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The effect of Betanin parenteral pretreatment on Jejunal and pulmonary tissue histological architecture and inflammatory response after Jejunal ischemia-reperfusion injury

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ABSTRACT

Intestinal ischemic-reperfusion (IR) injury has detrimental effects on both local and distant organs in the body. Betanin is known for its antioxidant properties, and it is found mostly in vegetables. Therefore, the aim of the present study was to test the hypothesis that betanin administration prior intestinal IR, may be beneficial in protecting jejunal mucosa and lung parenchyma against IR damage. Male specific pathogen-free Charles River Wistar rats were used (n = 42). Betanin (50 mg/kg) was administered intraperitoneally 30 min before ischemia of the superior mesenteric artery lasting 1 h, followed by 1, 4 and 24 h of reperfusion. Immunohistochemical as well as histomorphometrical analysis indicated a protective effect of betanin pretreatment on jejunal tissue. Regarding morphometrical analysis betanin significantly ($p < 0.01$) augments intestinal villus height after 24 of reperfusion comparing to early stages. Betanin application reduced number of mast cells population in early reperfusion periods ($p < 0.05$). The protective effect of betanin on lung parenchyma, was detected in late reperfusion period (24 h) with improvement of histopathological injury index and morphometric analysis ($p < 0.001$ for both). The improvement of histopathological injury index ($p < 0.001$) and morphometric analysis ($p < 0.001$) during the late reperfusion period, suggests a protective effect of betanin on lung parenchyma. Moreover, suppression of the inflammatory response was mirrored by the reduction of myeloperoxidase (MPO) positive cells within lung parenchyma after 1 and 4 h of reperfusion ($p < 0.001$). Especially, during the first 4 h of reperfusion after betanin administration, a reduction of 74% of the polymorphonuclear neutrophils infiltration (MPO positive cell population) and of a nearly 46% of active MCs was observed. Upon morphometric examination, the lung histological architecture after 24 h of reperfusion appeared to be almost 100% better following betanin treatment, with 25% thinner interalveolar septa and 20% larger alveolar surface for respiratory gas exchange. The results suggest that betanin pretreatment protects the jejunal mucosa and the lung parenchyma, as well as reduces the inflammatory cell density after intestinal IR injury.

Abbreviations: ALI, acute lung injury; AS, alveolar surface; ATP, adenosine-triphosphate; BM, basement membrane; CD, crypt depth; COX-2, cyclooxygenase-2; H&E, haematoxylin–eosin; IST, interalveolar septum thickness; IR, ischemia-reperfusion; LII, lung injury index; MCs, mast cells; MII, mucosal injury index; MPO, myeloperoxidase; ROS, reactive oxygen species; SMA, superior mesenteric artery; VH, villus height

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1. Introduction

The onset of intestinal ischemia-reperfusion (IR) initiates the challenging condition of IR injury since it may cause not only local damage, but also acute lung injury (ALI) due to oxidative stress (Carden and Granger, 2000), thus potentially progressing towards a systemic inflammatory response syndrome and finally to multiple organ dysfunction syndrome (Ceppa et al., 2003).

During the process of IR injury of the jejunum, destructive molecules that are produced locally enter the bloodstream and are able to initiate damage in numerous tissues and organs in the body. Such molecules are Reactive Oxygen Species (ROS) including hydrogen peroxide and superoxide, and inflammatory cytokines that pose a threat to healthy state of distant organs. The pathophysiological mechanism of IR injury can be summarized as such: the initial damage caused by the ischemic attack results in cellular injury and later necrosis or apoptosis. The reperfusion activates leukocytes overproduce ROS that induce further tissue damage. Interestingly, the ROS mediated damage has been reported as more severe than the ischemic (Cerqueira et al., 2005).

The most susceptible segment to this kind of injury is the small intestine (Chu et al., 2015). Intestinal IR is a clinical phenomenon which is observed in many diseases. Acute blood flow obstruction whether form occlusive disease (arterial of venous) or in case of severe hypoperfusion (splanchnic vasoconstriction, sepsis, cardio-vascular surgery) ultimately leads to severe intestinal damage (Grootjans et al., 2016). In the past, many attempts have been made to minimize the mucosal damage that follows a number of pathological and iatrogenic events. Such attempts include ischemic preconditioning, anti-complement, anti-inflammatory therapy and, lastly, antioxidant supplementation. In this regard, antioxidants such as allopurinol and quercetin have been proven to display a protective effect in IR injury secondary to trauma (Lakhanpal and Rai, 2008).

Pathophysiological basis of IR injury is well known and more or less in its principle uniform for all affected tissues with difference based on specific tissue function. Ischemic insults diminish adenosine-triphosphate (ATP) storage which alters ionic pump function dependent on ATP delivery. That results in intracellular movement of calcium and sodium along with water. Catabolism of ATP results in hypoxantine production which upon oxygen restitution leads to production of reactive oxygen species (ROS) mostly superoxide (O_2^-) and hydrogen peroxide (Granger and Kvietys, 2015). Production of ROS launch cascade of events including promotion and expression of proinflammatory mediators (interleukins, adhesion molecules, cytokines) leading to activation and attraction of inflammatory cells into tissue. Leukocytes adhere with endothelium and translocate into tissues and release toxic ROS such as various enzymes (e.g. protease, elastase) leading to increased microvascular permeability, oedema, thrombosis, and parenchymal cell death (Collard and Gelman, 2001). Intestinal IR injury results in loss of mucosal barrier which augments mucosa permeability and translocation of luminal content into systemic circulation. Knowledge about increased gut permeability, bacterial translocation and systematic inflammatory response on development of multiple organ failure are well known. Not only spreading of bacteria into systemic circulation, but also their local impact on activation of inflammatory system, production of mediators leading to further increase of gut permeability. Data from literature support knowledge that intestinal IR impairs distant organ function (Onder et al., 2012). Among those lung is the most susceptible to intestinal IR induced injury. There are many factors playing a role in the damaging of the lungs during IR. In ischemia stage, decreasing of oxygen pressure and inability to supply increased metabolic needs alongside severe cytokine release during reperfusion stage all together cause an increase in ROS production and led to damage in both cells and tissues (Mao et al., 2013).

Betanin (betanidin 5-O-β-D-glucoside, red), a member of the betacyanin family, is a water soluble and nitrogen containing molecule, found mainly in *Beta vulgaris* var. *rubra* but also in *Amaranthus*, *Opuntia*, *Cereus* (Nemzer et al., 2011). Betanin is the most abundant betalain component, making up 75–95% of total betalains (Gliszczyńska-Świgło et al., 2006). The betanin molecule includes phenolic and cyclic amine groups which act as electron donors. Scavenging activity is based on the potential of betanin to attract and to provide an electron to the ROS, thus stabilizing them. Interestingly 10 years before *Beta vulgaris*, has been ranked within the top ten vegetables displaying the most potent antioxidant effect *in vitro* (Azeredo, 2009). Its antioxidant effects were firstly revealed in 1980 and since then, this substance has been widely studied but not on its potential protective effect in mesenteric IR injury.

The objective of this study was to investigate the effect of betanin on the inflammatory infiltration that follows IR injury in both primary affected organs, i.e., jejunum and lung. To this purpose, inflammatory cells – mast cells (MCs) and neutrophilic granulocytes - participating in ROS-production through their secretory granules containing myeloperoxidase (MPO), were immunohistochemicaly evaluated. Protective antioxidant and scavenging activity of betanin parenteral pretreatment was then tested. This study provides a detailed immunohistochemical analysis of the aforementioned effect, utilizing quantitative and qualitative methods of cell investigation with the focus on inflammatory response.

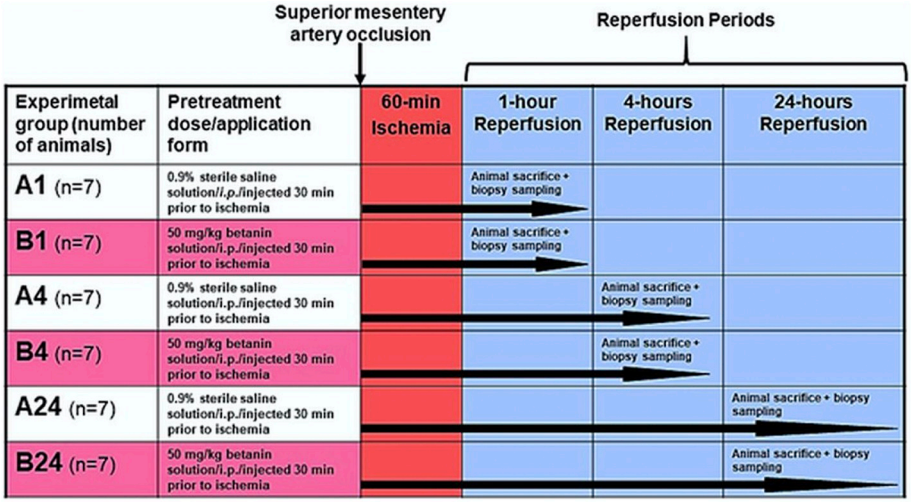


Fig. 1. Schematic diagram of the experimental design. The time courses of the pharmacological manipulations/pretreatment with administration time point, surgical procedures, and periods of ischemia-reperfusion are shown for the 6 included groups (3 control groups without betanin application + 3 experimental groups with betanin application), number of animals, time of animals sacrifice end points with biopsy sampling (jejunum and lungs).

2. Methods

2.1. Ethics approval

This experimental study was approved by the Ethics Committee on Animal Experiments of the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and the experimental protocol was confirmed by the State Veterinary and Food Administration of the Slovak Republic (No. 3004/14-221). The experiments were performed in Laboratory of Research Bio-models (No. SK 4013) of the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and all fitted with the relevant guidelines for the care and use of laboratory animals.

2.2. Chemicals

Betanin $C_{24}H_{26}N_2O_{13}$ (Red Beet extract diluted with Dextrin, Molecular Weight 550.47 g/mol, CAS number: 7659-95-2; Fig. 2B) was purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA). For betanin dissolution, the 0.9 normal saline was used as vehicle and the dose of 50 mg/kg was applied intraperitoneally for animal pretreatment (Fig. 1). (See Tables 1 and 2.)

2.3. Study population

A total of 42 adult male outbred pathogen-free Charles River Wistar rats weighing 250–350 g were used. The animals were housed in standard conditions with controlled temperature (21 °C) and had free access to commercial chow and water *ad libitum*. Animals were fasted for 12 h before surgery but with free access to water. Rats were randomly divided into two experimental groups as follows:

2.3.1. Experimental group with betanin pretreatment

(B, n = 21): 30 min before ischemia, betanin solution in 0.9% sterile saline solution (50 mg/kg) was injected intraperitoneally. Rats were subjected to total occlusion of superior mesenteric artery (SMA) by using an atraumatic vascular clamp for 60 min interval, followed by reperfusion period of 1 h (B1, n = 7), 4 h (B4, n = 7), and 24 h (B24, n = 7).

2.3.2. Control group without betanin application

(A, n = 21): the same volume of 0.9% saline solution was injected intraperitoneally 30 min before the start of ischemia. Rats then underwent total occlusion of SMA by using an atraumatic vascular clamp for 60 min interval, followed by reperfusion period of 1 h (A1, n = 7), 4 h (A4, n = 7), or 24 h (A24, n = 7).

2.4. Surgical procedures and sampling

Animals were fasted overnight in cages with raised floors to minimize coprophagy. They were then anesthetized with intraperitoneal injection of ZOLETIL 100 A.U.V. INJ (Tiletaminum and Zolazepamum, 50 mg/kg, Virbac S A., France). The SMA was isolated and ischemia was induced as mentioned above. The atraumatic vascular clamp was carefully removed after 60 min of ischemic period. A reperfusion period lasting 1, 4 and 24 h, respectively, came after. The abdominal incision was closed in two layers with Silon 2.0 EP suture (ChiRmax, Prague-Modřany, Czech Republic). Body temperature was maintained at 37 °C by a heating pad set until the animals were revived. After expiration of reperfusion period, animals were sacrificed.

2.5. Tissue specimen preparation

Small intestine (*jejunum*) samples 1–2 cm long were taken 10 cm below the Trietz ligament. A bioptic sample from the middle lobe of the right lung (*lobus medius pulmonis dexter*) was also harvested as the most representative of parenchyma damage. Specimens were washed with cold saline and fixed in 4% paraformaldehyde. Tissues were then embedded in paraplast, cut into 4–5 μm thick sections, and mounted. After deparaffinization, tissue sections were stained for routine histological, histochemical and immunohistochemical analysis.

2.6. Histological and morphometrical analysis of intestinal and lung damage

Routine haematoxylin–eosin (H&E) staining method was used for evaluation of jejunal mucosa damage according to our scoring system published elsewhere (Kovalčinová et al., 2014). Mucosal injury index (MII) was graded as follows: 0-normal (no pathological defects), 1-intestinal villi epithelium damage (swelling, vacuolation, progressive epithelial lifting from the basement membrane particularly at the villus tip known as “Gruenhagen's space”), 2-intestinal villi epithelium and *lamina propria mucosae* damage (local necrosis of the epithelium, apical villi denudation, dystrophy of connective tissue in the villi, capillary dilatation, epithelial detachment from basal membrane at the villus base), 3-damage of intestinal gland epithelial lining and *lamina propria mucosae* (massive denudation with necrotic lesions and haemorrhages in middle region of the villi), 4-complete destruction of mucosa with damage to the *lamina muscularis mucosae* (absence of connective tissue of the villi, distinct basophilic tissue staining and dystrophy of the epithelium of intestinal glands, capillary dilatation and congestion, necrotic lesions and haemorrhages in connective tissue around intestinal glands, discontinuity of the *lamina muscularis mucosae*).

Morphometry was performed using an Olympus BX50 light microscope equipped with Olympus Camera SP350 (Olympus, Tokyo, Japan) and QuickPHOTO Industrial 2.3 image analyser software (Promicra, Prague, Czech Republic). Villus height (VH) from the villus base to the tip and crypt depth (CD) from the villus base to the muscular layer were measured in 12 axially-oriented villi in at least three different high-power fields of each intestinal sample. All measurements were done using magnification 200×. Density of inflammatory cells (mast cells and MPO-positive cells) in the *lamina propria mucosae* was calculated as the number of cells per unit of mucosal tissue area (mm^2). The mucosal tissue area (*lamina propria mucosae*) was measured from the tip of villi to the *lamina muscularis mucosae*.

For the assessment of lung injury index (LII), the grading system described by Tassiopoulos et al. (1997) was used, based on the degree of leukocyte infiltration, congestion, interstitial oedema and alveolar haemorrhage, as follows: 0-no changes; 1-focal, mild, subtle changes; 2-multifocal mild changes; 3-multifocal prominent changes; 4-extensive prominent changes.

In addition, interalveolar wall/septum thickness (IAT) and alveolar surface (AS) were measured in 10 randomly arranged areas of each lung tissue sample. All measurements were done using magnification 200×. Density of inflammatory cells (mast cells and MPO-positive cells) in lung parenchyma was calculated as the number of cells per unit of lung tissue area (mm^2).

Table 1
Histological analysis of inflammatory cell populations in the jejunal mucosa.

Experimental groups	MPO-positive cells population (M ± SEM)	MAST CELLS population (M ± SEM)
A1	810.99 ± 46.05	439.62 ± 35.19[†]
B1	951.39 ± 42.07[**]	320.74 ± 24.93
A4	812.51 ± 47.48	420.32 ± 35.59
B4	759.97 ± 36.47	314.35 ± 19.44
A24	857.09 ± 84.14	314.02 ± 33.31
B24	700.87 ± 39.28	264.47 ± 15.99

[**] B1 vs. B24 $p < 0.01$.
[†] A1 vs. B1 $p < 0.05$.

Table 2
Histomorphometric analysis of pulmonary parenchyma.

Experimental groups	Interalveolar septa thickness (M ± SEM)	Alveolar surface (M ± SEM)
A1	13.48 ± 0.25***	1084.49 ± 32.36***/†††
B1	14.16 ± 0.32***	1479.97 ± 51.56**/***
A4	15.20 ± 0.28	647.79 ± 28.46***/†††
B4	15.23 ± 0.35***	1848.32 ± 56.83***
A24	14.29 ± 0.28†††	876.73 ± 32.03†††
B24	10.57 ± 0.20	1241.41 ± 37.65

** $p < 0.001$

*** A1 *vs.* A4 & A24 $p < 0.001$; B1 *vs.* B24 $p < 0.001$; B4 *vs.* B24 $p < 0.001$; A4 *vs.* A24 $p < 0.001$; B1 *vs.* B4 & B24 $p < 0.001$.

††† A24 *vs.* B24 $p < 0.001$; A1 *vs.* B1 $p < 0.001$; A4 *vs.* B4 $p < 0.001$; A24 *vs.* B24 $p < 0.001$.

2.7. Histochemical and immunohistochemical analysis

The population of MCs in 1 mm^2 of intestinal *lamina propria mucosae* and in pulmonary parenchyma was detected using metachromatic Cresyl Fast Violet (Merck Millipore, Billerica, Massachusetts, United States; #15947) staining protocol (Cook, 1961). The hallmark of this staining technique is the presence of distinct brilliant red/purple secretory metachromatic granules in the cytoplasm of MCs. The number of MCs was determined in 10 different randomly selected high-power fields using magnification ×400. The population of MPO positive cells (anti-MPO, RB-373-A1, dilution 1:300, Thermo Scientific, MA, USA) in 1 mm^2 of jejunal *lamina propria mucosae* and pulmonary parenchyma was detected in 10 different fields by immunohistochemical analysis. Positive cells were visualized with diaminobenzidine (Sigma-Aldrich; 32,750-1G-F) and counterstained with Mayer's haematoxylin. Omitting the primary antibody was considered as the negative control (Figs. 3C-B24, 5B). Tissue sections were examined and the pictures were taken using an Olympus BX50 light microscope with an Olympus SP350 camera (Olympus, Tokyo, Japan). Tissue samples were evaluated by two blinded and independent Pathologists. In each sample at least 10 randomly selected visual high-power fields were observed at magnification ×400. All positive cells were counted in each of them using cytometric software Quick-PHOTO Industrial 2.3 image analyser software (Promicra).

2.8. Statistical evaluation

Statistical analysis was performed using GraphPad In-Stat version 3.01 (GraphPad Software, San Diego, CA). Quantitative results (histochemical and immunohistochemical analyses) were determined using the one-way ANOVA test with a multiple comparison Tukey–Kramer *post hoc* test. Semiquantitative results (MII, LII) were determined using Kruskal–Wallis non-parametric ANOVA test with a multiple comparison non-parametric Dunn's test. Results were expressed as mean (M) ± standard error of the mean (SEM). P Values < 0.05 were considered to be significant.

3. Results

3.1. Jejunum

3.1.1. Histopathological injury index

The evaluation of the MII yielded noteworthy results. Despite the lack of statistical significance due to large deviations from given results

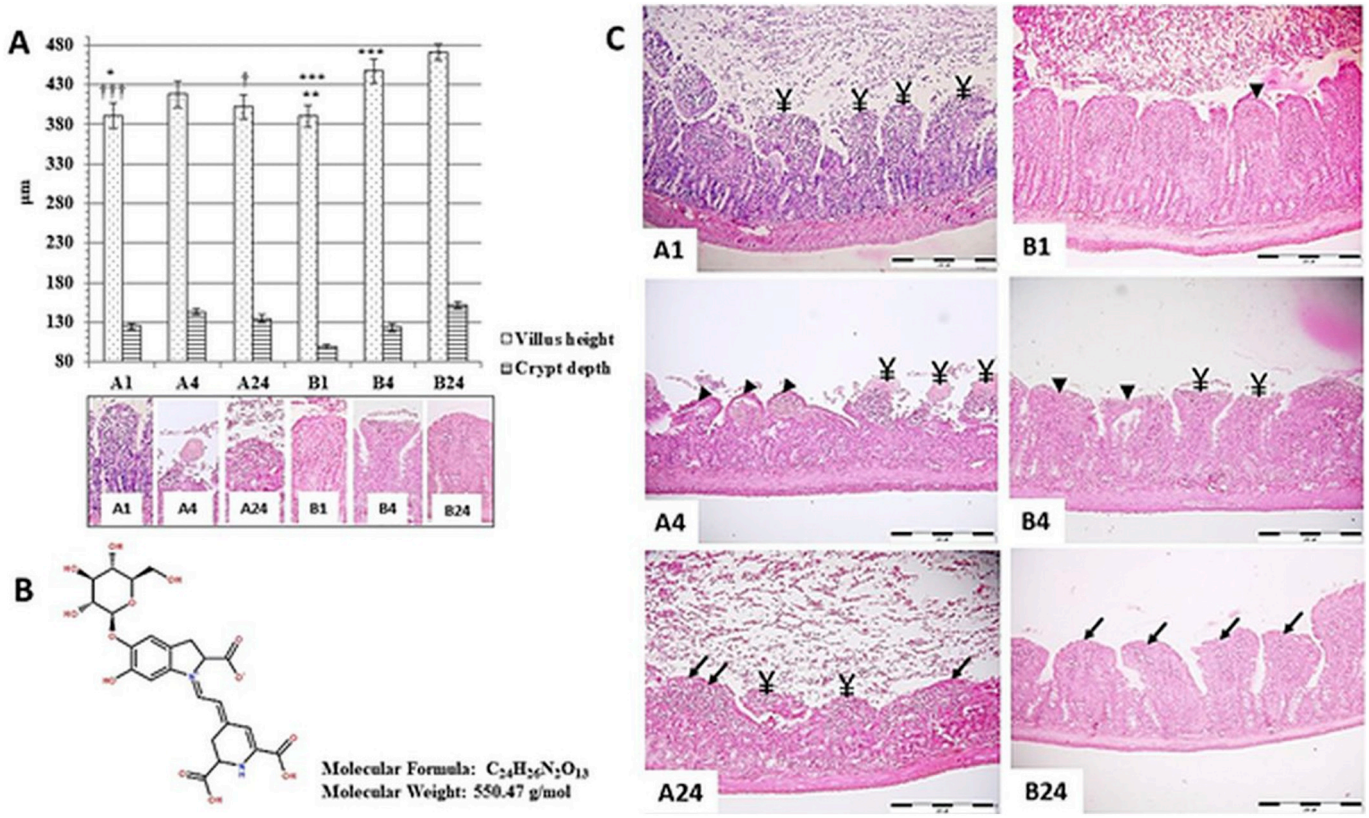


Fig. 2. Jejunal Injury Index. A. Histomorphometric analysis of jejunal mucosa (*A1 *vs.* A4 $p<0.05$; †A24 *vs.* B24 $p<0.05$; †††A1 *vs.* B1 $p<0.001$; **B1 *vs.* B24 $p<0.01$; B1 *vs.* B4 $p<0.01$; ***B1, B4 *vs.* B24 $p<0.001$). B. Chemical skeleton structure of betanin. C. Mucosal Injury Index. Images showing histopathological changes of the jejunal wall in each untreated groups (A) and betanin pretreated groups (B); ¥significant loss of intestinal villi structure, apical denudation, local necrotic lesions with destroyed villi typical architecture, ▼superficial epithelium detachment from basement membrane, ↓reepithelization of intestinal mucosa; (H&E, ×200 original magnification, Bar = 500 µm).

in each group, a reduction ranging from 40 to 57% of damage in all preconditioned groups (B1, B4, B24) was observed in comparison to the control ones (A1, A4, A24) (Fig. 2A,C). In all control groups, damaged intestinal mucosa (Fig. 2C-A1,4,24), destroyed villi architecture (Fig. 2C-A1,4), apical denudation of the villi (Fig. 2C-A1,4,24), and detachment of the epithelium from the BM (Fig. 2C-A1,4) were observed. In comparison, in B groups only swelling, vacuolization, and epithelium detachment limited to the apical areas of the villi known as "Gruenhagen's space" was found (Fig. 2C-B1,4). These histopathological findings are in harmony with changes observed at histomorphometric analysis of the jejunal mucosa (Fig. 2A). Significantly longer intestinal villi and deeper intestinal glands/crypts were measured in the B24 experimental group. Specifically, villi were 15% longer than those villi in the A24 control group (B24 *vs.* A24: $p < 0.05$). Intestinal glands/crypts ratios were 19% to 35% significantly deeper in B24 group in comparison to both B1 (B24 *vs.* B1: $p < 0.001$), and B4 (B24 *vs.* B4: $p < 0.001$) group. Moreover, intestinal crypts of the B24 group were 12% deeper than those of the A24 control group.

3.1.2. Mast cells

MCs count provide evidence of a significant decrease only when comparing the results of the B1 with those of the A1 group ($p < 0.05$, Fig. 3A). This free connective tissue cells population was identified according to its localization and presence of metachromatic secretory granules in the cytoplasm as mucosa-related type of mast cells (Fig. 3B,C).

3.1.3. Myeloperoxidase positive cells

As regard as the results of MPO cells, a stable elevation was observed, although not statistically significant, in the control untreated A groups. (Fig. 3A,B,C). By contrast, a significant decrease of MPO cell infiltration was evident when comparing B1 with B24 groups ($p < 0.001$), indicating a lower degree of inflammatory reaction and consequently, less mucosal damage (Fig. 3C-B1,24).

3.2. Lung

3.2.1. Histopathological inury index

Distinct multifocal mild pathological changes were found in lung parenchyma of control group A24 in contrast to those observed in the B24 one ($p < 0.001$). Upon calculation of the LII, the damage appeared up to 100% worse in A24 control group in comparison to experimental B24 (Fig. 4A,B,C-A24,B24). Specifically, tissue lesions were evident both in the interalveolar septa and pulmonary interstitium, with leukocyte infiltration, congestion of blood vessels, interstitial oedema and alveolar haemorrhage (Figs. 4C, 5C). Particularly, in the perivascular connective tissue, cell infiltration and local haemorrhagic lesions were presented at histological examination (Figs. 4C-A24, 5C-A1,4,24). Among the B groups, LII was significantly higher only in the B4 one in comparison to the B24 (Fig. 4C-B1,24). This is probably related to the culmination of the inflammatory response at this time point. Notably, this finding fully parallels the changes of the morphometric parameters observed in pulmonary parenchyma. However, overall considered, these parameters performed better in almost all (particularly in B24 group) preconditioned groups (Fig. 4A, C-B24). In detail, a significant reduction in thickness of the interalveolar septa was detected in the experimental group B24 (B24 *vs.* B1 & B4; $p < 0.001$ for both, Fig. 4C-B1,4,24). On the other hand, significant alveolar septa thickening was detected in group A4 in comparison to group A1 ($p < 0.001$). A larger AS for respiratory gases exchange, was found in the B4 group, as morphometrically evaluated (B4 *vs.* A4: $p < 0.001$).

3.2.2. Mast cells

A similar significant increase of the number of metachromatically

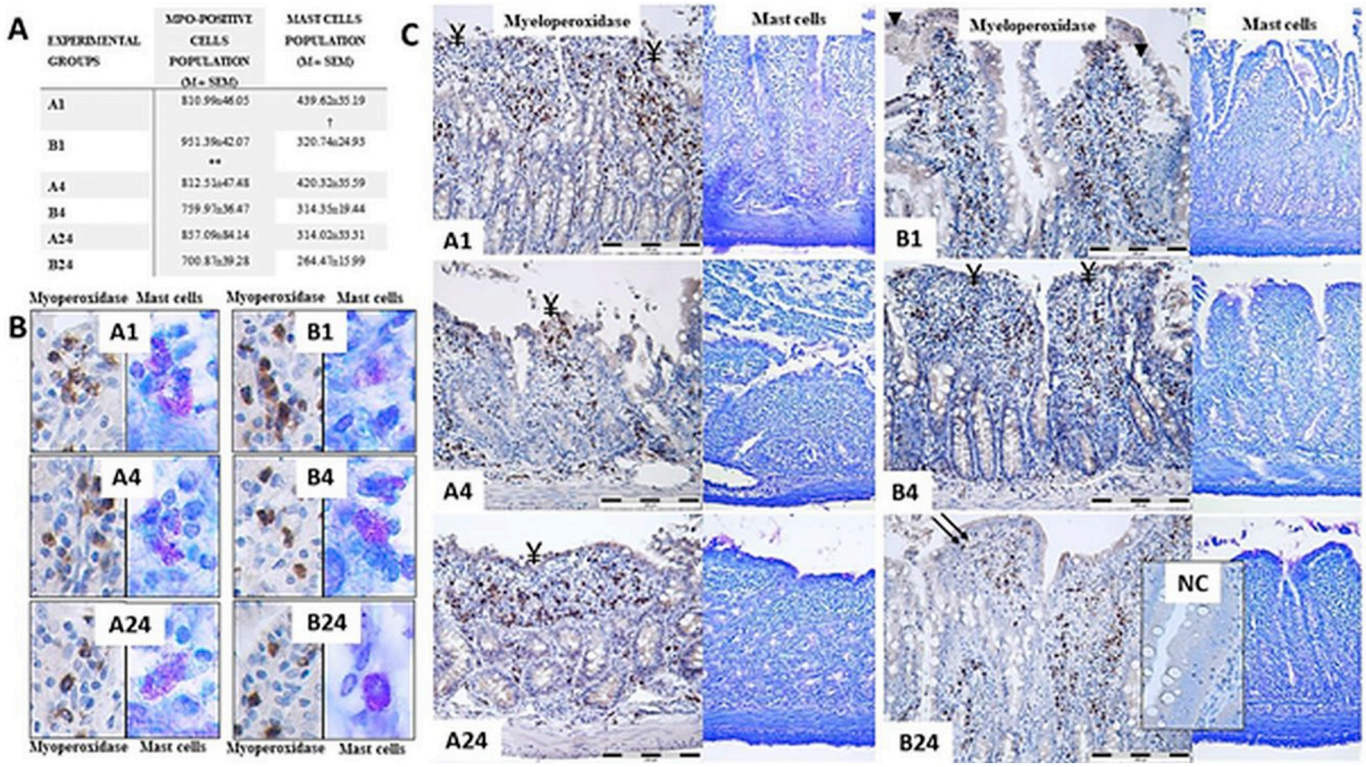
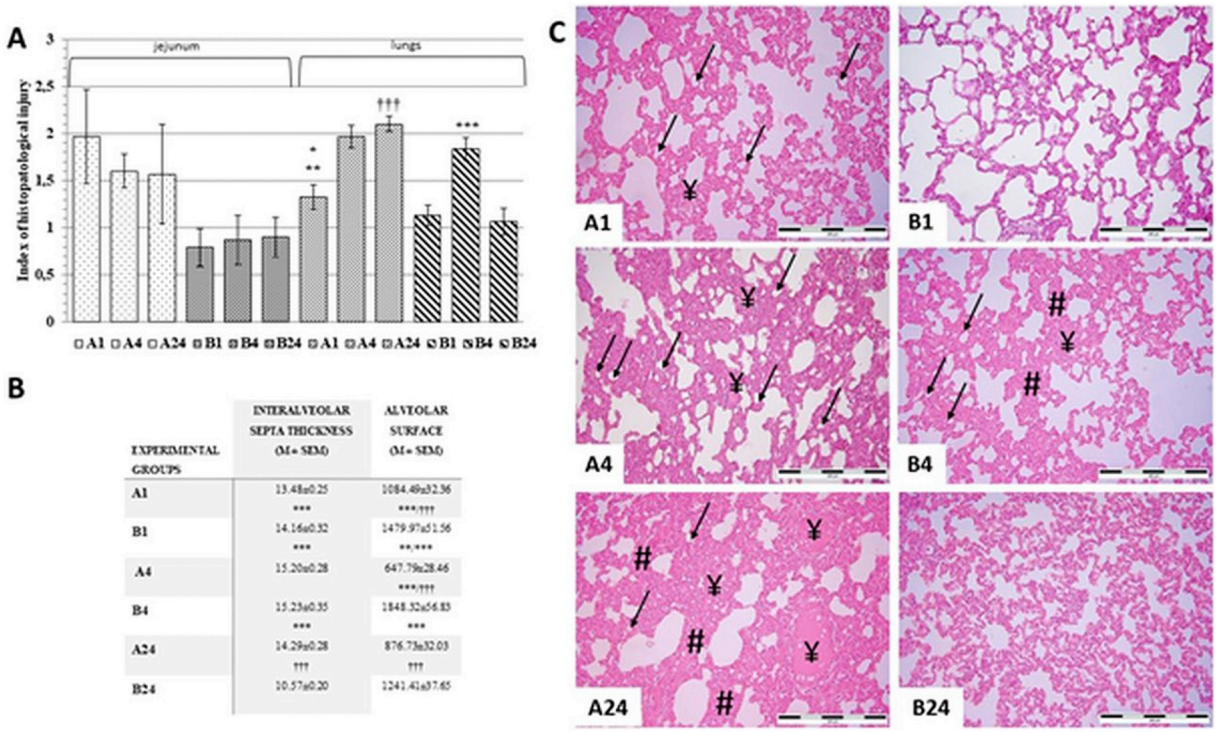


Fig. 3. Histochemical and immunohistochemical analyses of jejunum. A. Table: Histological analysis of inflammatory cell populations in the jejunal mucosa (**B1 *vs.* B24 $p^{<}0.01$; †A1 *vs.* B1 $p^{<}0.05$). B. & C. Images showing histopathological changes with MPO-positive cells population and mast cells (MCs) population of the jejunal wall in each untreated groups (A) and betanin pretreated groups (B) with negative control (NC) for immunohistochemistry; ¥apical denudation with destroyed villi architecture, ▼swelling, vacuolation, progressive epithelial lifting from basement membrane particularly at the villus tip "Gruenhagen's spaces", ↓prominent re-epithelization of intestinal mucosa; (anti-MPO antibody, Cresyl Fast Violet, details: 1000× magnification, ×400 original magnification, Bar = 200 μm).



EXPERIMENTAL GROUPS	INTERALVEOLAR SEPTA THICKNESS (M ± SEM)	ALVEOLAR SURFACE (M ± SEM)
A1	13.48±0.25 ***	1084.49±32.36 ***,†††
B1	14.16±0.32 ***	1479.97±51.56 **,***
A4	15.20±0.28	647.79±28.46 ***,†††
B4	15.23±0.35 ***	1848.32±56.83 ***
A24	14.29±0.28 †††	876.73±32.03 †††
B24	10.57±0.20	1241.41±37.65

Fig. 4. Lung Injury Index. A. Comparison of time-dependent changes of histopathological injury in jejunal mucosa and lung parenchyma (*A1 *vs.* A4 $p^{<}0.05$; **A1 *vs.* A24 $p < 0.01$; †††A24 *vs.* B24 $p < 0{,}001$; ***B4 *vs.* B24 $p^{<}0{,}001$). B. Table: Histomorphometric analysis of pulmonary parenchyma (***A1 *vs.* A4 & A24 $p < 0.001$; ***B1 *vs.* B24 $p < 0.001$; ***B4 *vs.* B24 $p < 0.001$; †††A24 *vs.* B24 $p < 0.001$; †††A1 *vs.* B1 $p < 0.001$; †††A4 *vs.* B4 $p < 0.001$; †††A24 *vs.* B24 $p < 0.001$; ***A4 *vs.* A24 $p < 0.001$; ***B1 *vs.* B4 & B24 $p < 0.001$). C. Lung Injury Index. Images showing histopathological changes in the lung parenchyma in each untreated group (A) and betanin pretreated groups (B); ¥congestion of blood vessels, #significant interalveolar septa thickening with interstitial oedema, ↓prominent alveolar surface reduction; (H&E, ×400 original magnification, Bar = 200 μm).

positive MCs in the group A1 was found in the lung tissue in comparison with the results in the A24 ($p < 0.01$; Fig. 5A, B, C-A1,24). By contrast, a 50% lower rate of MCs was found in the B1 group in comparison to the control group A1 (Fig. 5B,C).

3.2.3. Myeloperoxidase positive cells

Finally, as far as the MPO positive cells are regarded, a significant increase of their density in the lung tissue of the control group A1 with respect to the experimental group B1 ($p < 0.001$) but also to both A4 and A24 groups ($p < 0.001$ for both; Fig. 5A, C-A1,24 and C-B1) was registered.

4. Discussion

The current study describes the impact of betanin parenteral infusion prior to intestinal IR injury on histopathological changes of both jejunal mucosa and lung tissue by means of histomorphometry and immunohistochemical evaluation.

Intestinal IR injury is widely recognized to have detrimental effects on jejunal mucosa because of activation of a complex cascade of events leading to local and systemic damage (Curgali et al., 2018; Tóth et al., 2017; Zhang et al., 2008). In this study we investigated whether betanin administration before induction of IR injury could be of benefit in preventing both intestinal and lung damage.

However, despite a trend in amelioration of the MII in pretreated groups, the results did not achieve a statistical significance because of a high variability. Nonetheless, a histomorphometrically significant increase of intestinal CD that was observed in early reperfusion period (A1 *vs.* B1) intestinal VH increased afterwards (A24 *vs.* B24). The intestinal villi were 15% longer, and the intestinal crypts were 12% deeper than untreated control groups after 24 h of reperfusion. This possibly means an improved absorptive and secretory activity of the jejunal mucosa pretreated with betanin.

In addition, the number of MPO positive cells progressively decreased over time in jejunal mucosa of B groups, achieving the statistical significance after 24 h. These results are in agreement with others showing that pretreatment with antioxidants has reversing effect in jejunal tissue damage upon IR injury (Curgali et al., 2018; Tóth et al., 2017; Impellizzeri et al., 2016).

In previous studies another molecule with antioxidant properties, such as quercetin, was able to suppress inflammatory response in jejunal tissue after intestinal IR by reduction of COX-2 and MPO positivity and by increase of the level of the anti-inflammatory cytokine IL-10 (Curgali et al., 2018; Tóth et al., 2017). In general, the antioxidant mechanism and its pathways include a decrease of malondialdehyde levels (MDA), an increase of antioxidant capacity by gluthatione (GSH) and a reduced production of inflammatory mediators such as MPO, TNF-α, IL-1α, interferon-γ (Impellizzeri et al., 2016; Turan et al., 2017; Yucel et al., 2011).

Betanin is a natural antioxidant substance with the potential to reverse oxidative stress and reduce ROS-mediated damage (Han et al., 2015; Tan et al., 2015; Vulic et al., 2014). Indeed, data from several studies showed that betanin and betalains possess antioxidant and anti-inflammatory properties (Livrea and Tesoriere, 2012). Betalain richness makes red beetroot one of the 10 most potent antioxidant vegetables (Azeredo et al., 2007), which was reported to protect mice during gamma-irradiation (Lu et al., 2009). On the other side, betanin also inhibits lipid peroxidation and heme decomposition *in vitro* even when applied in extremely small concentrations (Kanner et al., 2001) and attenuates carbon tetrachloride-induced liver injury in fish (Han et al., 2014). Studies performed in conditions where oxidative stress played an important role in triggering and sustaining tissue damage, have supported the idea of a protective betanin's effect on IR injury. This was proved in acute myocardial infarction, where betanin application

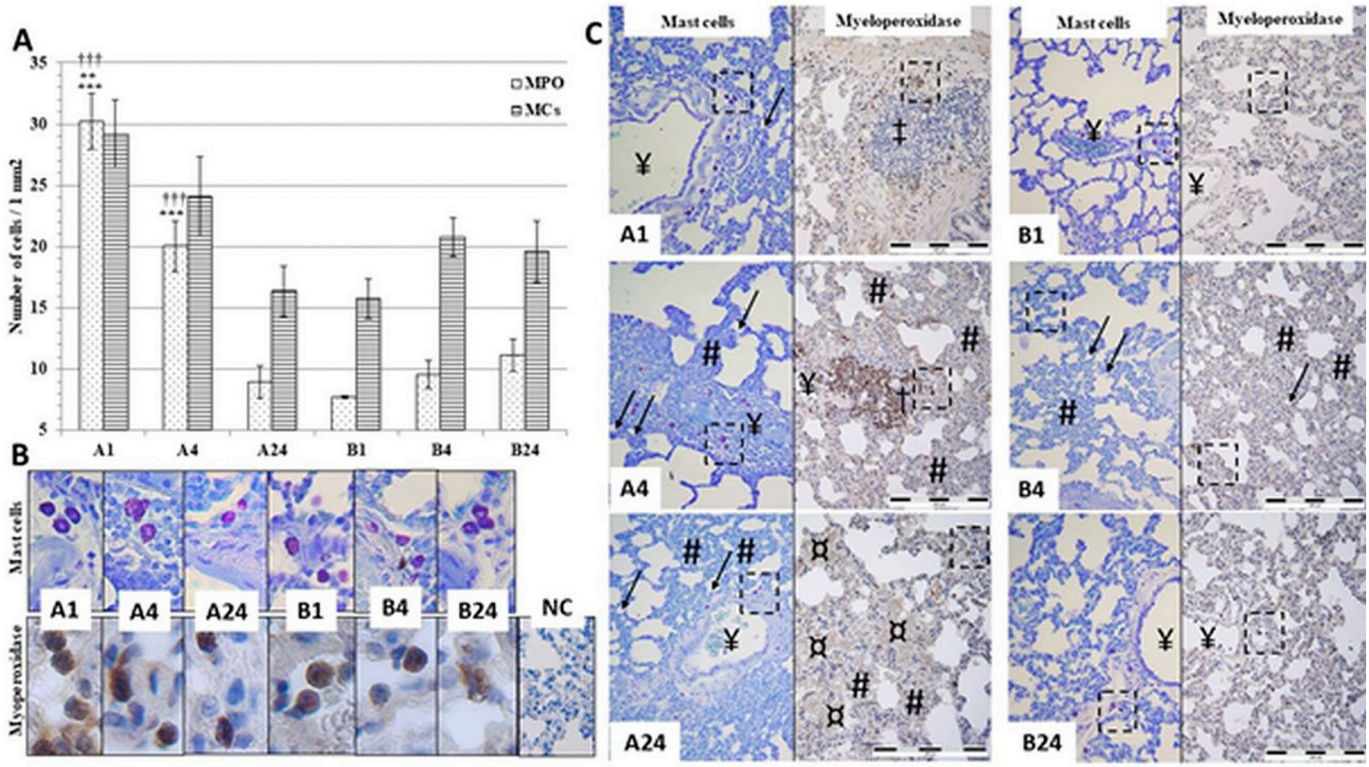


Fig. 5. Histochemical and immunohistochemical analyses of lungs. A. Imunohistochemical analysis of inflammatory cell populations in lung parenchyma (***A1 *vs.* A4 $p < 0.001$; †††A1 *vs.* B1 $p < 0.001$; ***A4 *vs.* A24 $p < 0.001$; †††A4 *vs.* B4 $p < 0.001$; **A1 *vs.* A24 $p < 0.01$). B. & C. Images showing histopathological changes with metachromatically positive MCs population and immunohistochemically MPO-positive cells population in each untreated group (A) and betanin pretreated group (B) with negative control (NC) for immunohistochemistry; ¥blood vessel; #thickening of interalveolar septa, ↓alveolar surface reduction, ¤haemorrhage, †MPO-positive cell infiltration, ‡bronchus-associated lymphatic tissue; (Cresyl Fast Violet, anti-MPO antibody, details: 1000× magnification, ×400 original magnification, Bar = 200 μm).

resulted in suppression of inflammatory cascade (MDA, MPO, inducible nitric oxide synthetase levels) and potentiation of antioxidant defence (superoxide-dismutase, catalase, glutathione) (Yang et al., 2016). Furthermore, another study showed a strong anti-inflammatory, antioxidant and thus protective effect of betanin through the inhibition of polymorphonuclear leukocytes population activity, a quantitative reduction of MPO, MDA, and pro-inflammatory mediators, such as TNF-α and IL-1β as well, all of which play an important role in oxidative stress (Han et al., 2014).

The observation that MCs are crucial in the development of mucosal injury was outlined by Gan et al. (2015) who demonstrated that during IR, MCs degranulation resulted in increased inflammatory response (MPO activity, Intercellular Adhesion Molecule 1 - ICAM, P-selectin expression), and that the inhibition of MCs activation diminishes the intestinal IR injury and the systemic inflammatory response as well. The current study introduces the first evidence of a beneficial effect of betanin pretreatment on both jejunal and lung tissue following intestinal IR injury. The histological results indicate that betanin may inhibit MCs activation also in intestinal mucosa and may reduce gut-induced lung damage.

It has been established that intestinal IR injury not only affects local and primary jejunal tissue but also has a complex and widespread outcome. Local inflammatory response driven by reperfusion alters gut-barrier integrity thus allowing bacterial translocation into systemic circulation whose detrimental effects are potentiated by the aforementioned inflammatory mediators. Consequently, distant secondary organ injury develops, affecting primarily lungs and kidney, and other organs to a lesser degree (Mura et al., 2007; Zhang et al., 2008). Lung injury is mainly mediated by an increased pulmonary vascular permeability due to the presence of inflammatory response triggered by ROS, cytokines and systemic endotoxemia (Kostopanagiotou et al., 2008).

The findings of the present study confirm the adverse effects of intestinal IR injury on lung tissue. Specifically, neutrophil recruitment and activation play a crucial role in gut-mediated lung damage (Koike et al., 1993). Betanin, other than displaying a local protective effect on intestinal IR injury, maybe more beneficial in protecting of distant organs such as lung. In fact, a progressive increase of both LII and IST was observed during the reperfusion period within untreated control groups, with the most striking effect found after 24 h, whereas betanin reversed this pattern. Accordingly, after 24 h of reperfusion, an almost 100% better lung histological architecture was evident, along with a 25% thinner interalveolar septa and 20% larger AS was available for respiration.

The inflammatory response, as assessed by MPO positive cell density within lung parenchyma, revealed its gradual decrease starting from early reperfusion period in the betanin pretreated groups. Moreover, betanin administration was also able to counteract the expansion of the MCs population. During the first 4 h of reperfusion, a nearly 46% lower population of perivascular MCs and 74% lower infiltration by MPO positive cells were recorded. The evidence suggests, a strong inflammatory response in lung tissue cells in early reperfusion period, whilst histopathological injury in the betanin pretreated groups was less pronounced in the late reperfusion period. One potential explanation lies in the fact that the inflammatory response (MPO and MCs) precedes histopathological changes (Kurt et al., 2015). In this regard, Mura et al. observed a late deterioration of lung damage, as assessed by evaluating apoptotic cells in bronchoalveolar lavage fluid, and lung permeability (Mura et al., 2007). The possibility of abating the injury of distant organs by intravenous treatment with hydrogen-rich saline before the onset of intestinal IR was supported by the results of Mao et al.

(2009), who observed a decrease of lung injury inflammatory mediators (*e.g.* neutrophil infiltration, the lipid membrane peroxidation, Nuclear factor-κB activation and the pro-inflammatory cytokine interleukin IL-1β and TNF-α) upon 4-hours reperfusion period. The histological results of the present study are consistent with the biochemical findings of Tian et al. (2010), who observed increased lung MPO levels after intestinal IR in early reperfusion period. In addition, time-dependent histopathological lesions of lung tissue after intestinal IR were also proved by An et al. (2007), who found that the most prominent changes of alveolar septa thickness were found in early reperfusion period (1 and 4 h).

Besides MPO, it was postulated that MCs, also play an important role in intestinal IR-induced lung injury. Study from Huang et al. (2012) implies that intestinal IR largely alters MCs protein expression and histamine release. During reperfusion, MCs act on various targets, including the vascular endothelium, resident monocytes/macrophages and infiltrating neutrophils, while mastocytes-derived TNF-α upregulates IL-6 expression (Frangogiannis et al., 1998). Taken together, the data demonstrated that MCs activation in association with the release of pro-inflammatory molecules largely contributes in the amplification of the lung tissue damage.

Our experimental study has several limitations. First, the betanin was administered before acute arterial occlusion. This experimental model partially trying to simulate surgical procedure where planned ischemia is performed. It is logical that in clinical practice where all ischemic situations are not,planned, this model does not meet the criteria for translation into practice. Nowadays we do not know how long term betanin application will affect intestinal metabolism and IR injury, nor the effect of different route of administration, timing and dosing of betanin. Betanin as natural plant substance has beneficial impact on human health which was proved by *in vivo* and also *in vitro* studies (Chhikara et al., 2018; Nowacki et al., 2015). Our current study needs more research with biochemical and molecular methods which will clarify mechanism of betanin impact on ischemic-reperfusion damaged tissue.

5. Conclusion

In summary, the results from our study highlight a beneficial effect of betanin on jejunal and lung tissue histological lesions after intestinal IR injury. This effect may be partially explained by the protection of intestinal mucosa, thus indirectly preserving also the lung parenchyma. Our analyses have also shown the ability of betanin pretreatment to dampen inflammation following intestinal IR injury.

Declaration of Competing Interest

Authors declare no conflict of interest.

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