Original Article
Glutamine protects intestinal mucosa and promotes its transport after burn injury in rats

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Abstract: Glutamine is an important energy source for intestinal epithelial cells (IEC); however, it is still controversial whether glutamine can be fully utilized under pathological conditions. In this study, we investigated the changes in glutamine transport after burns and assessed the effects of exogenous glutamine administration. Finally, the potential underlying mechanisms were explored. Experimental rats were randomly divided into three groups: control group (C); burn group (B); burn+glutamine group (B+G). Rats in groups B+G and B received intragastric administration of isodose glutamine or alanine, respectively. At days 1, 3 and 5 after burns, the structure of intestinal mucosa and brush-border membrane vesicles (BBMV) were observed. The glutamine transport capacity of IEC and BBMV was detected. The synthesis of glutamine transporter ASCT2 and B0AT1 was determined. Moreover, the intestinal mucosal blood flow (IMBF), diamine oxidase activity, and the glutamine and ATP content were measured. The results showed that burn injury caused structural damage to IECs and BBMV, and significantly impaired the ability for glutamine transportation. Moreover, the mRNA and protein expressions of ASCT2 and B0AT1 as well as the glutamine and ATP content were markedly decreased. Compared with group B, most of these indicators in group B+G showed significant improvement, and approached normal levels. We conclude that glutamine administration can relieve intestinal damage, improve IMBF, promote energy synthesis and alleviate endoplasmic reticulum stress after burn injury. Finally, the synthesis and modification of ASCT2 and B0AT1 are promoted, which ultimately enhances intestinal glutamine transport.

Keywords: Glutamine, glutamine transport, BBMV, intestine, burn

Introduction
Large-area deep burn is a severe traumatic injury, which can lead to major damage of the skin and subcutaneous tissue and varying degrees of injury to multiple organs; the condition may eventually result in persistent inflammatory reaction and multiorgan dysfunction syndrome (MODS) or even death [1]. Increasing research evidence has confirmed the critical role of the intestine in traumatic stress. Indeed, intestines are considered as “one of the central organs in traumatic stress” [2]. Studies have shown that burn injury is associated with impaired structural and functional integrity of the intestinal mucosa [3-5]. Ischemia/hypoxia is the most important mechanism of direct intestinal injury, while impaired cellular energy metabolism results in delayed repair of intestinal damage [6, 7]. Therefore, maintenance of normal energy metabolism is one of the key factors to reduce intestinal epithelial cell (IEC) damage and promote repair.

Glutamine, the most abundant amino acid in plasma and cells, is considered a conditional essential amino acid. Unlike most other amino acids, it has two nitrogen side chains: an amino group and an amide group [8, 9]. Glutamine provides carbon and nitrogen for rapid growth and differentiation of cells, such as immune cells and enterocytes, and thereby contributes to the maintenance of structural and functional integrity of the intestine [10]. Several clinical and animal studies, including our previous research, have demonstrated that administration of glutamine abates intestinal mucosal damage, accelerates repair of damaged mucosa, improves nitrogen balance, ameliorates wound healing and helps shorten the hospital stay of
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The role of glutamine in the maintenance of intestinal mucosal barrier is increasingly recognized in the field of critical care medicine. Numerous treatment guidelines for critical care and severe burns recommend routine use of glutamine preparations [14-16].

The pharmacologic effects of glutamine have been recognized. However, the ability of intestines to utilize glutamine in various pathological states is uncertain. Intracellular uptake of glutamine from the intestinal lumen is an essential first step for its utilization; it is also a crucial step in glutamine metabolism in the intestine. Previous studies have shown a significant decrease in intestinal glutamine transport in the setting of abdominal trauma, systemic infection, and sepsis [17-19]. In our previous clinical study, the increase in plasma glutamine concentration in burn patients after enteral administration was remarkably milder and slower than that observed in healthy volunteers [20]. The findings suggest that burn injury may compromise the ability of the intestines to transport and use glutamine.

The transport of extracellular glutamine into the cytoplasm occurs via specific amino acid transport systems. The specific glutamine transport systems have been defined on the basis of their amino acid selectivity and physico-chemical properties. Based on their dependence on sodium ions, glutamine transporters are categorized into two types: sodium-dependent and sodium-independent [21]. It is now known that the glutamine transport in intestinal mucosa is mainly dependent on the sodium-dependent amino acid transport system, of which the main members are ASCT2 and BOAT1 [22]. These carriers are responsible for most of the intracellular uptake of glutamine. This is an energy consuming process, which is mainly provided by Na⁺/K⁺ ATPase [23].

Some studies have found a significant decrease in the expressions and synthesis of ASCT2 and BOAT1 in a variety of pathologic states, which impairs glutamine transport [24, 25]. Currently, the change in expressions of ASCT2 and BOAT1 after burn injury, and the effect of these changes on intestinal glutamine transport is not well characterized. In this study, we investigated the changes in ASCT2 and BOAT1 post-burn, and examined the effects of glutamine on their expression and synthesis. On this basis, we explore the potential underlying mechanisms.

Materials and methods

Reagents

Standard ATP preparation and ASCT2 antibody were purchased from Sigma-Aldrich (St, Louis, MO, USA). BOAT1 antibody was purchased from Santa Cruz (CA, USA). Antibody to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from KangChen Shanghai (Shanghai, China). [3-H]-Glutamine was purchased from Isotope & Radiation Corporation (Beijing, China). Pentobarbital and buprenorphine were purchased from Chongqing Pharmaceutical industry (Chongqing, China). Unless otherwise specified, all other reagents were obtained from Sigma-Aldrich (St, Louis, MO, USA).

Animal experiment

Seventy two adult male Sprague-Dawley rats (age: 6-8 weeks; weight: 200-250 g, each) were purchased from the Animal Experimental Center of the third Military Medical University. The animals were reared in an independent cage with 22-25°C in a 12 h light-dark cycle; a standard diet was provided for a week before the experiment. Rats were randomly divided into three groups: control group (C); burn group (B); burn+glutamine treatment group (B+G). All rats were anesthetized with pentobarbital 40 mg/kg, and administered buprenorphine (1 mg/kg body weight) for analgesia. After shaving and fixing a 30% total body surface area exposed mould, rats in group C were placed in 37°C water for 18 seconds for sham burn; rats in group B and group B+G were placed in 97°C water for 18 seconds to induce burns. Intraperitoneal injection of Ringer’s solution (1.5 mL/kg/1% burn surface area) was immediately administered for resuscitation. After waking up, rats in the group B+G were administered intragastric glutamine (1 mg/kg), and rats in other groups were administered equivalent dose of alanine once a day. All animal experiments were performed in strict compliance with the principles of care and use of experimental animals at the First Affiliated Hospital of Third Military Medical University (NIH publication number 8023, revised 1978).
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**Histological assessment**

At different time points after burn injury, jejunal tissues of rats in the three groups were harvested under general anesthesia. The harvested tissues were fixed in neutral formalin solution (pH 7.4) and embedded in paraffin. Then serial sections were prepared for histopathological examination. Hematoxylin-eosin (HE) staining was used to observe histologic changes.

**Brush-border membrane vesicles (BBMVs) preparation**

The intestines were washed with 30 mL of saline containing 0.1 mM PMSF and scraped into debris with a cold slide. Cell suspensions were prepared by adding 65 mL of solution A (containing 300 mM mannitol, 5 mM EGTA, 12 mM Tris·Cl and 0.1 mM benzenesulfonyl fluoride). These were homogenized for 2 min, placed for 5 min, 65 mL of frozen double-distilled water and 18 mL of 100 mM MgCl₂ were added, and stirred on ice for 15 min. The supernatant was collected and then centrifuged for 30 min at 4°C at 20,000 g. The precipitate was retained, and 3 mL of solution B (containing 150 mM mannitol, 2.5 mM EGTA, 6 mM Tris·Cl and 0.1 mM benzosulfonyl fluoride) was added by gentle blowing; subsequently, 30 mL of solution B and 4 mL of 100 mM MgCl₂ were added again, and stirred on ice for 15 min; the supernatant was collected after centrifugation at 4°C for 15 min under 3000 g. Then the supernatant was centrifuged for 30 min at 4°C at 20,000 g, and the precipitate was retained and suspended in 500 mL of solution C (containing 300 mM mannitol and 5 mM Tris·Cl). Finally, a suspension of BBMVs was obtained.

**Morphology of BBMVs**

A membrane with a copper net was placed in BBMVs suspension for 10 min; a filter paper was used to absorb excess liquid around the copper mesh. Negative dyeing water (potassium phosphotungstate) was dripped onto the filter paper and quickly blotted up after 10 seconds; subsequently, the copper mesh was observed under transmission electron microscope.

**IECs preparation**

Intestinal epithelial cells were isolated from the small intestine of rats by calcium chelation technique. In brief, 30 cm of intestine was thoroughly washed with D-Hanks solution. Then it was cut into tissue masses and collected in a buffer containing 20 mL of cell separation solution (0.15 mM EDTA, 112 mM NaCl, 25 mM NaHCO₃, 2.4 mM KH₂PO₄, 0.4 mM K₂HPO₄, 2.5 mM glutamine, 0.5 mM dithiothreitol, pH 7.4). Thirty minutes later, tissues were gently palpitated to facilitate cell dispersion at 37°C. Then the buffer solution was collected, phenylmethyl sulfonyl fluoride was added, and the suspension was centrifuged at 100 g for 5 min.

**Uptake studies of [3-H]-glutamine**

40 μL of rat IECs or BBMVs suspension (4-5 mg/mL) was dripped into a 24-well plate, followed by addition of 1 mL of [3-H]-glutamine (0.5 μCi/mL, 20 μmol/L) uptake buffers (containing choline chloride or sodium chloride). After shaking for 1 min, the IECs or BBMVs were washed and filtered onto filter paper, and its ejection activity was detected by liquid scintillation method. The transport rate of [3-H]-glutamine was expressed as pmol/mg protein/10 s.

**Western blot**

The extracted protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, incubated overnight with the primary antibodies at 4°C [ASCT2 (1:1000 Sigma); B0AT1 (1:200 Santa); GRP78 (1:1000 CST)], and washed with Tris-Buffered Saline Tween. Subsequently, these were incubated with the corresponding secondary antibodies, and washed with Tris-Buffered Saline-Tween. Protein bands were detected by enhanced chemiluminescence ECL.

**Fluorescence quantitative PCR**

Total RNA was extracted from IECs using TRIzol reagent. The first-strand cDNA was synthesized by reverse transcription from 2 μg of total RNA with First Strand cDNA Synthesis Kit (TOYOBO, OSAKA JAPAN). The primer sets for GAPDH were 5'-CTG AGA ATG GGA AGC TGG TC-3' (forward) and 5'-AGC CCA GTA GAC TCC ACG AC-3' (reverse). The primer sets for ASCT2 were 5'-CCT AGA CCT GGG ATC ACG GA-3' (forward) and 5'-CAG ATC AGG ACG TAG CCG AC-3' (reverse). The primer sets for B0AT1 were 5'-TGA CAT GTG CCT GGA GTG TT-3' (forward) and 5'-CCA CGG ATG AGA AAG ATG GT-3' (reverse).
Primers were designed and synthesized by Sangon Biotech (CN). Quantitative-PCR was performed using the SYBR Green Realtime PCR Master Mix (TOYOBO, OSAKA JAPAN) on an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). The delta cycle threshold (CT) method was used to analyze the relative expressions of the target genes.

Glutamine concentration in plasma and IECs

The glutamine concentration in plasma and IECs was quantified using high-pressure liquid chromatography (HPLC). 0.5 mL of plasma or IECs were mixed with 4% sulfosalicylic acid and centrifuged at 10,000 rpm for 15 min to precipitate protein. The supernatant (100 mL) was injected into the HPLC (Gilson, France). Detailed methodology has been described previously [26].

Intestinal mucosal blood flow (IMBF)

IMBF was detected by microcirculatory Doppler blood flow meter. Laparotomy was performed under superficial anesthesia. Then 0.5 cm of intestinal wall on the opposite side of the hollow mesentery was cut, and the probe gently placed in contact with the side wall of the mesentery side. Results after stabilization were recorded, and average value was calculated after repeating 8 times.

ATP content in IECs

A mortar and pestle was used to mull intestine tissue into a powder, which was transferred into a test tube containing 0.6 N perchloric acid. After extracting the metabolites, the extraction was neutralized with a mixture of KOH and K$_2$CO$_3$. Then the extraction was centrifuged at
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8000 rpm for 15 min at 4°C. 10 mL of supernatant was subjected to high performance liquid chromatography with UV/VIS-152 (Gilson, France). The results were expressed as micromole ATP per gram protein.

Intestinal mucosal damage index

Histopathologic examination of jejunum was performed by H & E staining; the slides were observed and photographed with an Olympus microscope. Index scoring method for mucosal injury: normal villi, 0 point; presence of a cystic space on the top of the villi, with hyperemic capillaries, 1 point; intraepithelial interstitial enlargement, moderate endothelial edema, central chylous dilation, 2 points; obvious edema in lamina propria, degeneration and necrosis of epithelial cells, abscission on the tip of villi, 3 points; epithelial cell layer degeneration, necrosis, shedding, partial villi abscission, nude lamina propria, capillary dilatation, hyperemia, 4 points; villi shed, disintegration of lamina propria, bleeding or ulceration, 5 points.

Diamine oxidase (DAO) activity in plasma

The DAO activity in plasma was determined as described previously [27]. In the final volume of 3.8 mL, containing 3 mL of phosphate buffer (0.2 M, pH 7.2), 100 μL (4 μg) of horse radish peroxidase, 100 μL (500 μg) of o-dianisidine, 500 μL of plasma, and finally 100 μL (175 μg) of cadaverine. After mixing thoroughly, the sample was incubated for 30 min at 37°C, and DAO activity was measured at the absorbance level of 436 nm.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Correlation between variables was assessed by two-way Analysis of Variance (ANOVA). Since the experimental design involved repeated measures, repeated measures analysis of variance was performed to test the significance of the two variables (variable 1: treatment; variable 2: time) and their interaction simultaneously. All statistical analyses
were performed using SPSS 19.0. P<0.05 was considered statistically significant.

Results

Morphologic changes of intestinal mucosa

Light microscopy revealed disordered villi, dilated interstitial blood vessels, hemorrhage, edema, and degeneration and necrosis of mucosal cells in intestinal tissues isolated from rats in the burn group. The morphology of the villi was slightly improved after 3 days of glutamine treatment, and nearly returned to normal after 5 days of administration, which indicates that glutamine helped maintain the structure of the intestinal mucosa after the burn (Figure 1).

Morphological changes of BBMVs

On electron microscopy, all rats in burn group showed signs of serious damage to the intestinal BBMVs, i.e., irregular shape, collapsed vesicle wall, broken vesicles, rough borders and broken vesicle pieces. Glutamine treatment for 3 days significantly improved the morphology of BBMVs. After 5 days of treatment, the membrane structure was complete, the cavity was full, and the border was clear (Figure 2). The present study showed that glutamine helped maintain the structural integrity of BBMVs after burns.

Changes of glutamine transport in BBMVs and IECs

The study results showed that the ability for glutamine transport in IECs and BBMVs was significantly reduced after burns and sodium-dependent glutamine transport in BBMVs was particularly attenuated. It dropped to 30% of the control group on post-burn day (PBD) 1; despite a subsequent rebound, it was significantly lower than that in the control group on PBD5. Compared with the burn group, glutamine administration significantly promoted its transport in IECs and BBMVs; in particular, sodium-dependent glutamine transport in BBMVs was doubled on PBD3 (P<0.05, Figure 3).

Changes in the expression and synthesis of glutamine transporter ASCT2 and BOAT1

The results showed that the mRNA and protein levels of ASCT2 and BOAT1 in IECs were decreased markedly after burns. Compared with burn group, glutamine treatment significantly enhanced the mRNA and protein expressions of ASCT2 and BOAT1, both of which returned to near normal levels after 5 days of treatment (P<0.05, Figure 4). The results showed that glutamine could promote the expression and synthesis of ASCT2 and BOAT1 in intestines after burns.

Endoplasmic reticulum stress in IECs

The expression of ERS marker protein GRP78 in IECs of rats increased significantly after burns, with 4 times the normal level on PBD3.
Compared with the burn group, glutamine supplementation reduced the expression of GRP78, which returned to near normal level after 5 days of treatment ($P<0.05$, Figure 5). The results showed that glutamine obviously reduced the ERS in IECs of rats with burn injury.

Changes in glutamine and ATP content, IMBF, intestinal mucosal injury index, and DAO activity.

The glutamine content in plasma and IECs decreased by different degrees from days 3 to
5 after burns. A significant recovery was observed after glutamine supplementation; in particular, the glutamine content in IECs was restored to a normal level on PBD3 (P<0.05, Figure 6A, 6B). In addition, the mucosal blood flow and the ATP content decreased to 50% of that in the control group on PBD1; both of these were restored to normal levels after glutamine treatment for 3 days (P<0.05, Figure 6C, 6D). The more obvious change was with respect to the intestinal mucosa damage index and DAO activity, which reflects the degree of intestinal damage after burns; its value increased considerably as compared to group C. After 5 days of glutamine treatment, intestinal mucosa damage index and the DAO activity were down by 55% and 25%, respectively (P<0.05, Figure 6E, 6F).

Discussion

In this study, we found that the intracellular uptake of glutamine was significantly decreased after burn injury. Analysis of the trend of change in different transporter subtypes revealed that the effects of burns on sodium-dependent transporters were more obvious than those on non-sodium-dependent transporters. The main reason for decrease in glutamine transport is the significant impairment of transmembrane transport due to an inverse glutamine concentration gradient, while the amplitude of variation of C1S concentration gradient is not very large. These results suggest that impaired energy metabolism in the intestinal epithelial cells may be an important reason for the decreased capacity of glutamine transport in the intestines. With respect to glutamine transport in different parts, the effects of burns and glutamine administration on BB-MVs were more significant than those on IECs. BBMV is a specialized structure for absorption of materials that is located in the intestinal villi, which is the most apical portion of the intestinal mucosa and is extremely sensitive to ischemia and hypoxia [28].

The results of this study showed marked reduction in intestinal blood perfusion after burn injury. The intestinal mucosal blood flow reached the lowest value on the first burn day. Subsequently, the blood flow recovered, but remained significantly lower than baseline levels at the fifth burn day. Supplementation of glutamine increased IMBF and improved intestinal blood perfusion as compared to that in the control group. In our previous studies, glutamine supplementation was shown to alleviate the degree of stress response to burn injury, reduce the secretion of adrenergic neurotransmitters, decrease intestinal vascular systolic state, and improve intestinal perfusion [20]. Another study showed that through transamination, glutamine could be converted to arginine, the substrate of nitric oxide, which promotes NO synthesis, and eventually improves intestinal blood flow [29].

Ischemic and hypoxia are the key factors that cause tissue damage after burn injury, and glutamine administration can alleviate tissue damage by improving blood flow. Furthermore, the physiological function of glutamine is to provide nitrogen and carbon which is critical for
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Our results showed that the pathologic changes in intestinal mucosa or BBMVs in the glutamine treatment group were obviously mitigated as compared to those in the burn control group. The damage index of intestinal mucosa and the plasma DAO activity were remarkably decreased after administration of glutamine. More importantly, glutamine can also improve energy metabolism and promote ATP synthesis in the IECs. It is of cardinal significance to improve IEC function by increasing ATP content, which can provide enough energy for glutamine transport, and reduce endoplasmic reticulum stress (ERS) after burn injury.

Recent studies have shown that burns may induce ERS in multiple organs, including intestines [30]. Protein misfolding is the main reason leading to ERS, and this process is closely related to disordered cell metabolism. Insufficient energy may block the process of protein translation, modification and folding, induce some important protein structural errors and cause ERS [31]. Our research found that the content of GRP78, the ERS marker protein, was significantly increased after burn injury. After supplementation of glutamine, the amplitude of change in GRP78 was obviously reduced and the recovery was faster than that in the B group. These findings indicate that burns induce ERS in IECs, and that administration of glutamine may significantly attenuate the severity of ERS. The trend of change in GRP78 was opposite to that of intestinal blood flow and ATP content.

ERS decreases the activity of some important protein modifier enzymes in the endoplasmic reticulum, and weakens its protein modification function. In this study we found that the ASCT2 and BOAT1, two major glutamine transporters in IEC, were decreased markedly after burn injury. Compared with the burn group, glutamine administration significantly promoted the synthesis of ASCT2 and BOAT1, both of which almost returned to normal levels after 5-day treatment. Increased levels of transporter enhanced the capacity for transmembrane

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**Figure 6.** The changes in glutamine and ATP content, IMBF, intestinal mucosal injury index and DAO activity. The glutamine content was quantified by HPLC in plasma (A), in IEC (B). (C) The mucosal blood flow was detected by Doppler meter. (D) The ATP content in IECs was quantified by HPLC. (E) The intestinal mucosa was observed and scored using Olympus microscope. (F) The DAO activity was detected by spectrophotometric method. Data are presented as mean ± SD and analyzed by two-way ANOVA. *P<0.05 versus control group (n=8); †P<0.05 versus burn group (n=8).
transport of glutamine, and finally increased the glutamine concentration in IECs and blood.

ASCT2 and B0AT1 were shown to be cysteine-containing transporter proteins with special space structure [22]. Upon peptide chain synthesis, cysteine residues in the two transporters are catalyzed by some special enzymes in the endoplasmic reticulum such as protein disulfide isomerase (PDI) to generate disulfide bonds and form a correct spatial structure [32]. Therefore, endoplasmic reticulum function has a direct bearing on the modification of glutamine transporter proteins. The present study demonstrated that severe burn-injury induced ERS in rat IECs, which led to impaired synthesis and modification of ASCT2 and B0AT1.

After burn injury, ischemia and hypoxia can directly damage IEC and BBMV, which decreases the amount of glutamine transporter and causes its dysfunction. Furthermore, disordered energy metabolism caused by ischemia and hypoxia is an important reason for ERS-induced glutamine transporter ASCT2 and B0AT1 synthesis and modification disorder. The final result is a significant decrease in the intestinal transport and utilization of glutamine after burn injury. Enteral feeding with glutamine alleviates intestinal tissue damage, improves mucosal blood flow, promotes energy synthesis, and abates ERS. Ultimately it increases intestinal glutamine transport and metabolism through improved endoplasmic reticulum function and promotion of glutamine transporter ASCT2 and B0AT1 synthesis and modification.

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Disclosure of conflict of interest

None.

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