Intestinal ischemia/reperfusion (IR) injury is associated with many clinical conditions including acute mesenteric thrombosis, traumatic or septic conditions, small bowel transplantation, and abdominal aortic surgery, and plays an important role in the pathogenesis of systemic inflammation and multiple organ dysfunction [1]. Small intestine IR injury includes neutrophil accumulation, increased myeloperoxidase (MPO) activity, apoptotic cell death, and accumulation of proinflammatory cytokines and massive histopathological changes of intestinal wall [2–4]. Besides small intestine distant organs such as kidney, liver and lung are affected, which may cause tissue damage including increased inflammatory, apoptotic, histopathological and biochemical alterations [5,6]. Intestinal IR with its severity and complexity remains the aim of clinical practice and research as well. Several methods and protective substances were tested in an effort to reduce damage after intestinal IR injury. These strategies include: antioxidants, ischemic preconditioning, enteral feeding, NO, glutamine supplementation and others [1].

Many studies have proved a positive effect of glutamine pretreatment in the form of dipeptide or glutamine alone in attenuation of organ injury after intestinal IR [1,3,7,8]. Alanyl-glutamine (ala-gln) dipeptide has proven its great clinical value in critically ill patients [9,10]. Conclusions were made that ala-gln administration prior to insult can reduce TNF-α release in experimental endotoxemia [11]. Warm liver ischemic injury can be attenuated by ala-gln administration with enhancing GSH content and regulating the expression of Bcl-2 and Bax in liver tissue [8].

Glutamine is the most abundant non essential amino acid in the plasma during physiological conditions while various injuries are associated with decrease in plasma glutamine levels [12,13]. Among the most important glutamine benefits belongs increase in heat shock protein level (Hsp) leading to increase in resistance of cells against various forms of damage including IR injury. It was discovered that increased expression in Hsp caused reduction in proinflammatory cytokine concentration (IL-1β, TNF-α) [14,15]. Glutamine can reduce expression of adhesive molecules, which play an important role by adhesion of leukocytes in damaged tissue and starting inflammation [16]. Reduction in inflammatory cell population such as neutrophils, and macrophages contributes to reduction of histopathological consequences of intestinal IR. Therefore the aim of our experimental study was to determine the impact of ala-gln dipeptide supplementation...
on intestinal IR induced tissue damage through proliferative and inflammatory changes in small intestine mucosa.

1. Materials and methods

1.1. Ethics

This experimental study was approved by our Committee for Ethics on Animal Experiments at the Faculty of Medicine, Pavol Jozef Šafárik University, Košice, Slovakia, and the experimental protocol was approved by the State Veterinary and Food Administration of the Slovak Republic No. 720/10-221/3. All experiments were carried out in accordance with the relevant guidelines for the human use and care of laboratory animals.

1.2. Experimental animals and surgical procedure

The experiment was performed on adult, male Wistar (n = 30) rats weighing 300–350 g. The animals were housed in standard conditions and had free access to commercial chow and water ad libitum. Animals were fasted for 12 h before surgery but given free access to water. Animals were randomly divided into three experimental groups as follows:

1. Control group (C, n = 6): rats underwent medial laparotomy with obtaining biopic samples of jejunum without any surgical or pharmacological interventions.
2. Ischemia/reperfusion group (IR1, IR24, n = 12): rats were subjected to total occlusion of cranial mesenteric artery (CMA) by using an atraumatic vascular clamp for 60 min interval, followed by adequate reperfusion period (in hours 1, 24).
3. Experimental group with dipeptide pretreatment (D + IR1, D + IR24, n = 12): 30 min before ischemia alanyl-glutamine dipeptide solution (Dipeptiven 20%, Fresenius Kabi AB, Sweden, 0.75 g/kg) was injected into the caudal cava vein slowly during 5–10 min. Thirty minutes after pretreatment IR injury was induced according to the scheme in IR1 and IR24 experimental groups.

1.3. Surgical procedures and sampling

The animals were anesthetized with intraperitoneal injection of ketamine 60–80 mg/kg (Narketan 10 inj. ad us. vet., Vétoquinol S.A., Lure Cedex, France), and xylazine 8–10 mg/kg (Xylariem inj. ad us. vet., Riems Arzneimittel, Greifswald-Insel Riems, Germany). Using sterile techniques, 6 cm midline laparotomy was carried out. The CMA was isolated and ischemia was induced as mentioned before. The atraumatic vascular clamp was carefully removed, and this was followed by a reperfusion period in accordance with the experimental design. The abdominal incision was closed in two layers with Silon 2.0 EP (ChIRmax, Prague-Modřany, Czech Republic) suture for all operations. 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.

1.4. Histopathological assessment and histochemical staining

All animals were sacrificed in appointed interval. Small intestine samples 1–2 cm long were taken 10 cm from the Trietz ligament. Biopsies were washed with cold saline and fixed in 4% paraformaldehyde. The tissues were then embedded in paraffin, cut into 4–5 μm thick sections, and mounted. After deparaffinization, the tissue sections were stained with hematoxylin & eosin (H&E) for histopathological and morphometrical evaluations, Alcian blue staining method for histochemical evaluation. H&E-stained sections of small intestine were scored using a semi-quantitative Park/Chiu grading system adapted from Quaedackers et al. [17] and expressed as the histopathological injury index (HII). The population of mucus producing goblet cells (GCs) present in the intestinal epithelium was detected using the Alcian blue histochemical staining method. Alcian blue 8GX solution (pH 2.5, Sigma-Aldrich, St. Louis, MO, USA) stains both sulphated and carboxylated acid mucopolysaccharides and sulphated and carboxylated sialomucins (glycoproteins). Alcian blue/nuclear red stained tissues were acquired and the number of Alcian blue positive GCs was determined in 10 intestinal villi and corresponding intestinal crypts in each sample.

1.5. Immunohistochemical procedure

Histological sections (4–5 μm) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% H2O2 with methanol. Pretreatment was performed by microwave at 600 W for 15 min in 0.01 M citrate buffer at pH 6.0. This yielded the best results in terms of antigen retrieval. Primary rabbit polyclonal antibodies were used: anti-PCNA (Biolegend, San Diego, CA, USA), anti-MPO (Thermo Scientific, Waltham, MA, USA). Primary antibodies were applied at the appropriate titer: PCNA in 1:100, and anti-MPO antibody in 1:100 and tissue sections were consecutively incubated for 1 h at room temperature. Biotinylated secondary anti-goat anti-mouse antibody IgG (H + L) (Millipore Bioscience Research Reagents, Billerica, MA, USA) was used in labeling with IHC Select® Immunoperoxidase Secondary Detection System (Millipore Bioscience Research Reagents, Billerica, MA, USA) for detection of proliferative activity. Biotinylated secondary anti-mouse IgG (H + L)/anti-rabbit IgG (H + L) was used in labelling with R.T.U. Vectastain ABC Reagent (Vector Laboratories, Burlingame, CA, USA) for detection of MPO-containing cells. Positive cell populations were visualized with diaminobenzidine, DAB (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and counterstained with Mayer’s hematoxylin. Omitting the primary antibodies was considered as the negative control. Number of cell is expressed as mean for 1 intestinal villus counting form 10 villi quantification. The tissue sections were examined and photographed using an Olympus BX50 light microscope with an Olympus SP350 camera (Olympus, Tokyo, Japan) and were evaluated by two blinded and independent histologists.

1.6. Statistical evaluation

The statistical analysis was performed using the GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA). The quantitative results (histological and morphometrical evaluation and immunohistochemical quantifications–PCNA, MPO) were determined using one-way ANOVA with a multiple comparison Tukey-Kramer post hoc test. All the results are expressed as mean ± SEM. p values less than 0.05 were considered significant.

2. Results

2.1. Histopathological and histochemical evaluation

The most evident histopathological changes were detected after 1 h of reperfusion in IR group. Histopathological injury scored by Park–Chiu revealed its increase in IR1 experimental group compared to control group (IR1 vs. C, p < 0.001; Table 1). The most prominent and significant decrease in HII was observed in pretreated group (D + IR1 vs. IR1, p < 0.001). The alterations of intestinal mucosa architecture in IR1 group were based on mucosal surface destruction, reduction of intestinal villi height with changes in intestinal crypt compartment. Less evident destruction and disintegration of intestinal villi architecture were observed after 24 h of reperfusion, in both experimental groups. In IR24 group we observed apical destruction of villi with mucosal hypercellularity in lamina propria. The difference between HII of both experimental groups was not significant.
Histopathologically, GCs population was significantly diminished in IR1 group (IR1 vs. C, p < 0.001) compared to control group (Table 1). In glutamine treated group after 1 h of reperfusion we observed significant increase in GCs population (D + IR1 vs. IR1, p < 0.001) compared to IR1 group. Quantification of GCs after 24 h of reperfusion pointed out a slight increase in D + IR24 in comparison with IR24 and with C group but without significance.

2.2. Immunohistochemical evaluation

Quantification of PCNA as a marker of proliferation and nuclear repair was observed in jejunal mucosa in both layers — epithelial layer and lamina propria (Table 2). In jejunal epithelial layer PCNA-positivity was observed in intestinal crypts exclusively and identified by distinct dark brown staining of cell nuclei. Total number of PCNA-positive cells in jejunal epithelium revealed decrease in proliferative/reparative rate after 1 h of reperfusion in IR1 (IR1 vs. C, p < 0.001) and D + IR1 (D + IR1 vs. IR1, p < 0.001) groups. On the other hand after 24 h of reperfusion we observed increase in epithelial PCNA-positivity in IR24 (IR1 vs. IR24, p < 0.05) and in D + IR24 (D + IR1 vs. D + IR24, p < 0.001).

In lamina propria PCNA-positive cell nuclei were visualized by dark brown color of proliferative/reparative cells. Immunoreactive PCNA-positive cells detected in intestinal lamina propria were particularly connective tissue cells (e.g. fibroblasts, fibrocytes, myofibroblasts) as well as smooth muscle cells in villous core (Fig. 1). In lamina propria we observed significant decrease in PCNA-positive cells in IR1 group compared to C, IR24 and D + IR24 groups (IR1 vs. C, p < 0.05; IR1 vs. IR24, IR1 vs. D + IR24, p < 0.001 both; Table 2). In glutamine pretreated group significant increase in PCNA-positivity was observed after 1 h of reperfusion (D + IR1 vs. IR1, p < 0.01) compared to IR1 group.

Inflammatory response in jejunal mucosal membrane was assessed by immunohistochemical quantification of MPO-positive cells. Immunoreactivity was evident as distinct brown stain pattern of cytoplasm in MPO-immunoreactive cells (Fig. 2). Ischemia—reperfusion induced increase in MPO-positive cell number after 1 h of reperfusion compared to control group (C vs. IR1 p < 0.001). Significant increase in MPO-immunoreactive cells was identified in IR1 group compared to group with ala-gln administration (IR1 vs. D + IR1 p < 0.001; IR1 vs. IR24, D + IR24 p < 0.001; Table 2). Distribution of MPO-positive cells in C group was regular along intestinal villus axis generally, compared with MPO-positive cells in IR1 group where these cells form groups in lamina propria mostly in lower half of intestinal villi. After 24 h of reperfusion MPO activity in IR24 was decreased and it was comparable to control group. In group with ala-gln administration after 24 h of reperfusion we observed only low, non-significant decrease in population of MPO-positive cells in comparison with IR24 group (IR24 vs. D + IR24 p < 0.01).

3. Discussion

The current study demonstrates impact of ala-gln dipeptide administration on histopathological, inflammatory and proliferative changes in small intestine induced by intestinal IR injury. Wide knowledge is known about adverse effect of intestinal IR and its impact on changes in small intestine, lungs tissue and distant organs such as kidney and liver [5]. These acute changes include increased apoptotic cell death [18,19], histopathological injury [20,21,4] and acute inflammatory changes, too [22,23].

Small intestine with its large cell populations of various origin and function is very susceptible to different kinds of injury, including IR injury. Significant part of IR damage in intestine tissue is mediated via oxidative stress and free oxygen and nitrogen radical production. Reactive oxygen species (ROS) acts on cellular components associated with proinflammatory agents release [24]. MPO as an enzyme with location in inflammatory cells (granulocytes, monocytes/macrophages) plays an important role in inflammatory processes and its content increasing indicates an activation of acute inflammatory response [25,26]. It was also reported that intestinal IR causes an increase in MPO content in postischemic tissue [27,28]. The fact that glutamine is capable of reducing MPO content was concluded by Mondello et al. [3] who on a biochemical level proved that glutamine administration strongly decreased the degree of polymorphonuclear leukocyte (PMNL) infiltration (determined as increase in MPO activity) in inflamed ileum. Similar results on morphological level were obtained in our experiment. MPO immunooexpression was significantly decreased in control group compared to IR1 group. Significant increase of MPO immunoreactivity was observed in IR1 compared to D + IR1. The fact that MPO as a marker or indicator of active and acute inflammatory process was underlined in many studies where MPO decrease was observed after using some protective, anti-inflammatory or antioxidant substances [29–32]. Similar pattern in glutamine protection was observed by Yeh et al. [33] who have proved glutamine suppressed MPO levels after gut derived-sepsis. The IR study indicates that glutamine may attenuate interactions between PMNL and vascular endothelium, and thus may decrease neutrophil infiltration into tissues. Therefore MPO content depression may result to decrease in tissue damage as well as suppression of inflammatory processes in post ischemic tissues. According to Coeffier et al. [34] glutamine has favorable effects on cytokine response in human intestinal mucosa by reduction in pro-inflammatory cytokine production (IL-6 and IL-8), and stimulation of the anti-inflammatory cytokine production (IL-10).

Table 1

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>IR1</th>
<th>D + IR1</th>
<th>IR24</th>
<th>D + IR24</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histopathological index of injury</td>
<td>4.5 ± 0.2 **</td>
<td>2.9 ± 0.23 **</td>
<td>0.75 ± 0.15</td>
<td>0.42 ± 0.1</td>
<td>0.31 ± 0.1</td>
</tr>
<tr>
<td>GCs in epithelial layer</td>
<td>16.84 ± 1.4 **</td>
<td>26.27 ± 1.5 **</td>
<td>29.11 ± 1.31</td>
<td>31.4 ± 0.63</td>
<td>28.6 ± 0.26</td>
</tr>
</tbody>
</table>

(*** p < 0.001 D + IR1 vs. IR1; IR1 vs. C; values are mean ± SEM).

Table 2

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>IR1</th>
<th>D + IR1</th>
<th>IR24</th>
<th>D + IR24</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA in lamina propria</td>
<td>8.05 ± 0.55 ***</td>
<td>11.73 ± 0.9 **</td>
<td>11.92 ± 0.59</td>
<td>13.6 ± 0.75</td>
<td>11.27 ± 0.75</td>
</tr>
<tr>
<td>MPO in lamina propria</td>
<td>8.46 ± 0.57 ***</td>
<td>12.5 ± 0.46</td>
<td>13.85 ± 0.46 **</td>
<td>11.09 ± 0.45 **</td>
<td>14.06 ± 0.52 ***</td>
</tr>
<tr>
<td>PCNA in epithelial layer</td>
<td>8.65 ± 1.17 ***</td>
<td>8.53 ± 0.73 ***</td>
<td>11.26 ± 0.57</td>
<td>13.72 ± 0.81</td>
<td>13 ± 1.47</td>
</tr>
</tbody>
</table>

(*** p < 0.001 IR1 vs. D + IR1, IR24, D + IR24; C vs. IR1; †† p < 0.01 IR24, D vs. D + IR24; † p < 0.01 IR1 vs. IR24, D + IR24; ††† p < 0.01 D vs. IR1; † p < 0.05 C vs. IR1; †††† p < 0.01 IR1 vs. C; D vs. IR1, vs. C; D + IR1 vs. D + IR24; † p < 0.05; IR1 vs. IR24; values are mean ± SEM).
Acute intestinal IR injury widely affects regeneration of tissue and proliferation of different cells in intestinal epithelium and connective tissue layer. In our study we showed that ala-gln dipeptide stimulates proliferation of cells within lamina propria. Results of Sukhotnik et al. [35] indicated that glutamine supplementation may positively stimulate intestinal stem cells for proliferation using anti-BRDU as a marker of proliferation. It was found that glutamine in small intestine can play a role as substrate for intestinal cell proliferation but also as a stimulator of intestinal stem cells for proliferation using anti-BRDU as a marker of proliferation. In our study we showed that ala-gln dipeptide stimulates proliferation of the intestinal crypt cells but longer time period results indicate that ala-gln dipeptide may stimulate and modulate regenerative ability of intestinal epithelium was confirmed by distinct increase in cell proliferation in intestinal crypt cells after 24 h of reperfusion compared to untreated experimental group which may indicate positive impact of glutamine on cell proliferation within mucosal connective tissue. In jejunal epithelium we observed significant decrease in PCNA-positivity in IR1 in D + IR1 group compared to C group. High regenerative ability of intestinal epithelium was confirmed by distinct significant increase in PCNA-immunoreactivity after 24 h of reperfusion both in IR24 as well as D + IR24 groups. Our experimental results indicate that ala-gln dipeptide may stimulate and modulate proliferation of the intestinal crypt cells but longer time period somewhere between 1 and 24 h of reperfusion is necessary for induction of pathways. Sukhotnik et al. [35] stated that after glutamine administration intestinal villus height and crypt depth increased suggesting increased absorptive surface area and strong increase in the proliferation of crypt cells. The enterocytes consume large amount of glutamine and it was proved to be essential for enterocyte proliferative response [37]. Further studies are needed to define the molecular mechanisms by which ala-gln mediates these cellular and tissue responses of critical importance to intestinal homeostasis and repair.

Our results may suggest that application of ala-gln dipeptide prior to IR insult of small intestine may reduce histopathological damage of tissue, overall MPO-immunoexpression in acute inflammatory cell population and significant increase in cell proliferation in intestinal crypt and lamina propria. All those aspects of glutamine action may help to stabilize and therefore reduce IR injury of small intestine.

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