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Erdosteine and Ebselen As Useful Agents in Intestinal Ischemia/Reperfusion Injury

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Background. **Reactive oxygen and nitrogen species generated during reperfusion of the tissue are characteristic of ischemia/reperfusion (I/R) injury. The purpose of the present study was to investigate whether erdosteine and ebselen, molecules with antioxidant properties and peroxynitrite scavenging capability, respectively, can reduce oxidative stress and histological damage in the rat small bowel subjected to mesenteric I/R injury.**

Materials and methods. **Forty Sprague–Dawley rats were divided into five groups equally: sham, I/R, I/R plus erdosteine, I/R plus ebselen, and I/R plus erdosteine and ebselen. Intestinal ischemia for 45 min and reperfusion for 3 d were carried out. Ileal specimens were obtained to determine the tissue levels of malondialdehide (MDA), protein carbonyl content (PCC), superoxide dismutase (SOD), glutathione peroxidase (GPx), nitrite/nitrate (NO_x) level and histological changes.**

Results. **Intestinal I/R resulted in increased tissue MDA, PCC, and NO_x levels and decreased SOD and GPx activities. Both erdosteine and ebselen alone significantly decreased MDA, PCC, and NO_x levels and increased antioxidant enzymes activities, but all values were different from control. These changes almost returned to control values in the group treated with erdostein and ebselen. Histopathologically, the intestinal injury in rats treated with erdosteine and ebselen as well as combination were less than I/R group.**

Conclusions. **Both erdosteine and ebselen were able to attenuate I/R injury of the intestine via inhibition of lipid peroxidation and protein oxidation, maintenance of antioxidant, and free radical scavenger properties. Nevertheless, combination treatment showed more promising results, suggesting that scavenging peroxynitrite nearby antioxidant activity is important in preventing intestinal I/R injury.**

Key Words: **intestinal; ischemia/reperfusion; erdosteine; ebselen; peroxynitrite; rat**

INTRODUCTION

Intestinal ischemia/reperfusion (I/R) is associated with a high morbidity and mortality in both surgical and trauma patients. It is of importance in situations such as the interruption of blood flow to the intestine as in abdominal aortic aneurysm surgery, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis, and intestinal transplantation [1–3]. Intestinal I/R also occurs in severe burns and septic and hypovolemic shock [4, 5].

Tissues subjected to a period of ischemia undergo morphological and functional damages, which increase during the reperfusion phase. Reperfusion of ischemic tissue increases the effects of early ischemic injury by release of reactive oxygen species (ROS) and accumulation of activated neutrophils [1, 5]. This cascade of events is known as reperfusion injury. Reperfusion injury is a feature of intestinal I/R. Activated neutrophils in the reperfused intestine release miscellaneous mediators, e.g., myeloperoxidase from neutrophils produces chloride ions from peroxide [2, 6]. Such reactions lead to excessive ROS generation, such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), which have the capability to damage the cell membrane through lipid peroxidation and protein oxi-

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dation [2, 7]. These oxygen metabolites and enzymes of neutrophils also cause serious damage to the surrounding tissue and distant organs by direct and indirect injury to the vascular endothelium [1, 4, 8].

Nitric oxide (NO) synthesized by NO synthase (NOS) has many physiological functions but can also be hazardous if produced abundantly [9]. It has the ability to react with $\cdot O_2^-$ released from inflammatory cells to produce the potent oxidant molecule peroxynitrite ($ONOO^-$) [6, 10]. NO also reacts directly with O_2 to form nitrite that produces nitrogen dioxide by myeloperoxidase. These reactive nitrogen species (RNS) have been thought to cause detrimental effects in various cells [11].

Endogenous antioxidant systems are critically important in limiting I/R injury. ROS are normally removed by antioxidant enzymes; superoxide dismutase (SOD) rapidly and specifically reduces $\cdot O_2^-$ to H_2O_2 and glutathione peroxidase (GPx) degrades H_2O_2 to water [1, 2, 7]. I/R can be evaluated by detection of various products resulting from injury. Malondialdehyde (MDA) is the end-product of lipid peroxidation caused by ROS and/or RNS. Direct reaction of proteins with ROS/RNS results in formation of protein carbonyl derivatives; thus, measurement of protein carbonyl content (PCC) can reflect protein oxidation. Both MDA and PCC are therefore commonly used biomarkers of oxidative stress [2, 7].

Erdosteine [*N*-(carboxymethylthioacetyl)-homocysteine thiolactone] is a mucolytic agent and contains two blocked sulfhydryl groups that are released following its metabolic process. It has been shown that its active metabolites have exhibited free radical scavenging and antiinflammatory activities [12, 13]. Ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] is a selenoorganic compound that was originally described as a GPx mimic [14]. Furthermore, it has been reported that ebselen can act as a peroxynitrite-reductant [15].

Experimental studies examined the role of erdosteine in the protection of kidney after renal I/R [7], testis after torsion and detorsion [16], and against lung injury after hind-limb I/R in rats [4]. Ebselen has been shown to be protective against ischemic injury in liver [17] and in brain [18]. However, the effects of erdosteine and ebselen have not been evaluated in intestinal tissue subject to I/R injury. Following these data, the aim of the present was to examine the potential protective effect of erdosteine and ebselen on I/R injury in small intestine tissue of rats.

MATERIALS AND METHODS

Animals and Experimental Design

The project was approved by the Experimental Animal Committee at Gulhane Military Medical Academy, and the National Institute of Health's Guide for the Care and Use of Laboratory Animals was followed.

Forty male Spraque-Dawley rats, provided by the Experimental Research Council of the Gulhane Military Medical Academy, weighing between 100 and 150 g and held under standard feeding conditions were randomly divided into five groups ($n = 8$ for each): (1) control/sham operated, (2) intestinal I/R, (3) intestinal I/R + erdosteine, (4) intestinal I/R + ebselen, and (5) intestinal I/R + erdosteine + ebselen. Animals were operated on at a room temperature of 24°C under intramuscular ketamine hydrochloride (50 mg/kg) plus xylazine (10 mg/kg) anesthesia. Erdosteine (100 mg/kg) [19] and ebselen (20 mg/kg) [20] were administered via oral gavage first 1 d before operation and continued daily throughout the experimental period.

Through midline laparotomy, superior mesenteric artery (SMA) was isolated at its origin and occluded with an atraumatic microvascular clamp (Bulldog Artery Clamp; Harvard Apparatus, Cambridge, MA) for 45 min. The body temperature was preserved at approximately 37°C. Following warm ischemia, the microvascular clamp was removed and confirmed that intestine had been restored prior to closing the incision. Control groups were sham operated without clamp application.

Following 3 d of reperfusion period, tissue samples were obtained from small intestine 5 cm proximal to the ileocecal area, and the rats were killed by cervical dislocation. The tissue specimens were rinsed with cold saline solution, and the distal 2 cm was fixed in 10% buffered formalin for histopathologic evaluation. The remainder of the specimens was saved at −70°C for biochemical examination.

Tissue Preparation and Biochemical Analysis

The frozen tissues were homogenized in phosphate buffer (pH 7.4) by means of a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany) on an ice cube. The supernatant was used for the entire assay. Initially, the protein content of tissue homogenates was measured by the method of Lowry with bovine serum albumin as the standard [21].

Tissue PCC was determined spectrophotometrically by the method based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone [22]. 2,4-Dinitrophenylhydrazine was the reagent originally used for proteins subjected to metal-catalyzed oxidation. Absorbances were measured with a spectrophotometer (Helios Epsilon; UNICAM, Cambridge, UK). The results were given as millimoles (mmol) carbonyl per gram protein (g-protein).

Lipid peroxidation level was measured with the thiobarbituric acid (TBA) reaction by the method of Ohkawa [23]. This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to thiobarbituric acid (TBA) with MDA at 535 nm. The calculated MDA levels were expressed as mmol/g-protein.

SOD activity was assayed by using the nitroblue tetrazolium (NBT) method of Sun *et al.* [24]. In this method, NBT was reduced to blue formazan by $\cdot O_2^-$, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The estimated SOD activity was expressed as U/g-protein.

The GPx activity was measured by using the method described by Paglia and Valentine [25] in which GPx activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically followed up at 340 nm at 37°C. The absorbance at 340 nm was recorded for 5 min. The activity was the slope of the lines as mmol of NADPH oxidized per min. GPx activity was presented as U/g-protein.

Nitrate plus nitrite (NO_x) levels, end products of nitric oxide degradation, were measured by using the method described by Miranda *et al.* [26]. Briefly, tissue homogenates centrifuged at 2000*g* for 5 min. The supernatant (0.5 mL) was added to 0.25 mL 0.3 M NaOH. After incubation for 5 min at room temperature, 0.25 mL of 5% (w/v) $ZnSO_4$ was added for deproteinization. This mixture was then centrifuged at 3000*g* for 20 min at +4°C, and the supernatants

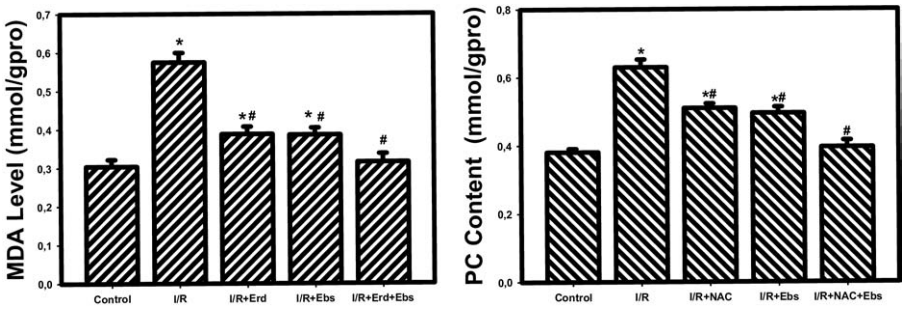

FIG. 1. Effect of erdostein (Erd) and ebselen (Ebs) on malondialdehyde (MDA) and proteine carbonyl (PC) contents level in the small intestine tissue. Data are the mean ± SEM; *Significantly different from control, #Significantly different from I/R group.

were used for the assays. A nitrate standard solution (100 μL) was serially diluted (generally from 200 to 1.6 mM) in duplicate in a 96-well plate. After loading the plate with samples (100 μL), addition of VCl_3 (100 μL) to each well was rapidly followed by addition of the Griess reagents, sulfanilamide (SULF; 50 μL), and N-(1-Naphthyl)ethylenediamine (NEDD; 50 μL). The Griess solutions may also be premixed immediately prior to application to the plate. Sample blank values were obtained by substituting diluting medium for Griess reagent. Nitrite was measured in a similar manner except that samples and nitrite standards were only exposed to Griess reagents. In either case, the absorbance at 540 nm was measured by using a plate reader following incubation (30 min). Tissue NO_x levels were expressed as μmol/g-tissue.

Histopathologic Evaluation

Tissue specimens were fixed in 10% formalin and embedded in paraffin and cut into 5-μm sections. Slides were prepared with hematoxylin and eosin stain and examined under light microscopy. Each slide was evaluated in a blind manner by two separate investigators. Injury was graded on a five-tiered scale define by Chiu *et al.* [27] as follows: Grade 0, no diagnostic change; Grade 1, moderate epithelial layer lifting from; Grade 2, significant epithelial cell layer lifting from lamina propria; Grade 3, loss of few villus with exposed lamina propria; and Grade 4, disintegration of lamina propria with ulceration and hemorrhage.

For evaluation of polymorphonuclear leukocytes (PMNL) infiltration, new slides were prepared and stained with Giemsa. PMNL were counted per high power field of Giemsa stained in 20 separate areas of each slide in the muscularis mucosa, and the mean number of PMNL per high power field was determined for each animal. A single pathologist, blind to the details of each specimen, performed the PMN counts.

Statistical Analysis

All statistical analyses were carried out by using SPSS statistical software (SPSS for Windows, Version 11.0; SPSS, Inc., Chicago, IL). All data were presented as means and the standard error of the mean (mean ± SEM). Differences in measured parameters among the three groups were analyzed by Kruskal–Wallis test. Dual comparisons between groups that present significant values were evaluated with Mann–Whitney *t*-test. Statistical significance was accepted a value of $P < 0.05$.

RESULTS

Oxidative and Nitrosative Stress Markers

Outcomes of MDA and PCC in the small intestine are displayed in Fig. 1. The rats subjected to intestinal I/R exhibited a strike increase in tissue MDA and PCC, suggesting increased lipid peroxidation and protein oxidation ($P < 0.001$ compared with I/R group). All three treatment modalities (agents alone and in combination) lowered these increased levels ($P < 0.05$ compared with sham group).

NO_x levels were significantly increased after I/R application ($P < 0.001$ compared with sham). These increased levels were significantly attenuated in treatment groups; however, their levels remained significantly higher than those of the sham-operated group ($P < 0.05$; Fig. 2).

Antioxidant Enzymes

SOD and GPx activities are shown in Fig. 3. Both SOD and GPx activities were significantly lower in the I/R group ($P < 0.001$ compared with sham group). In treatment groups, this fall was reversed; there were no

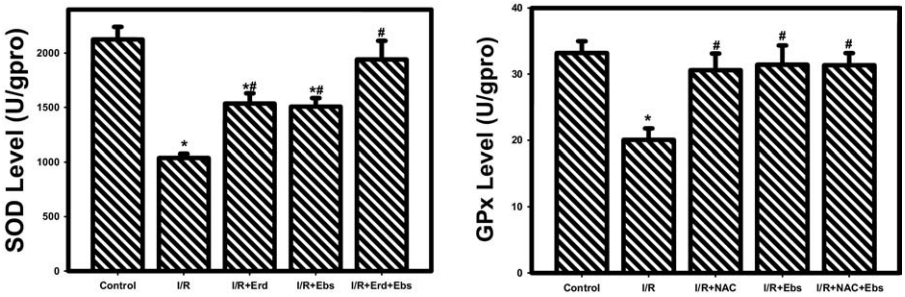

FIG. 2. Effect of erdostein (Erd) and ebselen (Ebs) on nitrite/nitrate (NO_x) level in the small intestine tissue. Data are the mean ± SEM; *Significantly different from control; #Significantly different from I/R group.

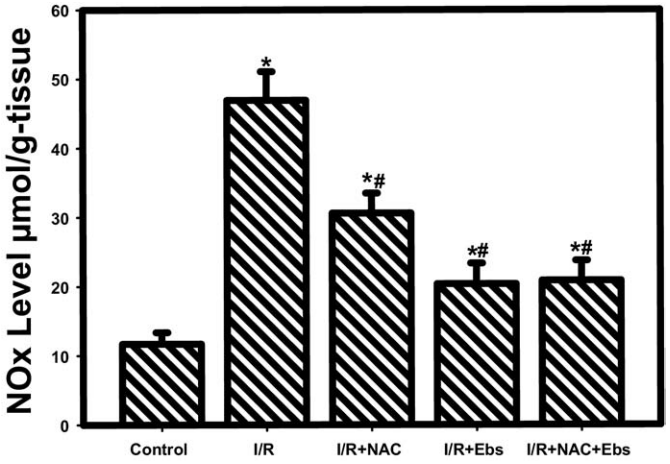

FIG. 3. Effect of erdoteine (Erd) and ebselen (Ebs) on superoxidase dismutase (SOD) activity and glutation peroxidase (GPx) activity in the small intestine tissue. Data are the mean ± SEM; *Significantly different from control. #Significantly different from I/R group.

significant differences between treated groups and sham-operated groups based on the GPx level. On the other hand, SOD activity in the erdosteine and ebselen alone treated groups were still found to be higher than sham animals ($P < 0.05$). Interestingly, SOD activity of the erdosteine and ebselen combination group was nearly the same as sham levels.

Histopathological Outcome

Histological grading of intestinal injury was displayed in Table 1. Significant mucosal injury with loss of villus, hemorrhage, and ulceration was seen in the rats subjected to I/R. The injury rate of treatment groups were found to be minimized. Representative histological samples from all groups are displayed in Fig. 4.

The number of PMNL per high power field was 0.6 ± 0.2 for sham-operated, 1.9 ± 0.3 for I/R, 1.1 ± 0.2 for I/R + erdostein, 1.2 ± 0.2 for I/R + ebselen and 0.6 ± 0.2 for I/R + erdosteine + ebselen group ($P < 0.05$ I/R *versus* treatment groups). This result was revealed that PMNL infiltration was suppressed with both drugs.

DISCUSSION

In this experiment, we evaluated the effects of erdosteine, a disulphide antioxidant [19], and ebselen, a molecule with peroxynitrite-scavenging capability [17, 28], in intestinal I/R injury. The results of the present study demonstrate that treatment with erdosteine and ebselen markedly attenuates the intestinal damage of rats subjected to I/R injury. Each agent has ameliorating effect on both oxidative status and histopathological changes. In addition, we found that combination of these two agents has more beneficial effect than each of them alone.

A number of experimental studies have indicated that I/R injury of the intestine occurs in a biphasic manner characterized by different time course: an early phase that immediately follows the ischemia and lasts for 2–3 h, and a late phase which begins 12–24 h from the ischemia and lasts for about 3–4 d [5]. The model of 45-min period SMA occlusion followed with 3 d of reperfusion was chosen to assess the changes in the late phase of reperfusion injury at intestine. Our model was reliable for rats to survive throughout the experiment period and to induce substantial intestinal injury.

ROS and RNS are believed to cause cellular injury and follow necrosis via different mechanisms, including peroxidation of cellular membrane lipids and oxidation of cellular proteins [5, 10]. In this study, we found that I/R caused a considerable increase in the intestinal tissue levels of oxidative stress markers, MDA and PCC. Moreover, the increase in these biomarkers after I/R was significantly ameliorated by treatment with erdosteine, ebselen, and, especially, combination of these agents. Erdosteine, a widely used mucolytic agent in clinics, contains two blocked sulfydryl groups which became free after hepatic metabolization. The reducing potential of these sulfydryl groups accounts for free radical scavenging and antioxidant activity of erdosteine [12, 29]. Consistent with present findings, previous experimental studies showed that erdosteine attenuated the increased MDA levels in kidneys after renal I/R [7] and in spinal cord after aortic clamping [30]. On the other hand, the specificity of ebselen for H_2O_2, smaller organic hydroperoxides, cholesterylester hydroperoxides, and peroxynitrite is displayed in various studies [14, 18, 31]. Animal studies related to I/R also showed the benefit of ebselen as a neuroprotective [18, 32], hepatoprotective [17], and cardioprotective [33] agent. Taken together, a possible explanation for decreased lipid peroxidation and protein oxidation in groups treated with ebselen is that ebselen might be a beneficial agent for prevention of membrane damage in cells.

TABLE 1

Histopathological Evaluation of Terminal Ileum

Groups	Mucosal injury score
Control	0
I/R	3.2 ± 0.4*
I/R + erdostein	1.2 ± 0.2*†
I/R + ebselen	1.1 ± 0.4*†
I/R + erdostein + ebselen	0.7 ± 0.3*†

Note. Data is the mean ± SEM;
*Significantly different from control;
†Significantly different from I/R group.

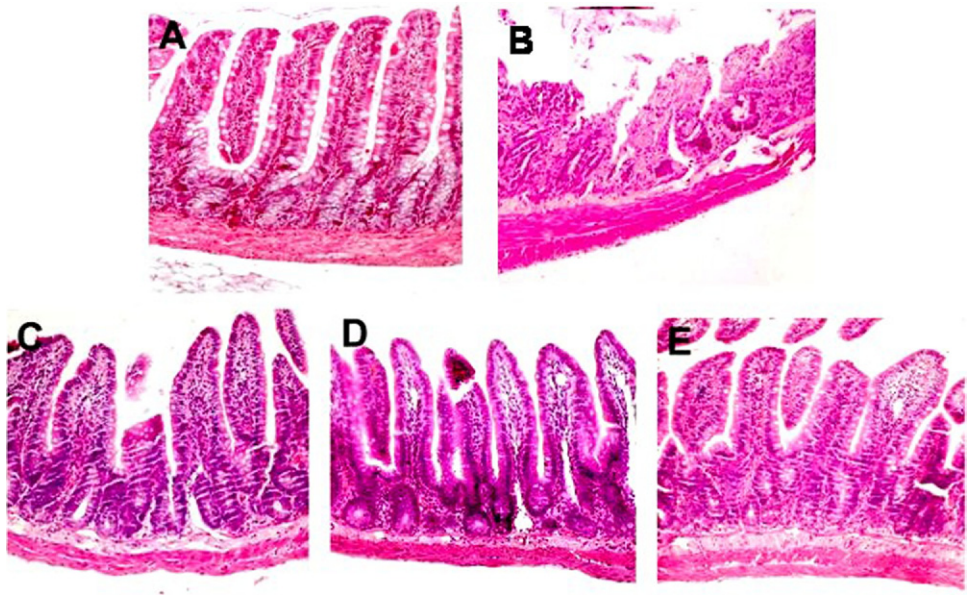

FIG. 4. Representative histopathology of terminal ileum stained with hematoxylin and eosin (H&S, 40× original magnification) from each experimental group. Ileal section of the sham-operated group revealed normal architecture (A). Ileal section of ischemia/reperfusion group (I/R) revealed marked ulceration and hemorrhage on the mucosa, loss of villus, and infiltration of polymorphonuclear lymphocytes (B). Ileal section of I/R + erdosteine (C), I/R + ebselen (D), and I/R + erdosteine + ebselen (E) groups showed minimal alterations characterized with moderate lifting of the epithelial layer from lamina propria.

Under physiological conditions, the damaging effects of ROS are prevented by endogen antioxidant enzymes such as SOD and GPx [11, 34, 35]. However, during reperfusion of intestine, the oxidant/antioxidant balance may change. In the present study, SOD and GPx activities were determined to evaluate tissue antioxidant system; both enzymes were found markedly decreased in I/R group. This finding can be accepted as an indirect indicator for excessive amount of $\cdot O_2^-$ and H_2O_2. Treatment groups resulted with reversely enhanced activities of SOD and GPx, suggesting an attenuation of intestinal I/R injury by detoxifying the oxygen free radicals. Erdosteine, owing to the presence of two sulfydryl group in its metabolites, can act as a free oxygen radical scavenger [13] and supply thiol molecules to the thioredoxin system, which serve to keep the redox state of a cell in balance. On the other hand, that ebselen quickly reacts with H_2O_2 to form water is a more likely explanation for increased antioxidant status in the ebselen-treated group [15].

Interestingly, ebselen significantly lowered the increased NO_x levels due to the I/R process. NO produced by iNOS reacts rapidly with O_2^- to produce the highly reactive peroxynitrite anion ($ONOO^-$). NO and $ONOO^-$ are eventually converted to nitrite (NO_2) and/or nitrate (NO_3) and nitrite plus nitrate is accepted NO_x. Therefore, NO_x levels can be used as an indirect but reliable indicator for NO and/or $ONOO^-$ production [20]. We could not determine iNOS expression or nitrotyrosine level (one of end product of peroxynitrite) in the intestine; however, our data are consistent with the findings that NO and/or $ONOO^-$ might demolish protein function and mediate intestinal injury [2]. It is not clear how ebselen prevents I/R-induced NO production; it may exert this effect by its scavenging activity for peroxinitrite and thereby secondarily decreasing NO production.

Another important finding of this study is that both antioxidant enzyme activity and tissue damage indices came back to near control level in the rats treated with combination of erdosteine and ebselen when compared with either drug alone. With erdosteine alone, ROS was scavenged from the environment; however, RNS (including peroxynitrite) might continue to cause tissue damage. On the other hand, although having GPx-mimicking properties [17], ebselen alone may be insufficient for the entire oxidative stress reaction, and erdosteine can support the fight against ROS. Hence, the combination of erdosteine and ebselen indicates that it may be more beneficial to use both agents together against intestinal I/R injury. Nevertheless, this beneficial effect might be related to either the synergistic or additive effect (due to receiving combination of both drugs) of the drugs used in the same pathway, and further studies are warranted.

In this study, we used ketamine as anesthetic drug. Ketamine, the most frequently used anesthetic in experimental studies, has been reported to have significant antiinflammatory effects via suppression of inflammatory cytokines [36]. It also has been demonstrated that ketamine could improve survival rates induced sepsis [37]. Because the usage of ketemine was standard in all

groups, we believe that it did not directly influence the significant differences among groups.

Additionally, to confirm our findings, we evaluated terminal ileum histologically. Histopathological injury scores and PMNL infiltration presented similarity with the degree of oxidative stress, namely lipid peroxidation and protein oxidation. Villus shortening is presumably due to decreased cell proliferation or to the decreased microvasculature, which results in a decreased oxygen tension gradient along the villi [38]. Treatment with erdosteine and ebselen significantly preserved mucosal injuries and decreased PMNL infiltration, suggesting that these agents are beneficial in attenuating intestinal I/R injury via suppressing PMNL infiltration and antioxidant effect of agents.

In conclusion, the present study demonstrated a possible protective effect of erdosteine and ebselen in intestinal I/R injury on the basis of oxidative and antioxidant systems, confirmed by histopathologic evaluation. The results also suggest that scavenging peroxynitrite has protective effect and should be taken into account to prevent intestinal I/R injury, which may occur during transplant surgery and mesenteric artery embolism. Nevertheless, more research is needed to further elucidate these efficacies and determine whether these results will apply in humans.

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