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RAPID COMMUNICATION

Enteral glutamine pretreatment does not decrease plasma endotoxin level induced by ischemia-reperfusion injury in rats

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Abstract

AIM: To investigate whether oral glutamine pretreatment prevents impairment of intestinal mucosal integrity during ischemia-reperfusion (I/R) in rats.

METHODS: The study was performed as two series with 40 rats in each. Each series of animals was divided into four groups. The first group was used as a control. Animals in the second group were only pretreated with oral glutamine, 1 g/kg for 4 d. The third group received a normal diet, and underwent intestinal I/R, while the fourth group was pretreated with oral glutamine in the same way, and underwent intestinal I/R. Intestinal mucosal permeability to ^{51}Cr-labeled EDTA was measured in urine in the first series of animals. In the second series, histopathological changes in intestinal tissue and plasma endotoxin levels were evaluated.

RESULTS: Intestinal I/R produced a significant increase in intestinal permeability, plasma endotoxin level and worsened histopathological alterations. After intestinal I/R, permeability was significantly lower in glutamine-treated rats compared to those which received a normal diet. However, no significant change was observed in plasma endotoxin levels or histopathological findings.

CONCLUSION: Although glutamine pretreatment seems to be protective of intestinal integrity, upon I/R injury, such an effect was not observable in the histopathological changes or plasma endotoxin level.

Key words: Endotoxin; Glutamine; Intestinal permeability; Ischemia-reperfusion injury

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INTRODUCTION

Intestinal injury as a result of ischemia and subsequent reperfusion plays an important role in a variety of clinical conditions such as shock, and in those undergoing cardiovascular surgery[1]. In these situations, the small intestine may suffer ischemia of varying duration. Intestinal ischemia-reperfusion (I/R) disrupts intestinal mucosal integrity[2,3], and causes an increase in intestinal permeability[4,5] and bacterial translocation[6]. Plasma endotoxin level also increases after I/R[6]. All these have been increasingly recognized as potential causes of multi-system organ failure in critically ill patients[7-9].

Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered test substances. Lactulose, various polymers of polyethylene glycol, ^{51}Cr-labeled EDTA and ^{99m}Tc diethylenetriaminepentaacetate are the most commonly used test substances. Test results may be influenced by a change in any of the pre- or post-mucosal factors, apart from intestinal permeability itself[10]. Measurement of intestinal permeability by some test substances may give an idea about some harmful factors such as microorganisms passing through the mucosal barrier, from the intestinal lumen to the systemic circulation. It is thought an increase in plasma endotoxin level is one of the responsible factors which may contribute to the clinical results of intestinal I/R injury[11]. Measurement of plasma endotoxin levels seems to be important in assessing the systemic effect of intestinal I/R.

Glutamine (Gln) is a non-essential amino acid, and the most abundant free amino acid in whole blood and the

intracellular amino acid pool[12]. It is an important respiratory fuel, and nucleotide precursor for the gastrointestinal tract[13]. Gln-supplemented parenteral nutrition protects rats against morphological and functional mucosal injury[14], and improves survival in animals after intestinal I/R[15]. Some other experimental studies have also shown that intraluminal injection of Gln protects the mucosa, and diminishes the accumulation of neutrophils in the lamina propria of the small bowel during I/R[16]. Generation of reactive oxygen intermediates (ROI) during reperfusion is thought to be one of the major causes of intestinal mucosal injury[17,18]. Gln is also essential for the synthesis of the intrinsic ROI scavenger glutathione. Therefore, this protective action may be due to augmentation of ROI scavenging in intestinal mucosa[19].

In this study, we aimed to investigate the effects of orogastric Gln pretreatment on intestinal mucosal permeability, plasma endotoxin level and intestinal histopathology during intestinal I/R injury in rats.

MATERIALS AND METHODS

Experimental design

The experiment was performed in female Wistar albino rats weighing 200-230 g. It was approved by the Ankara University Ethical Committee, and was conducted according to the European Community guidelines for the use of experimental animals. Animals were fed with their ordinary diet and allowed to drink water *ad libitum*. Owing to the time difference between urine collection and blood sampling, the study had to be completed in two series of experiments (Table 1).

Table 1 Characteristics of study groups

Groups	Measurement of intestinal permeability by ^{51}Cr-EDTA		Measurement of plasma endotoxin levels and histopathological changes	
	Gln	I/R	Gln	I/R
Ⅰ (Control)	–	–	–	–
Ⅱ (Gln)	+	–	+	–
Ⅲ (I/R)	–	+	–	+
Ⅳ (I/R and Gln)	+	+	+	+

Time-matched, sham-operated animals undergoing laparotomy and dissection of the SMA without occlusion served as controls (group Ⅰ). The Gln group (group Ⅱ) was pretreated with Gln (1 g/kg per day) by the orogastric route for 4 d[20,21]. Gln was prepared in 0.9% NaCl for daily use. Intestinal I/R group (group Ⅲ) underwent 1 h intestinal ischemia, and 1 h reperfusion. In the I/R and Gln group (group Ⅳ), I/R periods and Gln administration were the same as in Gln (Ⅱ) and I/R (Ⅲ) groups.

Induction of intestinal I/R

A rat model of transient mesenteric occlusion was used to obtain intestinal I/R. Rats were anesthetized with ketamine (80 mg/kg, im) and xylazine (10 mg/kg, im). Intestinal I/R was induced by 60 min occlusion, followed by 60 min reperfusion[22]. During the 2 h of the surgical procedure, animals were kept at room temperature, and given intraperitoneal fluid as 0.9% NaCl (10 mL/kg). The superior mesenteric artery (SMA) was exposed through a midline abdominal incision, and both this artery and the collateral branches coming from the celiac axis, and the inferior mesenteric artery were occluded with atraumatic vascular clamps (Vascu-Statt Ⅱ original No. 1001-532-3; Scanlan International, St Paul, MN, USA) for 1 h, followed by 1 h reperfusion. Existence of pallor and absence of pulsation ensured mesenteric occlusion during the ischemic period. Recovery of pulsation and pink color were controlled in each animal when the clamps were removed. The existence of intestinal I/R in this model was also confirmed in our laboratory by the appearance of pulses at the marginal arteries (direct vision of mesenteric circulation by microscopy), as well as by fluorescein angiography in preliminary experiments[23].

Measurement of intestinal mucosal permeability

Intestinal mucosal permeability was measured on the basis of urinary radioactivity levels following oral administration of ^{51}Cr-EDTA. ^{51}Cr-EDTA was employed as a well accepted marker of mucosal integrity[24,25]. After 60 min reperfusion, rats were given 5 μCi ^{51}Cr EDTA in 0.5 mL saline solution by the orogastric route. Urine samples were collected in metabolic cages for 6 h following the reperfusion period. During urine collection, animals did not receive any food; however, they were allowed to access tap water. The level of radioactivity in the urine samples of 500 μL was then determined by counting on a gamma counter (DPC Gambyt CR, Los Angeles. USA). The amount of ^{51}Cr-EDTA excreted in urine during 6 h was calculated as a percentage of the ingested dose.

Measurement of plasma endotoxin level

Plasma endotoxin level was measured by the colorimetric Limulus amebocyte lysate (LAL) test. The test was performed by using the Pyrochrome test kit (Pyroquant Diagnostik, Mörfelden, Germany). All glassware, solutions and surgical instruments used in the experiment were autoclaved at 121℃ for 15 min. The non-pyrogenicity of solutions was tested using the LAL test (Charles River Endosafe, Charleston, SC, USA).

Venous blood samples (3-4 mL) were collected using heparin-coated pyrogen-free disposable syringes. Platelet-rich plasma (PRP) was prepared from the blood by centrifugation at 150 g for 10 min. Fifty microliters of PRP was transferred into a polystyrene plastic tube and kept frozen at -80℃ until the assay. Frozen PRP samples were kept at room temperature for about 30 min before the assay. Fifty microliters of 0.18 mol/L NaOH was added to 50 μL PRP, and incubated at 37℃ for 5 min. Next, 50 μL 0.32 mol/L perchloric acid was added, and incubated at 37℃ for a further 10 min. To dissolve the formed precipitate, 100 μL 0.18 mol/L NaOH was added, and vortexed. Twenty-five microliters of the solution was transferred into sterile non-pyrogenic microplates (Pyroquant Diagnostik), and 25 μL 0.2 mol/L Tris/HCl buffer (pH 8.0) was added to the wells[26]. Finally, 50 μL pyrochrome test solution was added to all wells, and mixed for 30 s. Plates were incubated at 37℃. Optical density was read at 405 nm. Standard curves from 0.04 to 1.28 EU/mL were used to evaluate the concentration of endotoxin.

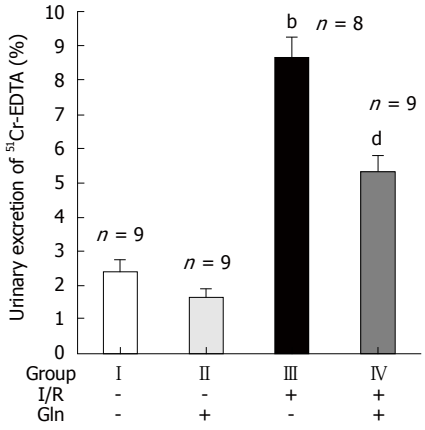

Figure 1 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on intestinal permeability during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in intestinal permeability were measured by urinary excretion of ^{51}Cr-EDTA after its oral administration in rats. Data are expressed as means ± SEM. $^{b}P < 0.001$ *vs* group Ⅰ, Ⅱ and Ⅳ; $^{d}P < 0.001$, *vs* groups Ⅰ, Ⅱ and Ⅲ.

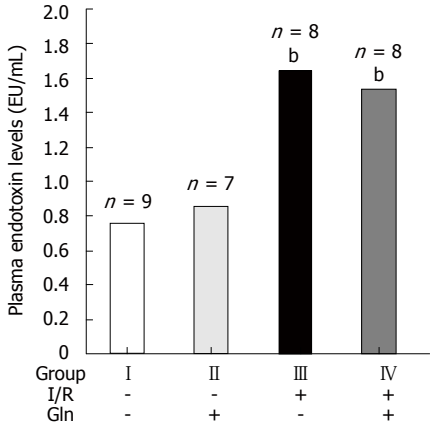

Figure 2 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on plasma endotoxin levels during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in plasma endotoxin levels were measured by LAL test. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

Results were calculated by using non-linear regression of a four-parameter logistic model.

Histopathological assessment of ileal tissues

After the collection of blood samples, ileal tissue samples, 10 cm proximal to the cecum, were harvested and evaluated for histopathological changes. Sections were stained with hematoxylin and eosin, and were examined by light microscopy by two pathologists in a blinded manner. Mucosal injury was scored on a scale from 0 to 5, as described by Chiu *et al*[2].

Statistical analysis

The SPSS program was used for statistical analysis. Comparison of the various protocols on the changes in intestinal permeability was made by one-way analysis of variance (ANOVA) following a Bonferroni post-hoc test. Changes in plasma endotoxin levels, and intestinal histopathology were determined using a Kruskal-Wallis test following a multiple comparison post-hoc test[27]. Data on changes in intestinal permeability were presented as means ± SEM. Data of plasma endotoxin levels, and intestinal histopathology were presented as medians. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Gln pretreatment on intestinal permeability during I/R injury

To investigate the effect of intestinal I/R and Gln pretreatment on intestinal permeability, renal clearance of ^{51}Cr-EDTA was assessed. Statistically significant differences were detected in intestinal permeability in the I/R group when compared to the control group (8.6% ± 1.7% *vs* 2.4% ± 1.1%, $P < 0.001$). Gln pretreatment significantly lowered the increased intestinal permeability due to intestinal I/R (8.6% ± 1.7% *vs* 5.3% ± 1.3%, $P < 0.001$). There was no statistically significant difference in intestinal permeability between the control and Gln groups (Figure 1).

Effect of Gln pretreatment on plasma endotoxin level during I/R injury

In the second series of experiments, plasma endotoxin level was evaluated after intestinal I/R injury. There was a statistically significant difference in plasma endotoxin level between the control (group Ⅰ), and intestinal I/R injury (group Ⅲ) groups (0.76 EU/mL *vs* 1.64 EU/mL, $P < 0.001$). Enteral Gln pretreatment lowered the plasma endotoxin level (group Ⅳ), which was increased due to intestinal I/R injury (group Ⅲ) (1.54 EU/mL *vs* 1.64 EU/mL, $P = 0.48$). However, this decrease failed to reach statistical significance. There was no significant difference in plasma endotoxin levels between the control (group Ⅰ), and Gln (group Ⅱ) groups (0.76 EU/mL *vs* 0.86 EU/mL, $P = 0.59$) (Figure 2).

Effect of Gln pretreatment on intestinal histopathological changes during I/R injury

Intestinal histopathological changes were also evaluated in the second series of experiments. There was significant intestinal injury due to intestinal I/R when the control group was compared to the I/R group (0 *vs* 3, $P < 0.001$). Gln pretreatment did not significantly decrease intestinal injury compared to the I/R group (3 *vs* 2, $P = 0.389$). There was no significant difference in histopathological changes between the control and Gln groups (Figure 3).

DISCUSSION

Ischemia and subsequent reperfusion is one of the major causes of cell injury. The mechanisms of I/R injury are complex, and are likely to differ with respect to the duration of ischemia, the specific tissue involved, and the species studied[3,28]. The small intestine may experience ischemia and reperfusion during septic shock[29], hemorrhagic shock[30] or cardio-vascular surgery[31,32]. These clinical situations can result in some serious postoperative complications such as delay in anastomotic healing[33]. Intestinal mucosa is known to be sensitive to I/R injury. Its basal high rate of oxygen use renders the intestine relatively incapable of increasing oxygen transport in cases of hypoxic stress, and thus is

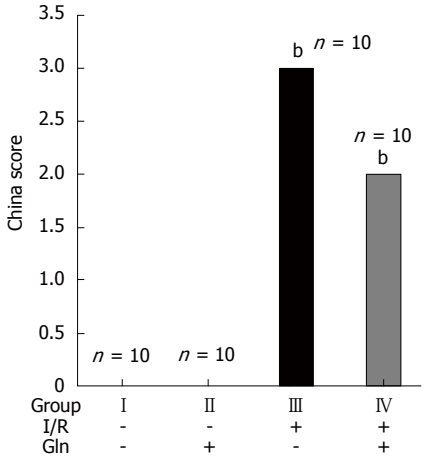

Figure 3 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on histopathological changes during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Histopathological changes were scored by the "Chiu" intestinal ischemia scoring system. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

more susceptible to ischemic injury. Intestinal ischemia predisposes the gut to subsequent necrosis. Restoration of blood flow and reintroduction of oxygen after deprivation accelerates tissue injury[34]. Reperfusion of ischemic intestine may lead to more severe functional and morphological changes than the injury produced by ischemia itself[3]. Oxygen free radicals are thought to be generated during the reperfusion phase, with the addition of oxygen to previously ischemic tissues causing the formation of reactive oxygen species[35].

Intestinal I/R disrupts the functional intestinal mucosal barrier, which results in an increase in mucosal permeability to ^{51}Cr-EDTA[5]. The results of the present study also demonstrated a significant increase in intestinal permeability to ^{51}Cr-EDTA, due to intestinal I/R injury (Figure 1). An increase in mucosal permeability also promotes bacterial translocation[36]. Bacteria colonizing the gastrointestinal tract can translocate from the intestinal lumen to the bloodstream, and this may cause both systemic infection and/or infections in distant organs, such as mesenteric lymph nodes, spleen and liver[9]. Intestinal I/R injury does not only result in bacterial translocation, but also promotes an increase in plasma endotoxin level[6,37]. Our study also demonstrated a significant increase in plasma endotoxin level parallel to disruption of intestinal tissue in the intestinal I/R group when compared to the control group. Bacterial translocation and increased levels of endotoxin in the circulation may initiate a systemic inflammatory response, and the secretion and activation of inflammatory mediators, including cytokines[38] and metabolites of arachidonic acid[39]. An increase in intestinal permeability, and the deleterious effect of endotoxin in non-steroidal anti-inflammatory drug (NSAID)-induced ulcers has also been observed in experimental NSAID-induced enteropathy[40]. Gut decontamination decreases plasma endotoxin levels, and attenuates the systemic injury in intestinal I/R in rats[11]. A beneficial effect of antibiotics was also observed in NSAID-induced enteropathy[41,42].

The results of our study demonstrated that glutamine pretreatment prevented the increase in intestinal permeability during intestinal I/R, which has been reported previously[6,43]. However, glutamine pretreatment itself did not change the intestinal permeability significantly when compared to that in the control group. It has previously been shown that administration of glutamine-enriched nutritional support protects the bowel from injury due to abdominal irradiation[44], chemotherapy[45] and sepsis[46].

Glutamine pretreatment also caused a decrease in plasma endotoxin level, which did not reach statistical significance in our study. Contrary to our result, Wu *et al* have reported that an increase in plasma endotoxin level due to intestinal I/R can be reduced by Gln-supplemented total parenteral nutrition[6]. However, our study differed from that of Wu *et al* regarding the dose, route and timing of Gln administration. Furthermore, Gln was delivered in a total parenteral nutrition solution containing various amino acids and lipids in the study of Wu *et al*. Entrance of ^{51}Cr-EDTA and endotoxin to the systemic circulation from the intestinal lumen may be driven by different mechanisms, which may also explain differential effects of Gln on the above-mentioned parameters. The possibility that colonic epithelial cells contain specific transport systems for endotoxin has been reported[47]. Intestinal I/R disrupted intestinal tissue significantly. Gln pretreatment did not prevent histopathological disruption. It has also been observed that there is no correlation between histopathological alterations and intestinal permeability during recovery after hemorrhagic shock which is accepted as a model of intestinal I/R[30].

In conclusion, although Gln pretreatment reversed increased intestinal permeability, it did not prevent an increase in plasma endotoxin levels or histopathological alterations in intestinal I/R. Further studies are necessary to clarify the effects of different doses and administration periods of Gln on plasma endotoxin levels and histopathological changes in intestinal I/R.

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COMMENTS

Background

Impairment of microcirculation during I/R in the gastrointestinal tract may diminish intestinal mucosal integrity and cause an increase in intestinal permeability. Increased plasma endotoxin levels after I/R are a major threat to many surgical patients. Different Gln treatments are known to have protective effects on mucosal integrity in intestinal I/R.

Research frontiers

Injury to the intestinal barrier results in an increase in permeability to intraluminal substances. Correlation between morphological alterations and the degree of increased intestinal permeability is uncertain. Recent studies have demonstrated that morphological alterations after intestinal mucosal injury cannot reflect the function of the intestinal barrier.

Innovations and breakthroughs

Recent studies suggest that gastrointestinal epithelial cells contain specific transport systems for lipopolysaccharides.

Applications

Intestinal permeability can be measured by many different *in vitro* and *in vivo* methods. Endotoxin passing through the intestinal mucosa into the circulation indicates loss of the intestinal barrier function. Measurement of plasma endotoxin level ensures the assessment of real pathogenic factors that arise in the systemic circulation as a result of intestinal mucosal injury.

Terminology

Bacterial translocation: indigenous bacteria that colonize the gastrointestinal tract can cross the epithelial mucosa to infect distant organs. Under normal conditions, the epithelial lining prevents the escape of these bacteria from the gut lumen. Intestinal permeability: relates to the properties and function of the epithelial barrier that enables unmediated passage of substances through the intestinal mucosa. The use of intestinal permeability tests for screening of intestinal disease and assessment of treatment efficacy, to understand the normal intestinal physiology and pathogenesis of disease, is described and reviewed. There is now a need for research into the basic mechanisms of regulatory control of the intestinal barrier function.

Peer review

Peer reviewers considered this to be a very interesting paper with a great deal of potential clinical benefit. These basic results may be of value in clinical applications in organ or cellular transplantion.

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