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Impact of alanyl-glutamine dipeptide on proliferative and inflammatory changes in jejunal mucosa after acute mesenteric ischemia

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ABSTRACT

Purpose: The aim of our study was to determinate the impact of dipeptide (alanyl-glutamine) administration on inflammatory and proliferative changes in jejunal mucosa after acute mesenteric ischemia.
Methods: Male Wistar rats (n = 30) were divided into three groups: ischemia/reperfusion (IR) group which undergoes 60 min of mesenteric ischemia and 1 or 24 h of reperfusion (IR1, IR24, n = 12). Groups with dipeptide administration (D + IR1, D + IR24, Dipeptiven con inf., i.v., 0.75 g/kg) prior to IR injury were followed by 1 and 24 h of reperfusion. At the end of reperfusion period jejunal bioptic samples were obtained for histological (H&E), histochemical (Alcian blue) and immunohistochemical (anti-PCNA, anti-MPO) evaluations.
Results: Our results pointed out a significant ($p < 0.001$) increase of histopathological injury score in IR1 group compared to D + IR1 group. Immunohistochemical evaluation showed that MPO-positivity was significantly increased in IR groups after 1 ($p < 0.001$) as well as 24 h of reperfusion ($p < 0.01$) compared to dipeptide pretreated groups. Proliferative/reparatory rate was assessed using anti-PCNA antibody and showed a significant increase ($p < 0.01$) in PCNA cell positivity in lamina propria in dipeptide treated group compared to IR group.
Conclusion: In conclusion we may suggest that administration of alanyl-glutamine dipeptide prior to IR injury may help to protect small intestine and its mucous membrane integrity against insult such as intestinal ischemic/reperfusion injury presents.

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

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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 bioptic 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., Riemser 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, Praque-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% H_2O_2 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 appropriated 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.