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Lithospermic Acid and Ischemia/Reperfusion Injury of the Rat Small Intestine Prevention

Kwas litospermowy a zapobieganie niedokrwieniu/uszkodzeniu reperfuzyjnemu jelita cienkiego u szczura

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Abstract

Background. Intestinal ischemia and reperfusion (I-R) injury of different causes, including cardiac insufficiency, sepsis, vasodepressant and cardiodepressant drugs, and complications of long-lasting surgery, represents a major clinical problem.
Objectives. The purpose of the present study was to investigate whether lithospermic acid (LA) can reduce oxidative stress and histological damage in the rat small bowel subjected to mesenteric I-R injury.
Material and Methods. The study was performed on three groups of animals, each composed of 7 rats: the SO (sham operation) group, the I-R/Untreated group and the I-R/LA (I-R plus LA pretreatment) group. Intestinal ischemia for 45 minutes and reperfusion for 60 minutes were applied. Ileum specimens were obtained to determine the tissue level of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and myeloperoxidase (MPO) activities and histological changes.
Results. Untreated intestinal I-R resulted in increased tissue MDA and MPO levels and diminished SOD and GPx activities. These changes were found to be almost reversed in the LA treatment group. Histopathologically, the intestinal injury in rats treated with LA was less than the untreated I-R group.
Conclusions. Lithospermic acid attenuates mesenteric ischemia reperfusion injury in rat intestines by increasing tissue SOD and GPx activities and decreasing MDA and MPO levels. Lithospermic acid also improves morphological alterations which occurred after periods of reperfusion (**Adv Clin Exp Med 2012, 21, 4, 433–439**).

Key words: small bowel, ischemia and reperfusion injury, lithospermic acid.

Streszczenie

Wprowadzenie. Uszkodzenie jelit spowodowane niedotlenieniem i reperfuzją (IR) wynikające z różnych przyczyn, w tym niewydolności serca, posocznicy, stosowania leków wazodepresyjnych i kardiodepresyjnych oraz powikłań dużych zabiegów chirurgicznych, jest ważnym problemem klinicznym.
Cel pracy. Zbadanie, czy kwas litospermowy (LA) może zmniejszyć stres oksydacyjny i uszkodzenie histologiczne jelita cienkiego u szczurów, u których wywołano niedotlenienie i reperfuzję krezki.
Materiał i metody. Badanie zostało przeprowadzone na trzech grupach zwierząt, z których każda składała się z 7 szczurów: grupa SO (operacja pozorowana), grupa IR nieleczona i grupa IR / LA (IR oraz podanie LA przed leczeniem). Niedokrwienie jelit utrzymywano przez 45 minut i reperfuzję przez 60 minut. Pobrano wycinki jelita krętego w celu określenia stężenia aldehydu malonowego (MDA), dysmutazy ponadtlenkowej (SOD), peroksydazy glutationowej (GPx), katalazy (CAT) i mieloperoksydazy (MPO) w tkankach i zmian histologicznych.
Wyniki. Nieleczone IR jelit spowodowało zwiększenie stężenia tkankowego MDA i MPO oraz mniejszą aktywność SOD i GPx. Zmiany te okazały się niemal odwrotne w grupie leczonej LA. Histopatologicznie uszkodzenie jelit u szczurów leczonych LA było mniejsze niż w grupie IR nieleczonej.
Wnioski. Podawanie kwasu litospermowego zmniejsza uszkodzenie jelit spowodowane niedotlenieniem i reperfuzją u szczurów przez zwiększenie tkankowej SOD i aktywności GPx, zmniejszenie stężenia MDA i MPO. Kwas litospermowy zmniejsza zmiany morfologiczne, które nastąpiły po okresie reperfuzji (**Adv Clin Exp Med 2012, 21, 4, 433–439**).

Słowa kluczowe: jelito cienkie, uszkodzenie spowodowane niedotlenieniem i reperfuzją, kwas litospermowy.

Intestinal ischemia and reperfusion (I-R) injury plays an important role in some clinical conditions, such as acute mesenteric ischemia, midgut volvulus, shock, cardiac surgical interventions and small bowel transplantation [1–4]. I-R injury of the intestine not only leads to loss of intestinal barrier functions but also may results in bacterial translocation and a systemic inflammatory response that causes multiple organ failure [5–7].

The development mechanisms of I-R injury are not fully known. However, it is known that reactive oxygen species (ROS) play a very important role in the pathophysiology of I-R injury. Hypoxia occurs during the period of ischemia, but reperfusion injury occurs after reconstitution of blood flow [8]. Reperfusion of ischemic tissue increases the effects of early ischemic injury by release of ROS and accumulation of activated neutrophils [6]. Superoxide, hydrogen peroxide, and hydroxyl radicals are generated and may cause cellular damage by direct action and by secondary activation of polymorphonuclear neutrophils (PMN) [9, 10]. Various anti-oxidant agents have been studied to prevent reperfusion injury after ischemia. Lithospermic acid (LA) is a biologically active component isolated from the aqueous extract of danshen, the dried root and rhizome of *Salvia miltiorrhiza* Bge (Labiatae) [11]. LA has a strong antioxidant effect in preventing the production of superoxide radical and lipid peroxide [12, 13].

In the present study, the authors aimed to determine if lithospermic acid has protective effects against mucosal injury induced by I-R in the rat small intestine.

Material and Methods

Animal Model and Experimental Design

Twenty-one Sprague-Dawley rats weighing between 200 and 225 g were divided into 3 groups, each containing 7 rats: the SO (sham operation) group, the I-R/Untreated (untreated ischemia-reperfusion) group, and the I-R/LA (ischemia-reperfusion plus LA pretreatment) group (10 mg/kg LA was administered intraperitoneally 30 min before the induction of ischemia).

The rats were kept under standardized conditions for food, water, light and temperature. After overnight fasting, each rat was anesthetized with ketamine (50 mg/kg) and xylazine (14 mg/kg) and underwent a midline laparotomy and bowel evisceration. After ligating collateral arcades from the right colic artery and the jejunal arteries, the superior mesenteric artery (SMA) was occluded with an atraumatic microvascular clamp as described by Megison et al. [14]. During this period of warm intestinal ischemia, each rat was cannulated via the jugular vein with a 24-gauge venula needle and lactated Ringer's solution (LR) was given (1 ml/kg/h) using an infusion pump. The bowel was placed in the abdominal cavity, and then the incision was closed. After 45 minutes of ischemia, the abdominal cavity was reopened. The occluding clamp was removed and the intestine was returned to the peritoneal cavity, then the abdomen was reclosed. After 60 minutes reperfusion period, the abdominal wall was opened once more and samples of ileum were obtained for biochemical and histopathological analysis. The animals were sacrificed when the procedure was completed. In the sham group, only laparotomy and preparation of SMA was performed, and SMA was not occluded with a clip.

Samples for biochemical analysis were frozen in liquid nitrogen and stored at –80°C until processing. Samples for histological analysis were fixed in (%10) formaldehyde.

Analytical Procedures of Oxidative Stress-Associated Parameters

All tissues were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.25 mM phenylmethylsulfonyl fluoride; pH 7.4), and a portion of the homogenate was measured immediately for malondialdehyde (MDA) (nmol/g) using a commercial kit. Another portion of the homogenate was centrifuged at 10,000 × g for 20 min at 4°C, and superoxide dismutase (SOD) (U/g protein) and glutathione peroxidase (GPx) (μmol/mg protein) activities in the supernatant were measured using commercial kits (Randox). The first-line defense mechanism includes antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH). These enzymes catalyze the conversion of ROS into less reactive species [15, 16]. Catalase, an oxidoreductase that catalyzes the conversion of hydrogen peroxide to water and oxygen, also can protect cells from damage induced by ischemia/reperfusion through scavenging ROS. GSH and SOD are the most important endogenous antioxidant defense mechanisms against oxidative stress [17, 18]. GSH and SOD are important antioxidants and the increased concentration of GSH in cells may be useful in the prevention of oxidative damage of endothelial cells [19]. Malondialdehyde (MDA), the end product of lipid peroxidation, is a good marker of free radical-mediated damage and oxidative stress [20]. Catalase (CAT) activity was determined using a method described by Beers and Sizer [21]. Myeloperoxidase (MPO) activities of all tissues were determined as a marker enzyme

for measuring neutrophil accumulation in tissue samples, because it is closely correlated with the number of neutrophils present in the tissue [22]. The homogenates were then centrifuged at 17,000 × g at 4°C for 15 min, and MPO activity (U/g protein) in the supernatant was measured as previously described [23].

Histopathology

The tissue specimens were fixed in 10% formaldehyde, then dehydrated and embedded in paraffin wax. The samples were sectioned and stained with hematoxylin and eosin (H&E) and assessed in a blinded fashion by pathologists. Mucosal lesions were graded on a system described by Chiu et al. [24].

Statistical Analysis

Values are expressed as the means ±SEM. To evaluate differences among the groups studied, a two-way analysis of variance (ANOVA) with Fisher's *post hoc* test was used. Differences were considered statistically significant when $P < 0.05$.

Results

The MDA, GPx, SOD and MPO values for the different groups are shown in Fig. 1a, b, c and d. The levels of MDA and MPO were significantly increased in the intestinal tissue of the I-R/Untreated group ($p < 0.001$) compared to the control group. However, in the I-R/LA treatment group, intestinal tissue MDA and MPO levels were significantly decreased compared to the I-R/Untreated group ($p < 0.5$). I/R caused a significant decrease in intestinal tissue GSH and SOD levels ($p < 0.05$) when compared to the control group. In the LA-treated I-R group, intestinal tissue GPx and SOD levels were increased ($p < 0.05$) compared to the untreated I-R/Untreated group.

Quantitative evaluation of hematoxylin and eosin stained histologic sections showed that the intestinal injury score increased significantly in the I-R/Untreated and I-R/LA group rats compared to the SO group ($p < 0.05$ and $p < 0.05$, respectively). On the other hand, the intestinal injury score was decreased in I-R/LA group rats compared to the I-R/Untreated ($p < 0.05$) (Fig. 2).

Using the Chiu scoring system, the ileal section of the sham-operated group revealed normal architecture (Fig. 3A), whereas in the rats subjected to I-R, significant mucosal injury with loss of villus, hemorrhage, and ulceration was seen (Fig. 3B). The injury rate of treatment groups was found to be minimized. Ileal sections of the I-R/LA group showed minimal alterations characterized with moderate lifting of the epithelial layer from lamina propria (Fig. 3C).

Discussion

Seven phenolic compounds isolated from *Salvia miltiorrhiza* as active components possess potent antioxidative activity against oxidative injury. Lithospermic acid B in these compounds is the most important component [25]. Lithospermic acid B has been shown to possess strong effects on antioxidative and free radical scavenging, on protecting renal dysfunction, hepatitis, lung fibrosis, and on improving blood circulation [13, 26–29]. Furthermore, lithospermic acid B showed endothelium-dependent vasodilator effects and an ameliorative effect on ischemia reperfusion induced acute renal failure in rats [13, 30, 31]. In this experimental study, we investigated the effects of lithospermic acid in intestinal I-R injury. As discussed below, the results of the present study demonstrate that treatment with lithospermic acid markedly attenuates the intestinal damage of rats subjected to I-R injury.

The intestine is known to be a tissue that is highly sensitive to ischemic injury. I-R injury has multifactorial pathophysiology: the major factors involved are leukocyte adhesion molecules, nitric-oxide, and free radicals [32]. LA has been widely studied and is known to exhibit numerous pharmacological activities such as antioxidant effect, improve effect on renal injury, protective effect on hepatitis, and antihypertensive effect [13, 33, 34]. Liu et al [35] investigated the *in vitro* xanthine oxidase (XO) inhibitory activity, and *in vivo* hypouricemic and anti-inflammatory effects of rats. They found that LA inhibited *in vitro* superoxide radical levels produced by phorbol-12-myristate-13-acetate (PMA), and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated human neutrophils in a dose-dependant manner, and had a marked inhibitory effect on edema in experimental gouty arthritis *in vivo*. Together with the direct superoxide scavenging effect, the suppressive effect on gouty arthritis by LA is mainly mediated through inhibiting the production of superoxide and direct superoxide scavenging. Liu et al. [35] suggested that LA is a competitive inhibitor of XO, able to directly scavenge superoxide and inhibit superoxide production *in vitro*, and presents hypouricemic and anti-inflammatory actions *in vivo*. Kang et al. [13] studied whether lithospermic acid B isolated from Salvia miltiorrhiza has an ameliorative effect on renal functional parameters in association

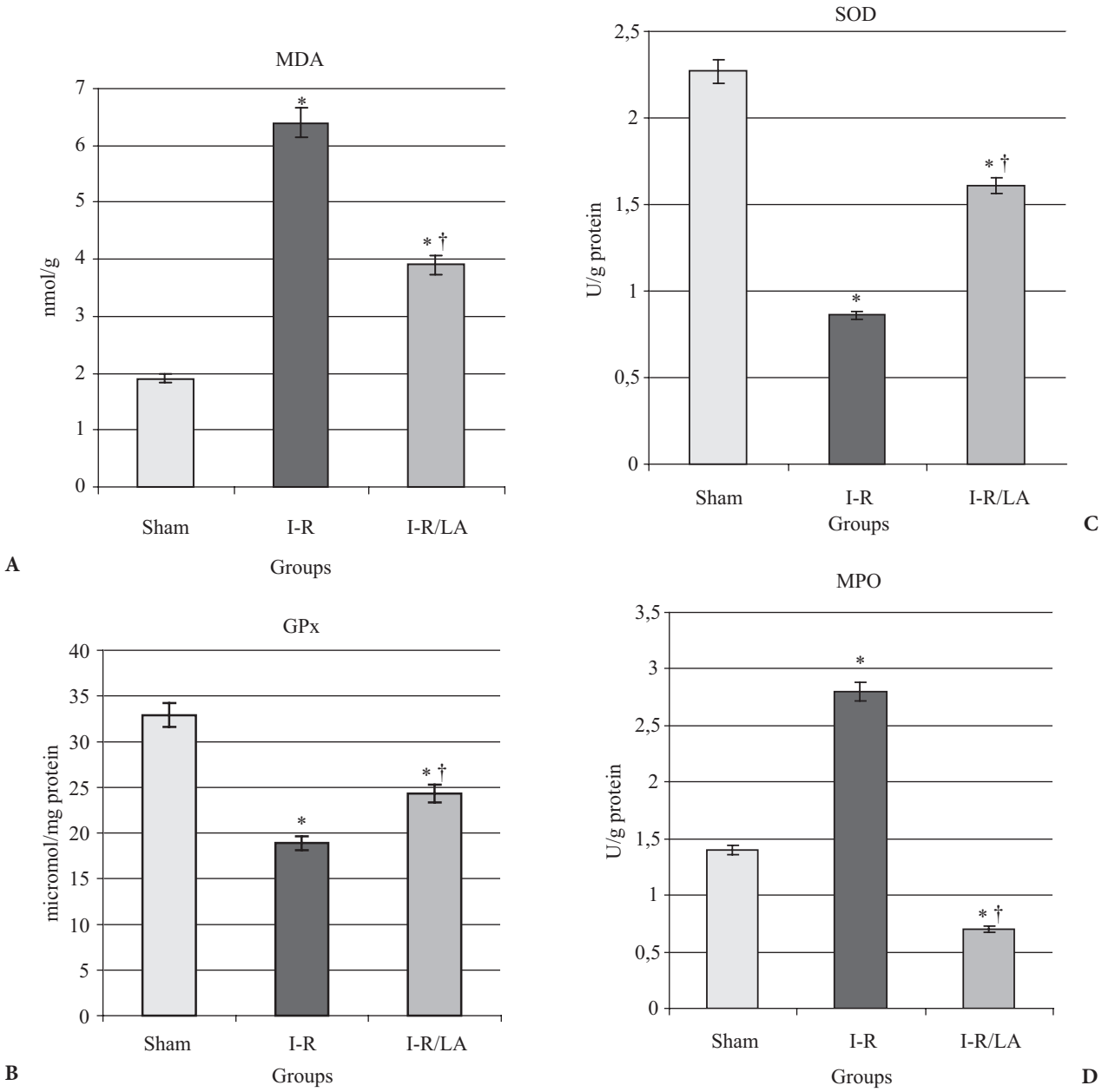

Fig. 1. (a) Malondialdehyde (MDA) and (b) glutathione peroxidase (GPx) levels, (c) superoxide dismutase (SOD) and (d) myeloperoxidase (MPO) activity in the intestinal tissue of sham-operated control groups, I-R/Untreated groups and I-R/lithospermic acid-treated groups. Each group consists of seven animals. * $p < 0.05$, compared to control group. † $p < 0.05$, compared to I-R/Untreated group. Group 1: sham-operated control; Group 2: I-R/Untreated; Group 3: I-R/lithospermic acid-treated

Ryc. 1. (a) Stężenie aldehydu malonowego (MDA) i (b) peroksydazy glutationowej (GPx), (c) dysmutazy ponadtlenkowej (SOD) i (d) mieloperoksydazy (MPO) w tkance jelit w grupie pozornie operowanej kontrolnej, IR / nieleczonej i IR / LA. Każda grupa składa się z siedmiu zwierząt. * P < 0,05 w porównaniu z grupą kontrolną. † p < 0,05 w porównaniu do grupy IR / nieleczonej. Grupa 1: kontrolna operowana pozornie; Grupa 2: IR / nieleczona, Grupa 3: IR / LA

with the expression of aquaporin 2 (AQP 2) and Na, K-ATPase in ischemia-reperfusion induced acute renal failure (ARF) in rats. Lithospermic acid B showed strong antioxidant activity against the production of reactive oxygen species (ROS), ROS-induced hemolysis, and production of lipid peroxide in a dose-dependent manner. They suggested that lithospermic acid B ameliorates renal defects in rats with ischemia-reperfusion induced ARF, most likely via scavenging of ROS.

Under physiological situations, the damaging effects of ROS are prevented by antioxidant enzymes such as SOD and GPx [36]. However, during reperfusion of the intestine, the oxidant/antioxidant balance may change. In the present study, SOD and GPx activities were determined to evalu-

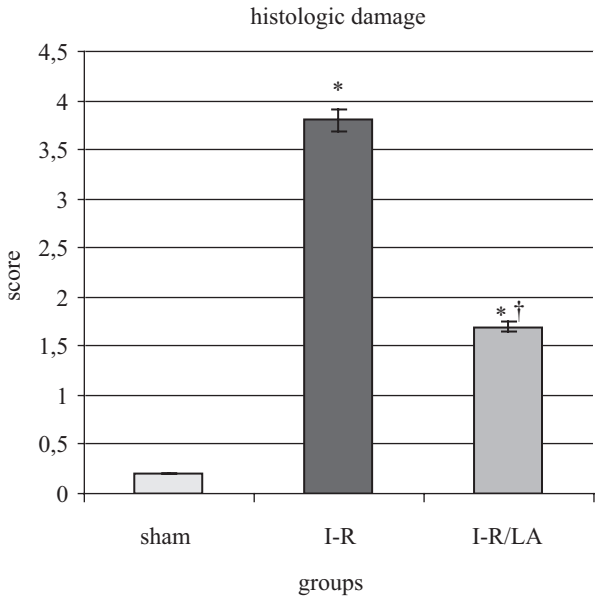

Fig. 2. Comparative histologic score measurements at the groups. *$p < 0.05$ compared with group 1. †$p < 0.05$ compared with group 3. Values are mean ± SEM. Group 1: sham-operated control; Group 2: I-R/ Untreated; Group 3: I-R/lithospermic acid-treated

Ryc. 2. Porównawcze pomiary histologiczne w grupach. * p < 0,05 w porównaniu z grupą 1. † p < 0,05 w porównaniu z grupą 3. Wartości są średnią ± SEM. Grupa 1: kontrolna operowana pozornie; Grupa 2: IR / nieleczona, Grupa 3: IR / LA

ate the tissue antioxidant system; the treatment group had reversely enhanced activities of SOD and GPx, suggesting an attenuation of intestinal I-R injury by detoxifying the oxygen free radicals. As mentioned above, LA is a well known ROS scavenger and contributes to the effects of antioxidant enzymes. Hence, in I-R/LA group rats, SOD and GPx may be higher. Additionally, we found that I-R caused a considerable increase in the intestinal tissue levels of oxidative stress markers, MDA and MPO. Moreover, the increase in these biomarkers after I-R was significantly ameliorated by treatment with lithospermic acid.

In conclusion, lithospermic acid attenuates mesenteric I-R injury in rat intestine by increasing tissue SOD and GPx activities and decreasing MDA and MPO levels. Lithospermic acid also improves morphological alterations which occur after periods of reperfusion. This study first offers evidence that LA protects the ileum from I-R induced injury, likely acting by multiple mechanisms including antioxidative, free radical scavenging and endothelium-dependent vasodilator effects. LA treatment may offer a new therapeutic alternative for I-R injury in some clinical entities, such as acute mesenteric ischemia, midgut volvulus, shock, cardiac surgical interventions and small bowel transplantation.

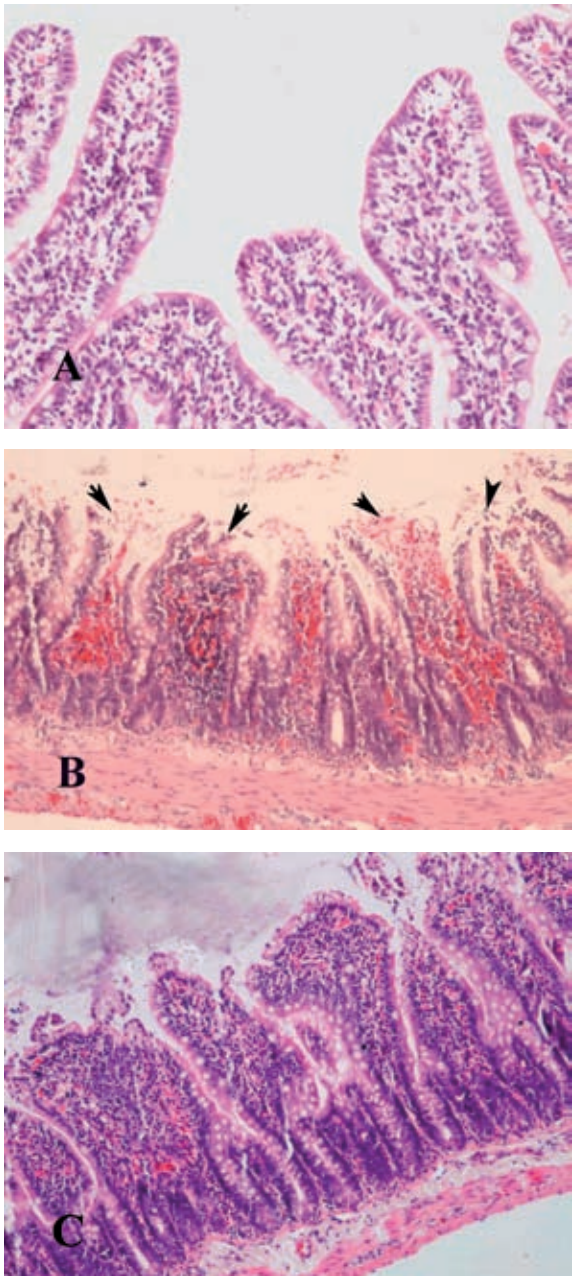

Fig. 3. Photomicrographs of hematoxylin and eosin stained sections of rat small intestine. (A) Normal appearance of mucosa in SO group. (B) Note that the mucosa is almost completely destroyed in the specimens from rats with untreated I-R (arrows). Massive subepithelial lifting, a denuded tip with lamina propria and increased cellularity (C) Moderate epithelial lifting confined to the tips of the villi is observed in animals treated with LA. (H&E, original × 10)

Ryc. 3. Obraz mikroskopowy barwiony hematoksyliną i eozyną odcinków jelita cienkiego szczura. (A) Normalny wygląd błony śluzowej w grupie SO. (B) Należy zwrócić uwagę, że błona śluzowa jest prawie całkowicie zniszczona w próbkach od szczurów z nieleczoną IR (strzałki). Masywna podnabłonkowe podniesienie, odsłonięta końcówka z blaszki właściwej i zwiększenie komórkowości. (C) Umiarkowane uniesienie nabłonka ogranicza się do wierzchołków kosmków u zwierząt leczonych LA (H & E, oryginalny 10 ×)

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