Surgical manipulation of the intestine and distant organ damage—protection by oral glutamine supplementation

Simmy Thomas, MSc, Ramamoorthy Prabhu, MSc, and K. A. Balasubramanian, PhD, Vellore, India

Background. The intestine is increasingly recognized as a primary effector of distant organ damage, such as the lung, after any abdominal surgery. Earlier studies have shown that surgical manipulation of the intestine induces generation of reactive oxygen species in the intestine, resulting in mucosal and lung damage. Because glutamine is preferentially used by the small intestine as an energy source, this study examined the effect of glutamine and glutamic acid on intestinal and lung damage after surgical manipulation.

Methods. Controls and rats were pretreated for 7 days with 2% glutamine or glutamic acid, or the isonitrogenous amino acids glycine or alanine in the diet and subjected to surgical manipulation of the intestine. The intestine and lung were assessed for damage, and protection offered by various amino acids was studied.

Results. Surgical manipulation resulted in oxidative stress in the intestine as evidenced by increased xanthine oxidase activity and decreased antioxidant status. Enterocyte mitochondria were also functionally impaired with altered calcium flux, decreased respiratory control ratio, and increased swelling. Gut manipulation also resulted in neutrophil infiltration and oxidative stress in the lung as assessed by an increase in myeloperoxidase activity, lipid peroxidation, and antioxidant status. Glutamine or glutamic acid supplementation for 7 days before surgical manipulation showed a protective effect against the intestinal and lung damage.

Conclusions. This study suggests that preoperative enteral glutamine or glutamic acid supplementation attenuates intestinal and lung damage in rats during surgical manipulation and that this effect might offer protection from postsurgical complications. (Surgery 2005;137:48-55.)

From the Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Vellore, India

The normal functions of the gut are digestion and absorption of nutrients, and exclusion of luminal bacteria and their metabolic products. Under certain circumstances the gut may lose its barrier function and serve as the source of systemic infections. Loss of gut barrier function and increased gut permeability have been reported after surgery, and our earlier work has shown that laparotomy and mild intestinal trauma (handling) leads to activation of the superoxide-generating enzyme xanthine oxidase (XO) in enterocytes, resulting in oxidative stress and considerable damage to intestinal mucosal structure and function. Surgical manipulation also affects the structure and function of the intestinal brush border membrane and mitochondria. Oxidative stress induced by reactive oxygen species may be a major cause of the cell and tissue damage. Mesenteric hypoperfusion during severe physiologic stress, such as shock, trauma, or burn injury, is associated with impaired mucosal barrier function, which, in turn, facilitates translocation of bacterial pathogens into the systemic circulation. The small intestine is increasingly recognized as a primary effector of distant organ damage in intestinal ischemia/reperfusion, it is likely that...
gut-derived chemical mediators are transferred to distant organs through the lymph.

Glutamine, traditionally considered a non-essential amino acid, appears to be a conditionally essential nutrient during serious injury or illness. In good health, this is the most abundant amino acid in plasma and skeletal muscle, but circulating and tissue concentrations decrease after injury, surgery, or infection. Glutamine is the preferred fuel for the small intestine, and clinical studies have revealed that both parenteral and enteral glutamine supplementation is beneficial in patients after multiple trauma and surgery. The uptake of glutamine by the cells of the intestine equals the rate of glucose uptake and, as an oxidative fuel for enterocytes, is even more important than glucose. The gut is well suited to metabolize glutamine because the ammonia thus produced diffuses readily into the portal blood and is extracted by the liver before it reaches the portal circulation. Utilization of glutamine by the gut increases after surgery and other stresses, and appears to play a vital role in gut maintenance during critical illness. These clinical studies are supported by experimental data showing that glutamine administration maintains gut barrier function. In view of this, the present study looked at the beneficial effect of oral glutamine and glutamic acid supplementation on the mucosal alterations and lung damage after surgical manipulation of the small intestine.

**MATERIAL AND METHODS**

Nicotinamide adenine dinucleotide; its reduced form (NADH); 1-chloro-2,4-dinitrobenzene; reduced glutathione (GSH); oxidized glutathione (GSSG); 5, 5′-dithio-bis-(2-nitrobenzoic acid); 1, 1′, 3, 3′ tetramethoxy propane; 2-thiobarbituric acid; triton-X100; xanthine; xanthine oxidase; bathocuproine disulphonic acid; 3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide (MTT); dimethyl sulfoxide; and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, Mo). All other chemicals used were of analytic grade.

**Animals.** Adult Wistar rats of both sexes (200-250 g), exposed to a daily 12-hour light-dark cycle and fed water and rat chow ad libitum, were used. The rats were divided randomly into 6 groups (n = 4): group I, glutamine-pretreated sham control; group II, surgical manipulation; group III, glutamine-pretreated surgical manipulation; group IV, glutamic acid-pretreated surgical manipulation; and groups V and VI, glycine- or alanine-pretreated surgical manipulation. This study was approved by the Animal Experimentation Ethics Committee of the institution.

**Amino acid supplementation in rats.** The rats were placed on a powdered rat chow supplemented with 2% glutamine, 2% glutamic acid, 2% glycine, or 2% alanine for 7 days. On the eighth day, a laparotomy and surgical manipulation was performed. One hour later, the animals were sacrificed.

**Surgical manipulation of the small intestine.** Surgical manipulation was carried out as described. Briefly, overnight-fasted rats were anesthetized and the abdominal wall opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileocecal junction identified. The intestine was handled along its entire length from the ileocecal junction proximally, simulating the "inspection" that occurs in a clinical setting. The intestine was then replaced back in the abdominal cavity; the whole process was completed within 1 to 2 minutes. Next the abdominal wall was sutured, and the animals were killed by decapitation 60 minutes after the surgical procedure. Our earlier work has shown that maximum alterations in the intestine occur 60 minutes after surgical manipulation.

**Enterocyte isolation.** Enterocytes were isolated from the different groups of rats by the metal chelation method. Briefly, overnight-fasted rats were washed with Krebs Hensleit (KH) buffer (pH 7.4), filled with the same buffer containing 5 mmol/L EDTA, and incubated at 37°C for 12 minutes in a beaker containing KH buffer alone. After incubation, the intestine was washed with cold KH buffer containing 0.25% albumin and filled with the same buffer. Enterocytes were isolated by gently rubbing the intestine along its entire length. After this maneuver, the isolated cells were centrifuged at 900g for 5 minutes. The cells were washed twice, and the final preparation of enterocytes was suspended in 25 mmol/L phosphate-buffered saline (pH 7.4). Cell viability of the isolated enterocytes was assessed by the dye-exclusion method with trypan blue. Cell homogenates were prepared with the use of a Porter-Elvehjem homogenizer and then used for enzyme activity measurements. Mitochondria were isolated from the homogenate by differential centrifugation as described. The final mitochondrial preparation was suspended in a solution containing 250 mmol/L sucrose and 5 mmol/L HEPES at pH 7.4.

**Preparation of the lung homogenate.** After surgical manipulation of the intestine, the heart and lungs were removed en bloc, and 20 mL sterile saline solution was infused into the right ventricle.
to wash out the residual blood. Both lungs were then separated from the heart and the hilar structures. The lung specimens were weighed and homogenized with the use of phosphate-buffered saline at pH 7.4. The lung homogenate was used to measure lipid peroxidation: The homogenate was centrifuged at 2000g for 4 minutes, and the supernatant was used for various enzyme assays.

**Collection of bronchoalveolar lavage fluid.** The trachea was cannulated, and the lungs were washed 3 times with 2 mL of saline to provide approximately 6 mL of bronchoalveolar lavage fluid (BALF). Protein concentrations were measured in the cell-free BALF obtained after centrifugation. Protein was estimated by Lowry’s method by using bovine serum albumin as the standard.\(^{18}\)

**MTT reduction assay.** An MTT reduction assay was done with the use of a microtiter plate as described.\(^{19}\) The amount of MTT formazan formed was calculated by using the molar extinction coefficient of MTT formazan \(E_{370}\) of 17,000 M\(^{-1}\) cm\(^{-1}\) at pH 7.4 to 8.

**Mitochondrial functional assessment.** Polarographic determination of oxygen uptake by isolated mitochondria was performed with the use of a Clark-type electrode in 2 mL of respiration medium (225 mmol/L sucrose, 5 mmol/L KH\(_2\)PO\(_4\), 20 mmol/L KCl, 10 mmol/L Tris, and 5 mmol/L HEPES at pH 7.4) containing 5 mmol/L succinate as the respiratory substrate. Mitochondrial swelling was determined by absorbance at 540 nm. Calcium flux was measured by quantitating the changes in the absorption spectrum of Arsenazo III at 675/685 nm. Mitochondria were suspended in reaction medium containing 250 mmol/L sucrose, 5 mmol/L HEPES, 5 mmol/L succinate, and 40 \(\mu\)mol/L Arsenazo-III (pH 7.4). A concentration of 10 \(\mu\)mol/L of calcium was added to the reaction medium to initiate the flux studies.\(^{20}\)

**Enzyme assays.** XO activity was measured spectrophotometrically based on the production of uric acid measured at 295 nm.\(^{21}\) Superoxide dismutase activity was measured at 540 nm, after the reduction of the tetrazolium dye MTT by a superoxide generated by the xanthine–xanthine oxidase system.\(^{22}\) Catalase activity was estimated by measuring the change in absorption at 240 nm with the use of H\(_2\)O\(_2\) as the substrate.\(^{23}\) Total glutathione peroxidase activity was determined by the oxidation of NADPH at 340 nm with the use of H\(_2\)O\(_2\).\(^{24}\) The activity of glutathione-s-transferase was measured spectrophotometrically at 340 nm with the use of the substrate 1-chloro-2,4-dinitrobenzene.\(^{25}\) Lung myeloperoxidase (MPO) was measured to quantify the degree of pulmonary neutrophil sequestration. The lung specimens were weighed, placed in potassium phosphate buffer (pH 7.4) with 0.5% hexadecyltrimethyl ammonium bromide, and homogenized. The homogenate was then centrifuged at 2000g for 4 minutes, and the supernatant was used for the MPO assay. The assay procedure consisted of 50 mmol/L phosphate buffer, pH 6, 0.167 mg of o-dianisidine hydrochloride, 0.1 \(\mu\)mol/L hydrogen peroxide, and an aliquot of the enzyme. The rate of decomposition of H\(_2\)O\(_2\) by MPO with o-dianisidine as the H\(_2\) donor is determined by measuring the rate of color development at 460 nm.\(^{26}\)

**Peroxidation parameters.** The total lung homogenate was used for assessment of lipid peroxidation. Malonaldehyde was measured with the thiobarbituric acid method.\(^{27}\) For conjugated diene measurement, total lipids were extracted as described,\(^{28}\) dissolved in 1 mL heptane, and read at 233 nm with the use of a Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated with the molar absorption coefficient of 2.52 \(\times\) 10\(^{4}\) and expressed as micromoles per milligram of protein.\(^{29}\)

**Glutathione estimation by HPLC.** Homogenate proteins were precipitated with trichloroacetic acid; glutathione in the acid supernatant was then quantitated by HPLC after derivatization. The derivatization was based on the initial conversion of S-carboxyl methyl derivatives of free thiols with iodoacetate followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives. Dinitrophenyl derivatives were separated by HPLC on an Ultrasil amino column (Shimadzu, Kyoto, Japan) with a gradient of methanol and sodium acetate, and detected at 365 nm.\(^{30}\)

**Statistical analysis.** Data are expressed as mean \(+\) SD. The Mann Whitney nonparametric tests were used for tests of significance of differences between groups. A probability of less than .05 was accepted as significant. Statistical calculations were performed with the use of SPSS software (version 9; SPSS Inc, Chicago, Ill).

**RESULTS**

Biochemical studies were carried out with the use of isolated enterocytes. Cell viability, as assessed by trypan blue exclusion, showed a decrease in viability after surgical manipulation compared to control (Fig 1, A). Prior treatment with glutamine or glutamic acid restored the cell viability, which was not seen with either glycine or alanine pretreatment. Superoxide anion, the main oxygen-free radical generated in the cell, is known to reduce
MTT, a tetrazolium compound, to its colored formazan. Enteroocytes isolated from rats after surgical manipulation showed an increase in MTT reduction compared to control; this increase was almost completely abolished by oral glutamine or glutamic acid supplementation before surgical manipulation but not by glycine or alanine (Fig 1, B). Superoxide generation during surgical manipulation was further confirmed by XO (source of superoxide) and xanthine dehydrogenase activity. Laparotomy and intestinal handling increased XO activity approximately 2.5 fold, which was associated with a concomitant decrease in xanthine dehydrogenase activity. These alterations were prevented by supplementation with glutamine or glutamic acid, but not by glycine or alanine (Fig 1, C, D).

Because glutamine is a precursor for cellular GSH synthesis, which is an important intracellular, non-enzymatic antioxidant, total cellular GSH was measured (Fig 2, A) after glutamine or glutamic acid feeding and compared with a normal control. Glutamine or glutamic acid feeding alone increased the GSH level in the intestine 3-fold. Surgical manipulation resulted in a decrease in the GSH level; prior treatment with glutamine or glutamic acid maintained the GSH level similar to that of the glutamine control after surgical manipulation.

Catalase and superoxide dismutase activities (Fig 2, B, C) were decreased significantly after surgical manipulation, which was prevented by pretreatment with either glutamine or glutamic acid.

Mitochondrial swelling increased after surgical manipulation, but the swelling was prevented by glutamine or glutamic acid supplementation (Fig 3, A). Studies on mitochondrial respiration showed that glutamine or glutamic acid pretreatment protected against the decrease in respiratory
control ratio after surgical manipulation (Fig 3, B). Enterocyte mitochondria after intestinal manipulation also showed an increase in MTT reduction compared to control; this was almost completely prevented by glutamine or glutamic acid pretreatment (Fig 3, C). Calcium flux studies on mitochondria showed altered flux after surgical manipulation. Pretreatment with glutamine or glutamic acid restored the calcium influx (Fig 4).

MPO activity in the lung was significantly higher after surgical manipulation. Glutamine or glutamic acid pretreatment restored the activity to control levels (Fig 5, A). Lung permeability, as measured by total protein content in the cell-free BALF, was significantly increased after surgical manipulation; glutamine or glutamic acid supplementation restored the protein level to control values (Fig 5, B).

DISCUSSION

The intestine is suggested to be the “motor” for damage to distant organs such as the lung. Our earlier studies have shown that surgical manipulation results in the generation of reactive oxygen species in the intestine leading to functional and biochemical alterations. Surgical manipulation also affects the intestinal mitochondrial structure and function;7 these changes may be the result of hypoperfusion of the intestine. One of the distant organs affected in this condition is the lung; oxidative stress–induced lung damage during surgical manipulation of the intestine has been studied recently.31 Acute lung injury is caused as a result of factors generated in the intestine and transported through the lymph into the systemic circulation. Oxidants are thought to be responsible for much of the cell and tissue damage that occurs in lung injury.32

Fig 3. A representative trace of swelling (A), respiratory control ratio (B), and MTT reduction (C) of enterocyte mitochondria isolated from control, after surgical manipulation, and after various amino acid treatments before surgical manipulation. Each value represents mean ± SD of 4 separate experiments (n = 4). All the assays were carried out as described in Material and Methods. $P < .05$ compared to control; *$P < .05$ compared to glutamine control; #$P < .05$ compared to surgical manipulation.

Fig 4. A representative trace of the calcium flux by rat enterocyte mitochondria from control, after surgical manipulation, and after various amino acid treatments before surgical manipulation. The values are a mean of 4 different experiments (n = 4). Assay was done as mentioned in Material and Methods; 10 μmol/L of calcium was added as indicated in the figure.

Lipid peroxidation (malondialdehyde and conjugated diene) was increased and the antioxidant enzymes (glutathione peroxidase, glutathione-s-transferase, and catalase) were decreased in the lung after surgical manipulation of the intestine compared to control; pretreatment with glutamine or glutamic acid reverted these changes (Fig 5, C, D; Fig 6, A–C). These protective effects on lung were not seen with pretreatment with glycine or alanine. Except for an increase in GSH in the intestine, all other study parameters showed no difference between normal control and glutamine-pretreated control, both in the intestine and the lung.
The amino acid glutamine has received considerable attention in recent years, particularly because of its role in mucosal turnover and function. In a number of injury models, improved survival has been demonstrated when enteral or intravenous feedings are supplemented with glutamine. Gut glutamine requirements are increased during critical illness; a number of studies suggest that glutamine may be essential for the maintenance of gut metabolism, structure, and function.

The present study shows that both glutamine and glutamic acid offer protection to the intestine as well as the lung from oxidative damage during surgical manipulation of the small intestine. Intestinal protection is evident both at the cellular and subcellular level. This protection was not seen with the other studied isonitrogenous amino acids glycine or alanine. Earlier studies have shown activation of the superoxide-generating enzyme xanthine oxidase in the intestine during surgical manipulation and use of inhibitors of xanthine oxidase, which indicate a role for xanthine oxidase in causing lung damage. Surgical manipulation not only intensifies oxidative stress by generating reactive oxygen species but also weakens the biological defense system against attack by these reactive oxygen species. The important cellular antioxidant glutathione and the superoxide and hydrogen peroxide–scavenging enzyme activities (superoxide dismutase and catalase) were decreased in the intestine after surgical manipulation. Both glutamine and glutamic acid supplementation could maintain the glutathione level and the enzyme activities to normal. This prevention of oxidative stress in the intestine by glutamine also protected the lung from oxidative damage as indicated by decreased BALF protein and MPO activity, decreased lipid peroxidation, and increased antioxidant levels. A similar protection occurred with glutamic acid suggesting that glutamine’s mechanism of protection during
surgical manipulation may possibly through its conversion to glutamate.

Glutamine can offer protection to the cells by different mechanisms. Glutamine is utilized as a major energy source by the intestinal mucosa and drives mitochondrial ATP formation by oxidative phosphorylation. Glutamine may also serve as a metabolic precursor for glutathione, the important cellular antioxidant. Glutamic acid is formed from glutamine by the action of glutaminase. This is further channeled to α-ketoglutarate, which enters the tricarboxylic acid cycle. Glutathione, a major antioxidant, is synthesized from glutamic acid, cysteine, and glycine by the consecutive action of γ-glutamyl cysteine synthetase and glutathione synthetase.

The small intestine is the principal organ of glutamine consumption, extracting approximately 20% to 30% of circulating glutamine in the postabsorptive state. The avid uptake of glutamine by the mucosal cells is due, in part, to the high activity of glutaminase, which is the first enzyme in a series of reactions that completely oxidize the carbon chain of glutamine to generate energy. Recently it has been shown that caco-2 cells supplemented with glutamine can prevent bacterial translocation due to glutamine acting as an energy supplement rather than through other mechanisms. Glutamine also acts as a key precursor for the intestinal synthesis of the antioxidant glutathione. Glutamine or glutamic acid feeding alone could increase the GSH content in the intestine by 3-fold; pretreatment of glutamine or glutamic acid maintained the GSH level even after surgical manipulation. The glutathione system is one of the major mechanisms of reducing oxidative stress. Others have shown that glutamine-supplemented nutrition can enhance tissue glutathione levels and is associated with improved survival after 5-flurouracil administration. Glutamine can act as a source of glutamate in many tissues such as liver or skeletal muscle and has been shown to preserve total glutathione levels after injury/ischemia to both hepatic and gut models. Apart from all these functions, GSH plays a general role in maintaining the structural integrity of the mucosal enterocytes. This role is proved by a study that showed a substantially disruptive effect on the mucosal architecture after pharmacologic inhibition of GSH synthesis. It has been shown that gut barrier protective action by glutamine could possibly be due to glutathione augmentation by scavenging reactive oxygen intermediates and decreasing the formation of free radicals. Glycine and alanine were used as controls in the diet since they are non-essential amino acids and have been used by others in similar experiments that studied the effects of glutamine.

CONCLUSION

This study has shown that oral glutamine and glutamic acid supplementation before surgical manipulation can prevent certain oxidative stress alterations both in the intestine and lung, and thereby offer protection from postsurgical complications. Although the present study used a 7-day pretreatment model, it is unknown if a shorter duration of treatment may be beneficial in a clinical setting. Since oxidative stress occurs in the intestine after surgical manipulation and glutamine or glutamic acid can increase the glutathione status in the intestine, upregulation of cellular antioxidants may be one of the mechanisms by which the intestine and lung are protected.

REFERENCES


