects the extent of lipid peroxidation in tissue. Activated neutrophils can release ROS and proteolytic enzymes, resulting in functional and structural damage in lung parenchyma [4]. Pretreatment with Sp provided a significant reduction in lipid peroxidation of the

FIGURE 2: The histopathological appearances of the study groups (H&E, X100). (A) Sham-operated control group: normal lung tissue with minimal alveolar damage and hemorrhage. (B) I/R group: lung tissue damage displaying hemorrhage along with patches of alveolar damage with inflammatory cell infiltration and bronchial epithelial cell damage. (C) Sp+I/R group: hemorrhage, interstitial edema and mild inflammatory cell infiltration along with mild alveolar damage in lung tissue. (D) Sp+sham group: minimal alveolar damage, hemorrhage, and inflammatory cell infiltration.

FIGURE 3: Immunohistochemical iNOS expression within lung tissue of the study groups (B-SA, 3,3'-diaminobenzidine tetrahydrochloride, X100). (A) Sham-operated control group: iNOS expression being minimal among interstitial space and more prominent in bronchial epithelium. (B) I/R group: intense iNOS immunoreaction, in both bronchial epithelium and interstitial space. (C) Sp+I/R group: moderate iNOS expression within lung tissue. (D) Sp+sham group: iNOS immunoreaction being mild among interstitial space and more prominent in bronchial epithelium.

lung tissue, preventing the development of lung injury. In accordance with our findings, a considerable reduction in oxidative stress was also observed previous studies in renal [21] and intestinal I/R injury [26], both of which were treated with Sp. Moreover, it has been demonstrated that the administration of aldosterone induces ROS generation by activating the enzyme NADPH oxidase in cultured ventricular myocytes and mesangial cells in rats [21]. In various animal models, Sp can function as an antioxidant factor and protect organs from oxidative damage by enhancing antioxidative defence systems while suppressing production of free radicals [32]. Similarly, Ji *et al.* demonstrated the therapeutical potential of Sp, which significantly reduces the pulmonary inflammatory response induced by bleomycin. Sp-treated lungs exhibited fewer macrophage, lymphocyte, neutrophil and eosinophils in alveoli compared with the untreated lungs [33]. Lieber *et al.* demonstrated that Sp treatment attenuates acute pulmonary inflammation induced by both bleomycin and lipopolysaccharide [19]. Similar beneficial effects of Sp have been demonstrated by Atalay *et al.* in acute respiratory distress syndrome of rats [18]. The studies mentioned above differs from the present one in that only a single dose of Sp was administered.

The activity of MPO is commonly used to measure the extent of inflammation since it is an indicator of neutrophil accumulation in the tissues. Intestinal I/R showed elevated tissue MPO activity, indicating the presence of enhanced leukocyte recruitment in the inflamed tissue; however, a significant decrease in MPO activity was detected in the group pretreated with Sp.

In addition, the pretreatment with Sp significantly inhibited the I/R-induced increase in iNOS immunoreactivity. NO, in mammalian cells, is produced by a family of nitric oxide synthases (NOS). NOS isoenzymes are classified as low output, calcium-dependent (cNOS) or high output, calcium-independent cytokine iNOS isoforms. iNOS expression is induced in the majority of mammalian cell types upon exposure to inflammatory stimuli, including cytokines, bacteria and bacterial products [34-36]. Previously published studies show that increased NO production in the lung is associated with increased iNOS expression and/or iNOS activity in various models of ALI [37-40]. The mechanisms of the cytotoxic actions of excessive NO production are not fully understood. It has been suggested that the superoxide ions react with NO to produce peroxynitrite, which then causes accentuated lipid peroxidation and protein and DNA modifications, resulting in alveolar-capillary damage [41]. Enhanced formation of NO by iNOS may also contribute to

inflammatory processes. The iNOS immunoreactivity of lung tissue appears to be increased in the I/R group. This finding is consistence with another study, which showed significant up-regulation of iNOS in I/R injury induced by intestinal I/R [9]; however, in the present study, pretreatment with Sp significantly decreased the immunoreactivity of iNOS in the tissue, implying that Sp reduces the generation of NO and eventually results in decreased peroxynitrite-induced lipid peroxidation.

GSH, an endogenous antioxidant found naturally in all animal cells, has the capability of reacting with free radicals. Glutathione precursors are protective for different types of free radical-mediated cellular injury [9]. Published studies have demonstrated that intestinal I/R depletes tissue GSH [42]; therefore, the decreased GSH content in lung tissue from the I/R group seems to be the result of oxidant injury. Pretreatment with Sp increased the GSH levels almost to sham control levels; thus, Sp pretreatment provides a significant protective effect against I/R-induced lipid peroxidation.

Based on our results, it is evident that intestinal I/R caused an elevation in MDA levels, MPO activity and iNOS immunoreactivity, as well as a reduction in GSH levels. These changes were accompanied by the significant lung injury. Pretreatment with Sp ameliorated these changes. These findings suggest that the administration of Sp may be an effective therapeutical approach for the prevention of I/R-induced lung injury. Furthermore, these potential therapeutic effects may be related to Sp's anti-inflammatory and antioxidant effects.

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