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Glutathione and Nitric Oxide Concentrations in Glutamine-Infused Rabbits with Intestinal Ischaemia/Reperfusion

Mahmut Başoğlu[1], *İlhan Yıldırgan*[1], *Fatih Akçay*[2], *Ahmet Kızıltunç*[2], *İbrahim Kavak*[1] and *Durkaya Ören*[1]

[1] Department of General Surgery, School of Medicine, Atatürk University Erzurum, Turkey
[2] Department of Biochemistry, School of Medicine, Atatürk University Erzurum, Turkey

Summary: Intestinal ischaemia/reperfusion causes formation of reactive oxygen intermediates which lead to mucosal cell injury. Glutathione, a scavenger of reactive oxygen intermediates, protects tissues from reactive oxygen intermediate-mediated cell injury. Nitric oxide is a lipophilic gas and its synthesis is stimulated by ischaemic conditions. In this experimental study, we aimed to investigate the role of i. v. *L*-glutamine infusion on mucosal tissue glutathione and serum nitric oxide concentrations in intestinal ischaemia/reperfusion. External jugular vein of albino rabbits was cannulated with catheter and infused with normal saline at 4 ml/h. After 3 days, they were randomly divided into two main groups. Group 1 (n = 30) received i. v. normal saline alone, group 2 (n = 30) received normal saline + 205 mmol/l glutamine at 4 ml/h for 24 hours. Next, mucosal glutathione and serum nitric oxide concentrations were measured after 0, 30, 60 min of ischaemia/60 min of reperfusion. Basal glutathione concentrations were similar in normal saline alone and normal saline + 205 mmol/l glutamine infusion groups ($p > 0.05$). At 30 and 60 min of ischaemia/60 min of reperfusion, glutathione concentrations were significantly lower in normal saline-infused rabbits compared to the normal saline + 205 mmol/l glutamine-infused rabbits ($p < 0.05$). In addition, serum nitric oxide concentrations were found to be significantly increased in rabbits 30 and 60 min after ischaemia/reperfusion when compared to mean basal nitric oxide concentrations obtained from control animals. However, the normal saline + 205 mmol/l glutamine group had lower serum nitric oxide concentrations than did the normal saline alone group. In conclusion, this study revealed that intestinal mucosal glutathione concentrations were significantly higher in glutamine-receiving rabbits than in non-receiving ones. Additionally, it was shown that nitric oxide concentrations increased in ischaemia both in normal saline alone and normal saline + 205 mmol/l glutamine receiving groups, while this increase in nitric oxide was more prominent in the normal saline alone group ($p < 0.01$). These findings show that glutamine supplementation may protect the small intestine from ischaemia/reperfusion injury and may play a regulatory role in the biosynthesis of nitric oxide.

Introduction

Multiple organ failure is still the major cause of mortality in surgical patients and dysfunction is a frequent occurence in the cascade of events leading to death (1). Bowel injury due to acute intestinal ischaemia continues to be a significant problem. Mortality in a patient with this problem approaches 80%, and even when bowel ischaemia is reversible, death frequently occurs due to multisystem organ failure (2). After prolonged bowel ischaemia, cell membrane lipid peroxidation secondary to oxygen-free radical injury occurs and may lead to cell death (3, 4).

Intestinal tissue hypoxia is generally accepted to play the key role in the pathogenesis of mucosal lesions, and in local intestinal ischaemia models demonstrated significant tissue damage that might occur after restitution of the intestinal blood flow (4–6). There is much evidence that oxygen-derived free radicals contribute to the cellular damage induced by ischaemia, and that exogenously added scavengers might be protective against post-ischaemic deterioration (7).

Glutathione is essential for the protection of endothelial cells and gastric chief cells against oxidative stress. Glutathione also has a major role in restoring other free radical scavengers and antioxidants such as vitamins E and C to their reduced state (8). The source of oxygen-free radicals in the ischaemic small intestine is the enzyme xanthine oxidase (9, 10). However, the superoxide anion and hydrogen peroxide formed can be degraded by superoxide dismutase and catalase, respectively, resulting in improved morphologic, anatomic, and cytologic alterations (9, 11–13).

Nutritional support specific for the bowel (e. g. glutamine) has been shown previously to protect the bowel from injury due to abdominal irradiation, chemotherapy, and sepsis (14, 15). Gut *L*-glutamine requirements are increased during critical illness, and *L*-glutamine may be essential for the maintenance of gut metabolism,

structure, and function (15). *L*-Glutamine is the principle fuel used by the gastrointestinal tract, with most of the uptake occurring in small intestinal epithelial cells that line the villi (16). *L*-glutamine is necessary for the synthesis of glutathione (2).

Nitric oxide is generated from *L*-arginine by the action of the enzyme nitric oxide synthase in vascular and neural tissues of many organs (17, 18). It has a role in vasodilation and platelet aggregation and acts as an effector molecule in mediating cytotoxicity (6, 17). Nitric oxide is a lipophilic gas (17) and its synthesis is stimulated by endotoxins and ischaemic conditions (19, 20). Although several studies have shown nitric oxide to be protective by acting as a vasodilator to prevent long-term ischaemia, some studies have suggested that nitric oxide could contribute to ischaemia/reperfusion injury (21). While nitric oxide, since it is physiologically a vasodilator, has a role in protecting tissue in ischaemic events, and since it is chemically a free radical, it may occasionally be harmful to tissue by producing secondary toxic products. In low tissue O_2 saturation conditions, nitric oxide reduces ischaemic damage by increasing blood flow secondary to vasodilating effect. In high tissue O_2 saturation conditions, it leads toxic metabolites to a cell by reacting superoxide and forming some toxic substances such as peroxynitrite (22).

The aim of this study was to investigate serum nitric oxide concentrations and mucosal glutathione content of ischaemic bowel, and whether glutamine prevents possible tissue damage produced by ischaemia/reperfusion.

Materials and Methods

This study included 60 adult male albino rabbits whose weights ranged from 1.5 to 3 kg. The rabbits were anaesthetized with ketamine hydrochloride injected intraperitoneally (10 mg/kg weight). After anaesthesia, the external jugular vein was cannulated with a catheter that was tunnelled subcutaneously, brought through the skin posteriorly between the scapulas, passed through a stainless steel catheter guard, and attached to a swivel on a runner above the animal cage. Through the catheters, normal saline at 4 ml/h was infused using a microinfusion pump. Following recovery, rabbits were allowed to drink water and to eat a regular diet. After 3 days, they were randomly divided into two main groups. Group 1 (n = 30) received i. v. normal saline alone, group 2 (n = 30) received normal saline + 3% (205 mmol/l) glutamine at 4 ml/h for 24 hours. Both of the groups were again divided into 3 subgroups, each containing 10 rabbits (10 exposed to 0, 10 to 30 and 10 to 60 min of ischaemia). The rabbits that underwent a sham-operation were taken as the control group.

After that, each animal was reanaesthetized and underwent midline abdominal opening. Next, the superior mesenteric artery was exposed. After a half-hour wait, for the production of intestinal ischaemia, the superior mesenteric artery was acutely occluded with a vascular clamp for 0, 30 or 60 min. In each animal, the ischaemic period was followed by 60 minutes of intestinal reperfusion. Then, the small intestine was excised and opened longitudinally scarping with a spatula. Following small bowel resection the animals were sacrified until which i. v. infusion of normal saline or normal saline + 205 mmol/l glutamine was continued. Blood samples for nitric oxide measurement were taken after the ischaemia/reperfusion period, (at 0, 30 and 60 min) and centrifuged at 2500 *g* for 10 minutes. Both mucosa and serum samples were frozen and stored at −70 °C until analyzed.

Analytical procedures

Total glutathione concentrations in the small intestine were assessed according to the method of *Griffith* (23). Briefly, 0.5 g of sample taken from small intestine was homogenized in a dilution reagent containing triton X-100 (volume fraction 0.05) and 1 mmol/l EDTA. After centrifugation at 10 000 *g* for 10 min at 4 °C, 400 µl of supernatant, 700 µl 0.3 mmol/l NADPH, 100 µl 6 mmol/l 5,5'-dithiobis (2-nitrobenzoic acid) and 500 µl buffer (0.2 mol/l sodium phosphate plus 10 mmol/l EDTA, pH: 7.5) were mixed carefully in a cuvette. Then 10 µl of glutathione reductase were added and incubated 10 min at room temperature. The absorbance of colour developed was detected at 412 nm. The results were presented as µmol/g wet tissue. Tests were performed in duplicate.

For the determination of nitric oxide concentrations, the serum samples obtained were deproteinized with sulphosalicylic acid (1.377 mol/l). Then, nitrate (NO_3^-) in a supernatant was reduced by cadmium column (100 mesh) to nitrite (NO_2^-) and the concentration of NO_2^- was determined with the *Griess* reaction (24). In this reaction, the *Griess* reagent consisted of one part 0.004 mol/l naphthylenediamine dihydrochloride and one part 0.057 mol/l sulphanilamide in 0.131 mol/l phosphoric acid, mixed together and kept chilled. After incubation in a waterbath at 60 °C, the colour of the product dye was developed and its absorbance was detected at 560 nm using a spectrophotometer (Shimadzu, Japan). Each sample was studied in duplicate. Standards of sodium nitrite and sodium nitrate from 10 to 60 µmol/l.

Statistical analysis

Data are represented as mean ± standard deviation. Statistical comparisons were performed using the ANOVA and linear regression analysis. Statistical significance was defined with a $p < 0.05$.

Results

Tissue glutathione concentrations were similar in the normal saline alone and in normal saline + 205 mmol/l glutamine infusion groups that did not undergo ischaemia/reperfusion injury ($p > 0.05$). In animals receiving normal saline alone and normal saline + 205 mmol/l glutamine exposed to 30 and 60 min of ischaemia, both followed by 60 min of reperfusion, tissue glutathione concentrations were found to be lower compared to the controls. In addition, tissue glutathione concentrations were significantly higher in normal saline + 205 mmol/l glutamine than in the normal saline alone group at 30 and 60 min of ischaemia/reperfusion (tab. 1).

For serum nitric oxide values, there was no difference between the animals not exposed to ischaemia in either normal saline alone or normal saline + 205 mmol/l glutamine groups ($p > 0.05$). In both groups serum nitric oxide levels were found to be significantly increased in rabbits that underwent 30 and 60 min of ischaemia when compared to mean basal nitric oxide concentrations obtained from control animals. However, the normal saline + 205 mmol/l glutamine group had lower serum nitric oxide concentrations than did the normal saline alone group (tab. 2).

Tab. 1 Mucosal tissue glutathione concentrations (μmol/g wet weight)

	Duration of ischaemia		
	0	30	60
Normal saline	4.16 ± 0.64[a]	2.86 ± 0.62[c]	2.73 ± 0.38[e]
Normal saline + 205 mmol/l glutamine	4.47 ± 0.64[b]	3.49 ± 0.32[d]	3.15 ± 0.20[f]

$p < 0.001$: a vs c, a vs e, b vs f; $p < 0.01$: b vs d; $p < 0.05$: e vs d, e vs f

Tab. 2 Serum nitric oxide concentrations (μmol/l)

	Duration of ischaemia		
	0	30	60
Normal saline	23.60 ± 5.71[a]	38.90 ± 4.97[c]	39.60 ± 5.98[e]
Normal saline + 205 mmol/l glutamine	23.30 ± 5.47[b]	31.40 ± 4.62[d]	30.80 ± 5.47[f]

$p < 0.001$: a vs c, a vs e; $p < 0.01$: b vs d, b vs f, c vs d, e vs f

In each group, glutathione and nitric oxide values at 30 and 60 min of ischaemia were not statistically different ($p > 0.05$). On correlation analysis, no significant relation was detected between nitric oxide and glutathione values in any group.

Discussion

Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine and under various experimental conditions, the glutamate portion of the molecule is derived from glutamine (10). *L*-glutamine is a major antioxidant and a vital component of host defences (6, 10). The small intestine extracts approximately 25% of circulating *L*-glutamine (16). The bonding of glutamate with cysteine is the rate-limiting step in the synthesis of glutathione, and it has been shown that depletion of glutamate leads directly to a decrease in cell glutathione content, suggesting that an adequate supply of *L*-glutamine is important for the synthesis of glutathione (2).

Total glutathione concentrations are known to progressively decrease with increasing duration of ischaemia (8). While during reperfusion, total glutathione concentrations slowly increase back toward baseline concentrations. In addition, it has been found that the severity of ischaemia/reperfusion cell injury is inversely proportional to the cell's pre-ischaemia total glutathione content (2, 7, 8). In *Stein* (8) and *Hirota* et al.'s (25) studies, they claimed that pretreatment with glutamine did not increase mucosal glutathione levels in the control group but protected against the ischaemia/reperfusion-induced drop in endogenous glutathione, which paralleled the development of mucosal injury in glutathione-depleted animals. These reports strongly support the important protective function of glutathione during reperfusion of ischaemic tissue.

Nutritional supplementation of *L*-glutamine can assist in protecting the small bowel during a variety of pathologic states (2). Glutamine is the main fuel used by the intestinal tract (16) but is absent from currently available amino acid solutions and many enteral diets (26). Oral administration of glutamine increases the glutathione concentrations of jejunal and colon mucosa and protects these tissue against *L*-buthionine-sulphoximine induced glutathione. This suggests that oral administration might be of therapeutic value in inflammatory disease, ischaemia, oxidative damage, chemotherapy, and radiation (26). Addition of alanyl glutamine dipeptide to the total parenteral nutrition solution improves intestinal *L*-glutamine metabolism and prevents mucosal atrophy and deterioration of permeability. In experimental animal models, the addition of free *L*-glutamine and glutamine dipeptide to total parenteral nutrition solution demonstrated a beneficial effect on intestinal morphology (16).

The etiology of bowel wall protection provided by glutamine is unclear, and may be due to maintenance of the cell antioxidant defence system. Ionizing radiation and some chemotherapeutic agents injure cells by increasing tissue oxidant activity. These data suggest that glutamine provides protection during oxidant injury by helping to maintain cell glutathione concentrations. *Ziegler* et al. (27) reported that *L*-glutamine supplementation improved nutritional status and shortened the hospital stay of patients with bone marrow transplantation. *Harward* et al. (2) studied intestinal ischaemia/reperfusion after *L*-glutamine supplementation and found an increased intestinal glutathione content. We also detected that intestinal mucosal glutathione concentrations were found to be higher in the normal saline + 206 mmol/l glutamine group than in the normal saline alone group at 30 and 60 min of ischemia/reperfusion, so glutamate concentration increased and that led to increased glutathione concen-

trations in the normal saline + 205 mmol/l glutamine infused rabbits when compared to the normal saline alone group. Glutathione concentrations both at 30 and 60 minutes of ischaemia/reperfusion were found to be lower in normal saline alone and normal saline + 205 mmol/l glutamine infused groups when compared to control groups. This finding was in agreement with the studies mentioned above. Glutathione concentrations increased to minimize the damage of increased free radicals produced during ischaemia/reperfusion. *L*-Glutamine, which is more easily transportable across cell membranes than glutamate, serves as the intracellular source of glutamate. *L*-Glutamine is deaminated in the mitochondria to glutamate and NH_3. Glutamate is then transported back to the cytosol where it is readily available for glutathione synthesis (10).

Studies have implicated nitric oxide as a mediator, messenger, or regulator of cell function in physiologic states that include vascular tone, platelet function, septic shock (19). *Masuda* et al. (28) have demonstrated that endogenous nitric oxide may be an essential protective factor in the pathogenesis of ethanol-induced gastric mucosal injury through improved mucosal haemodynamics. There is a alternative explanation of the protective mechanism of nitric oxide – may function as a chemical barrier to cytotoxic free radicals because nitric oxide could be a natural extracellular scavenger of O_2^-. It seems that superoxide radicals, possibly from endothelial cells themselves and/or *Kupffer* cells, contribute to the endothelial cell injury (6). The nitric oxide radical is increasingly recognized as an important intra- and intercellular messenger that is identical to endothelium-derived relaxing factor and is the principal signal for relaxation of vascular smooth muscle cells. A role for nitric oxide has been implicated in a variety of biological processes such as neutrotransmission, tumour cell killing, immunity, and inflammatory processes. Both cytoprotective and cytotoxic effects of nitric oxide have been reported (29). Nitric oxide is important in the regulation of mucosal blood flow, acid secretion and mucus secretion and contributes to mucosal protection of the stomach (29). *Takeuchi* et al. (30) showed that the inhibition of endogenous nitric oxide production stimulates duodenal HCO_3^- secretion and rather protects the duodenal mucosa from damage caused by the duodenal ulcerogen, mepirizole.

In the present study, serum nitric oxide concentrations both at 30 and 60 minutes of ischaemia/reperfusion were found to be higher in normal saline alone and normal saline + 205 mmol/l glutamine infused groups when compared to controls. However, serum nitric oxide values of the normal saline + 205 mmol/l glutamine group were lower than those of the normal saline alone group. According to *Sessa* et al., this decrease shows that *L*-glutamine is the inhibitory principle present in culture medium that prevents the intracellular generation of *L*-arginine and inhibits the release of endothelium-derived relaxing factor from cultured endothelial cells (31). In these two groups, the increase in nitric oxide concentrations may be secondary to the free radicals produced during ischaemia/reperfusion. Decrease in nitric oxide concentrations in the normal saline + 205 mmol/l glutamine group compared to the normal saline alone group may be explained as follows: glutamine infusion may lead to increased production of glutathione that protects the tissue being exposed to ischaemia/reperfusion from oxygen radicals, thus nitric oxide production may be rather less in the glutamine-infused group.

In conclusion, this study revealed that intestinal mucosal glutathione concentrations were significantly higher in glutamine-receiving rabbits than in non-receiving ones. Additionally, it was shown that nitric oxide concentrations increasing in ischaemia. Nitric oxide concentrations were high in the glutamine-receiving group but lower than those of the normal saline group. These findings show that glutamine supplementation may protect the small intestine from ischaemia/reperfusion injury.

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Corresponding author: Dr. Mahmut Başoğlu, Department of General Surgery, School of Medicine, Atatürk University, Erzurum, Turkey