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Original Article

Effects of glutamine on intestinal mucus barrier after burn injury

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Abstract: Severe burns may cause intense stress and persistent inflammation, resulting in intestinal mucosal barrier damage. In this study, we evaluated the effects of glutamine (Gln) on intestinal mucus barrier after burn injury. The results showed that glutamine could improve intestinal mucosal blood flow (IMBF), decrease diamine oxidase (DAO) activity, and reduce intestine damage, thereby alleviate intestinal mucous permeability. Severe burn was associated with subsequent decrease in mucus thickness, levels of hexose, sialic acid, and protein. Glutamine administration might partially reverse these changes. Additional experiments showed that supplementation with glutamine could markedly raise the content of glutamine, glutathione (GSH), and ATP in intestinal tissue. Moreover, the levels of mRNA and protein expression of MUC2, intestinal trefoil factor (ITF) were increased remarkably, but contrary to the trend of GRP-78, CHOP. These results suggest that glutamine can improve tissue perfusion and increase energy synthesis in enterocytes, decrease endoplasmic reticulum stress (ERS) and improve mucin and ITF synthesis. Finally, lessen intestinal mucus barrier damage after burn injury.

Keywords: Glutamine, mucus barrier, intestine, burn

Introduction

The importance of glutamine to metabolic function of the intestinal mucosa was established by the pioneering work of Windmueller and Spaeth [1]. Over the past years, both basic and clinical research has proven repeatedly that glutamine was essential for intestinal function [2-6]. Early studies found that the proliferation and differentiation of intestinal mucosal cells were inhibited by glutamine deprivation [7, 8]. The physiological function of glutamine is mediated largely by special molecular radicals. Glutamine has two nitrogen side chains: an amino group and an amide group. This structure provides carbon and nitrogen for the rapid growth and differentiation of cells, including immune cells and enterocytes [9, 10]. A series of studies, including previous research performed in our laboratory, showed that the treatment with glutamine could alleviate intestinal injury, accelerate intestinal repair, prevent villous atrophy, and minimize mucosal immunosuppression which contributing to immune homeostasis. Thus, glutamine contributes to maintenance of the intestinal structure and function [11-16]. Studies published to date have focused on mechanical and immune barriers, neglecting the effects of glutamine on the intestinal mucosal barrier.

The epithelial surface of the intestine is covered by a layer of mucus, which is a hydrated polymeric gel with thickness of 50-800 μm. Mucus is secreted by goblet cells and comprises proteins, carbohydrates, and lipids; its core component is mucin [17]. The mucus serves as a lubricant to prevent food or feces from abrading the epithelia. The layer of mucus serves as a barrier to bacteria, their products, and various digestive enzymes [18]. Some studies found that mucus may prevent direct contact between enterocytes and intraluminal bacteria (including associated antigens) [19, 20]. Thus, the mucus layer plays an important role in maintaining the integrity of the intestinal muco-

sa. Growing evidence shows that loss of mucus barrier may result in intestinal disease or disease progression [21, 22].

Previous studies of trauma have reported that loss of the mucosal layer contributes significantly to dysfunction of the gut barrier in patients with conditions associated with ischemia-reperfusion-mediated gut injury or intestinal hypoperfusion [23, 24]. Our previous research found that intestinal perfusion was markedly decreased after severe burn. The effects observed were attributed to burn stress and blood redistribution. Pathological evidence also showed post-burn damage throughout the intestinal epithelium, including goblet cells [16]. These findings indicate damage to the intestinal mucus barrier may be damaged after burn injury. No previous study has investigated whether glutamine may improve intestinal mucus barrier function. The cytoprotective effect of glutamine on intestinal epithelial cells indicates that glutamine protects the structure and function of goblet cells, thereby maintains the intestinal mucus barrier after burn injury. However, the specific details and mechanisms remain unclear, which is indeed focused in the present study.

Materials and methods

Animal model

Preparation and grouping: One hundred and twenty male C57BL6 mice (6 weeks old, 25-28 g) were purchased from the Fujian Medical University Laboratory Animal Centre. Animals were reared in independent cages maintained at 22-25°C with a 12-h light-dark cycle. A standard diet was provided for 1 week prior to the experiment. Mice were randomly assigned to three groups: control group (C), burn group (B), and glutamine treatment group (Gln). All mice received pentobarbital 40 mg/kg for anesthesia and buprenorphine (1 mg/kg body weight) for analgesia. After shaving and fixing an area equivalent to 20% total body surface area, mice in group C were placed in 37°C water for 10 seconds for sham burn; mice in group B and group Gln were placed in 90°C water for 10 seconds to induce full-thickness burn. An intraperitoneal injection of Ringer's solution (50 mL/kg) was immediately administered for resuscitation. After resuscitation, mice in the Gln group received intragastric glutamine (1 mg/kg.d), and mice in other groups received an equivalent dose of alanine. Mice in all three groups were anesthetized, and jejunal tissue was harvested after 1, 3, 5, 7 and 10 days. All animal experiments were performed in strict compliance with the principles of care and use of experimental animals at the Union Hospital of Fujian Medical University.

Reagents: Standard ATP preparation and ITF antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). MUC2 antibody was purchased from Santa Cruz (CA, USA). Antibody of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from KangChen Shanghai (Shanghai, China). Kits for detection of GSH were purchased from Nangjing Jiancheng Biochemical Ltd (Nangjing China). [^{99m}Tc] DTPA-HAS was purchased from Isotope Radiation Corporation (Beijing, China). Pentobarbital, buprenorphine, and glutamine granules were purchased from Chongqing Pharmaceutical Industry Company (Chongqing, China). All other reagents were obtained from Sigma-Aldrich (St, Louis, MO, USA).

Histological assessment: Samples of jejunal tissue (5 cm in length) were harvested from mice under general anesthesia, at different time points after burn injury. Harvested tissues were fixed in neutral formalin solution (pH 7.4) and embedded in paraffin. Serial sections were prepared for histopathological examination. Histologic changes were observed with hematoxylin-eosin (HE) and mucus staining.

Mucus sample collection: The distal 30-cm segment of terminal ileum was identified and transected from the intestinal tract and underlying mesentery. Debris was removed from the external portion of the segment by washing with PBS. Fecal matter was subsequently flushed from the jejunal lumen with PBS. The exterior of the segment was gently compressed in order to collect intestinal mucus, as described by Vesterlund et al [25]. We conducted pilot studies in order to identify a method for collection of mucus that would minimize injury to intestinal villi. First, the intact mucus layer was removed. The intestinal segment was subsequently stained to measure the extent of villous injury. Samples of mucus were homogenized in 500 µl of PBS over ice prior to centrifugation for 30 min at 5000 rpm (Denville 260D; Denville Scientific, Metuchen, NJ).

Supernatant was removed and stored at -80°C until further use [24].

Study indices

IMBF: IMBF was detected by microcirculatory Doppler blood-flow meter. Laparotomy was performed under superficial anesthesia. After 0.5 cm of intestinal wall was obtained from the opposite side of the hollow mesentery, the probe was gently placed in contact with the mesenteric side wall. Results after stabilization were recorded, and average values were calculated after measurements had been performed in triplicate.

DAO activity in plasma: The level of DAO activity in plasma was determined as described previously [26]. For each measurement, a final volume of 3.8 mL, contained 3 mL of phosphate buffer (0.2 M, pH 7.2), 100 μL (4 μg) of horseradish peroxidase, 100 μL (500 μg) of odianisidine, 500 μL of plasma, and 100 μL (175 μg) of cadaverine. After samples were mixed thoroughly, they were incubated for 30 min at 37°C. DAO activity was measured at absorbance of 436 nm.

Mucosal thickness: The Alcian blue combination method was used to infer mucosal layer thickness Buffer 0.2 M NaH_2PO_4, 0.1 M citric acid, pH 5.8) was combined with 1 g/L Alcian blue solution buffer, ten centimeters of ileum was measured, then washed in isotonic saline solution and then immersed in 10 mL of staining solution. After incubation at room temperature (RT) for 2 h, the intestinal segment was removed, and approximately 5 mL of staining solution was centrifuged at 3000 rpm for 15 min. The OD value of the supernatant was measured at λ = 615 nm, using buffer as blank and Alcian blue solution as standard.

Alcian blue combination (mg) = 10 - (OD_{sample}/$OD_{standard}$) × 10

Measurement of mucosal hexose levels: Mouse intestinal mucus was diluted and centrifuged to remove impurities. Mucosal hexose levels were determined with phenol sulfone. 1 mL of sample was added to 1 mL of 6% phenol. Ten minutes later, 5 mL of 98% sulfuric acid was added. Colorimetry was performed 30 min later, at a wavelength of 495 nm. The standard curve was plotted using D-galactose as standard.

Determination of mucosal sialic acid levels: To measure levels of sialic acid in mucus, 0.1 mL of the mucus sample was added to 0.8 mL of 0.06 N sulfuric acid. After incubation at 100°C for 10 min and cooling with running water, the mixture was added to 0.1 mL of 50 g/L sodium wolframate, mixed thoroughly, and centrifuged at 3000 rpm for 5 min. Then 0.1 mL of supernatant was added to 10 mL of periodate potassium. Samples were then incubated at 37°C for 30 min, combined with 0.2 mL of 60 mmol sodium arsenite, mixed, and allowed to settle for 5 min. When the color of the mixture turned to iodic yellow and then disappeared completely, 1.0 mL of 0.15 M thiobarbituric acid was added. The mixture was then immersed in boiling water for 8 min. After cooling with running water, 2.5 mL dihydroxyethyl ether. After mixing, the solution was allowed to settle for 5 min. Then colorimetry was performed at a wavelength of 495 nm. The standard curve was plotted using N-acetylneuraminic acid as standard.

Measurement of protein concentration: Protein concentrations in mucus samples were measured by Thermo Scientific Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. Results are expressed as mg/mL.

Glutamine concentration in plasma and intestinal mucosa: The glutamine concentration in plasma and intestinal mucosa was quantified using high-pressure liquid chromatography (HPLC). To precipitate protein, 0.5 mL of plasma or intestinal epithelial cells (IEC) were mixed with 4% sulfosalicylic acid and centrifuged at 10,000 rpm for 15 min. The supernatant (100 mL) was injected into the high-performance liquid chromatography (HPLC) device (Gilson, France). Detailed methodology has been described previously [27].

Fluorescence quantitative polymerase chain reaction (PCR): Total RNA was extracted and purified from intestinal mucosa with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only samples for which the RNA integrity number was higher than the internal threshold value were included in the study. First-strand cDNA was synthesized by reverse transcription from 1 μg of total RNA with the

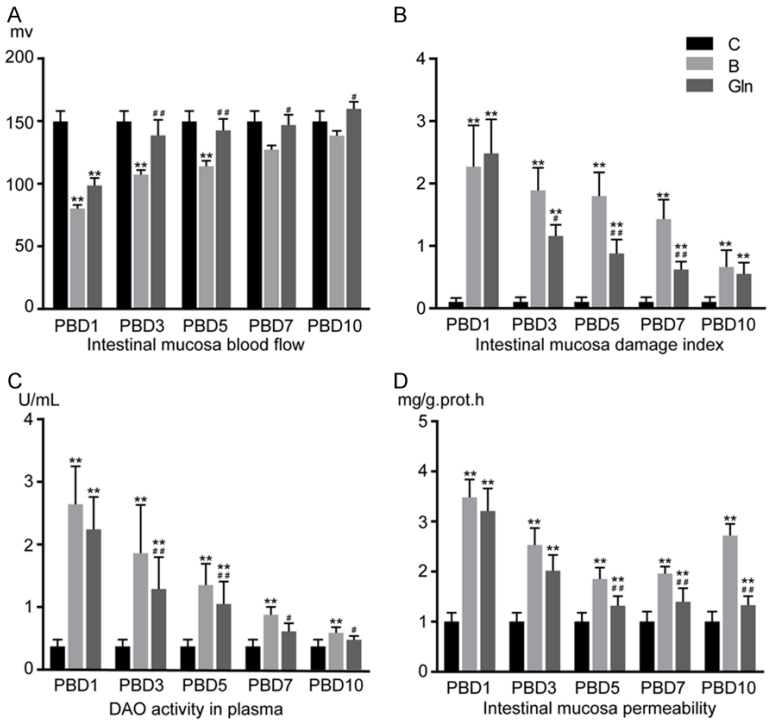

Figure 1. IMBF, intestinal mucosal damage index, DAO, permeability of the intestinal mucous membrane were monitored on days 1 through 10 postburn. All the four indexes of group B and group Gln were lower than those in group C during the experiment. otherwise, IMBF, DAO of group Gln Significantly lower than group B during day 3 through day 10 after burn. The Intestinal mucosal damage index and the permeability of intestinal mucous membrane of group Gln were lower than group B on postburn 5 to 10 days. Data are expressed as mean ± SD. There were n = 8 animals per group. $^{**}P < 0.01$ versus C groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus B groups.

High Capacity cDNA Reverse Transcription Kit (Toyobo, Osaka, Japan). The following primer pairs were used: for GRP-78, 5'-CAAGAACCAACTCACGTCCA-3' and 5'-ACCACCTTGAATGGCAAGAA-3' (forward, reverse); for Chop, 5'-CTGGAAGCCTGG TATGAGGA-3' and 5'-GGGA TGCAG GG TCAAGAGTA-3' (forward, reverse); for ITF, 5'-GGGGCTG CTGCTTTGAC-3' and 5'-AGGTGCATTCTGCTTCCTG-3' (forward, reverse); for MUC2, 5'-CATGTTCGTGTCCTTCATGG-3' and 5'-GTTCTGGTTGAGTGGGCA TT-3' (forward, reverse); for GAPDH, 5'-CTGCGACCACCAACTGCTTAGC-3' and 5'-CTTCACCACCTTCTTGATGTC-3' (forward, reverse); Real-time polymerase chain reaction (Q-PCR) was performed with SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) on an Applied Biosystems 7500 Real-time PCR System (Foster City, CA, USA). Data were quantified according to the comparative cycle threshold (CT) method. Relative mRNA expression levels were determined using the comparative CT method after normalizing to GAPDH expression levels.

Western blotting: Total protein was extracted from intestinal mucosa with RIPA lysis buffer supplemented with protease inhibitors. Equal amounts of protein extract were separated by SDS-polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membranes (Healthcare, Buckinghamshire, UK), probed overnight at 4°C with antibodies against: CHOP (1:1000 CST), GRP-78 (1:1000 CST), ITF (1:1000 Sigma), MUC2 (1:1000, Abcam, UK), and GAPDH (1:5000; Kangchen, Shanghai, China). PVDF membranes were washed with TBST and incubated with corresponding secondary antibodies (1:5000). Membranes were washed with TBST, then revealed with the ECL Western Blotting Analysis System (Healthcare, Buckinghamshire, UK).

High iron diamine-Alcian blue (HID-AB) staining: Diamine solution was prepared by dissolving N, N-dimethyl-*p*-phenylenediamine monohydrochloride (20 mg) and N, N-dimethyl-*m*-phenylenediamine dihydrochloride (120 mg) in distilled water (50 mL). High-iron diamine solution was also prepared as described by using 40% (w/v) ferric chloride prepared from anhydrous resublimed salt and the diamine solution. After staining for 24 hr at RT, sections were rinsed 3 times with tap water. Sections were then dehydrated, cleared, and mounted after exposure to 1% (w/v) Alcian blue in 3% acetic acid for 30 min at RT. HID-AB stain was graded on a three-point scale from - to ++ (- = no glands positive; + = only luminal mucin positive; ++ = intracytoplasmic mucin positive).

Periodic acid Schiff (PAS) staining: PAS and Alcian blue staining were performed with a Periodic Acid-Schiff kit (395B-1KT, Sigma-Aldrich, St Louis, MO, USA). After deparaffiniza-

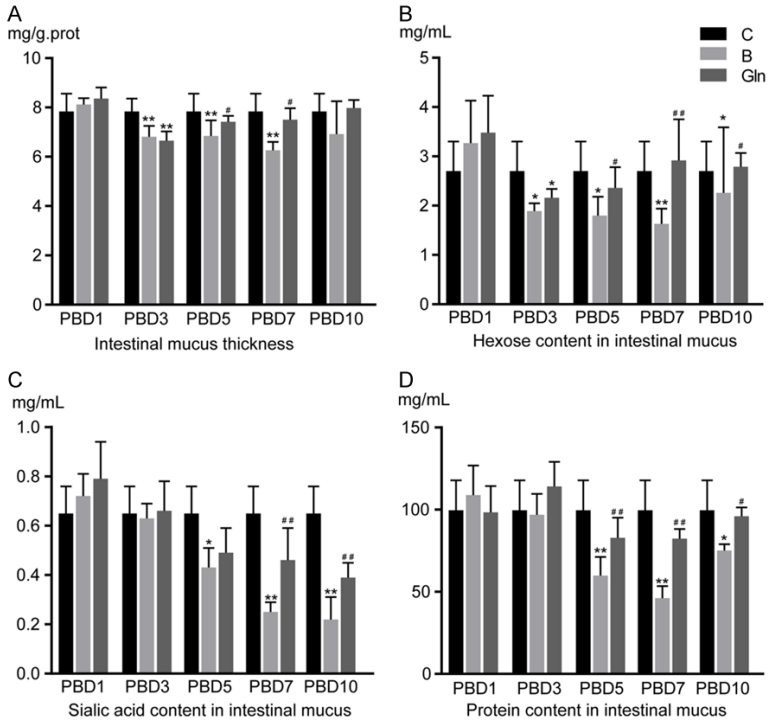

Figure 2. After burn injury, intestinal mucus shows decreased mucosal thickness and lower levels of hexose, sialic acid, and protein. A. The thickness of the mucus layer was indirectly reflected by the Alcian blue combination method. B. The mucus hexose content was determined by the phenol sulfone method. C. The mucus sialic acid content was determined by high efficiency liquid chromatography. D. The concentration of protein in the mucus samples was measured by the Thermo Scientific Pierce BCA Assay. Data are expressed as mean ± SD; n = 8 animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus C groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus B groups.

tion and rehydration, periodic acid solution was applied for 5 min at RT. Schiff reagent was then applied for 15 min at RT, followed by hematoxylin counter staining. PAS-Diastase staining was performed using the same kit, with the addition of a-amylase (0.2 g to 40 mL water). Slides were then placed in a microwave at 600 W for 25 s and rinsed in water for 5 min.

Examination of intestinal tissue sections using aldehyde fuchsin (AF) staining: Paraffin sections were washed with AF dye solution for 5 min, then gently washed in distilled water for 20 min or until the dye was removed. Normal dehydration yielded transparent sections, and the dewaxing water used for paraffin sections was washed with AF dye for 5 min. AF has strong affinity for the sulfate group on mucopolysaccharide. Therefore, strongly sulfated mucus is dark purple, while weakly sulfated mucus is purple.

Other methods (*1 Index of intestinal mucosal damage. 2 Measurement of intestinal mucous membrane permeability. 3 GSH levels in intestinal mucosa. 4 Levels of ATP in intestinal mucosa. 5 Hematoxylin and eosin (HE) staining*) are shown in the annex (Supplementary Data).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Correlations between variables were 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 inter-action simultaneously. All statistical analyses were performed with SPSS 19.0. $P < 0.05$ was considered statistically significant.

Results

Effects of glutamine on IMBF, intestinal mucosal damage index, DAO, and permeability

In order to assess whether glutamine decreased the extent of damage to the intestinal mucosa, a mouse model of severe burn injury was created. IMBF was detected by microcirculatory Doppler blood-flow meter. One day after burn, IMBF had decreased to 50% of baseline. IMBF then increased slowly, nonetheless remaining lower than normal levels at 10 days postburn. Compared with group B, group Gln had significantly higher IMBF from days 3 to 10 postburn (**Figure 1A**). The intestinal mucosal damage index used for histopathologic examination of jejunum was determined with HE staining. After burn injury, the intestinal mucosa appeared hyperemic and edematous, with mucosal exfoliation and ulceration. Compared with group B, group Gln had significantly less intestinal mucosal damage on postburn days 3 and 7 (**Figure 1B**).

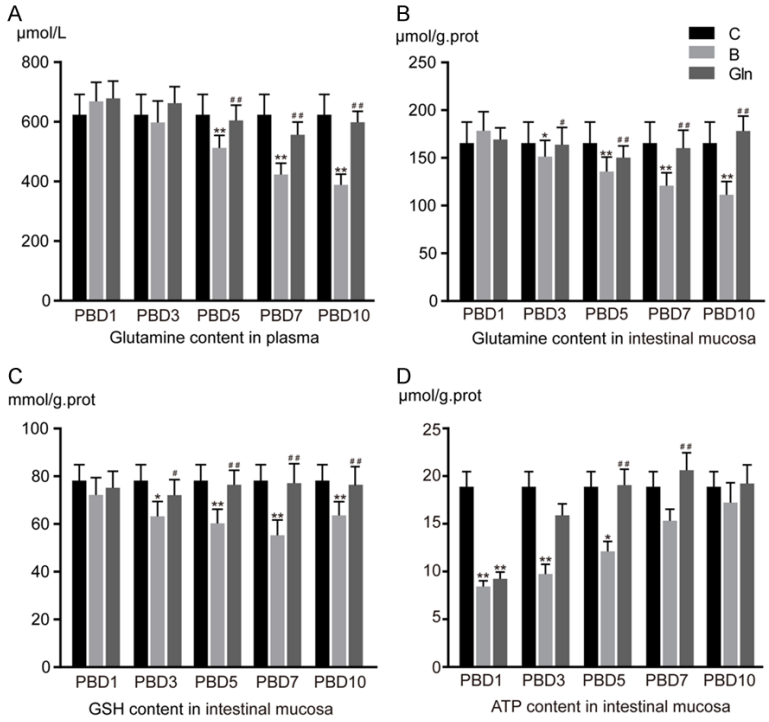

Figure 3. Analysis of plasma and intestinal mucosa samples showed decrease in levels of glutamine, GSH, and ATP postburn. Levels of glutamine, GSH, and ATP were increased following treatment with glutamine. A. Glutamine in plasma. B. Glutamine in intestinal mucosa. C. GSH in intestinal mucosa. D. ATP in intestinal mucosa. Data are expressed as mean ± SD, and there were n = 8 animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus C groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus B groups.

DAO is a sensitive indicator of intestinal damage, plasma DAO activity is significantly increased after damage to the intestine. The results showed that DAO activity reached a peak 1 day postburn, with a 3.8-fold increase in group B and a 3.3-fold increase in group Gln, compared with the group C. DAO activity decreased over time but remained significantly higher than that observed in group C until 10 days postburn. DAO activity was significantly lower in group Gln than in group B during days 3 through day 10 postburn (**Figure 1C**). After burn, the permeability of the mice intestinal mucous membrane increased significantly, peaking on day 1 (2.48-fold increase in group B and 2.21-fold increase in group Gln, compared with group C). Permeability declined from that point on but remained significantly higher than in group C until 10 days postburn. Permeability was significantly lower in group Gln than in group B during days 5 through 10 postburn (**Figure 1D**).

Changes in mucosal thickness and levels of hexose, sialic acid, and protein in intestinal mucus

Thickness of the intestinal mucus layer decreased after burn injury, except for a transient increase 1 day postburn. The mucus layer was significantly thicker in group Gln, compared with group B on postburn days 5 and 7 (**Figure 2A**). Levels of hexose and sialic acid reflect the degree of mucus maturity. After burn injury, levels of hexose and sialic acid in intestinal mucus tended to decline, relatively, these indicators were significantly higher in group Gln than in group B on days 5 through 10 postburn (**Figure 2B**, **2C**). After burn injury, levels of protein in intestinal mucus decreased, Gln administration could increase protein content, the general change trend was consistent with hexose and sialic acid (**Figure 2D**).

Changes in levels of glutamine, GSH and ATP

Plasma glutamine content decreased from days 5 to 10 postburn, remaining significantly higher in group Gln than in group B over the same period ($P < 0.01$, **Figure 3A**). The glutamine content in intestinal mucosa decreased from days 3 to 10 postburn, remaining significantly higher in group Gln than in group B during days 3 through day 10 postburn ($P < 0.01$, **Figure 3B**). After burn injury, Gln supplemention could increase GSH levels, the general change trend was consistent with the glutamine content in intestinal mucosa ($P < 0.05$ or 0.01, **Figure 3C**). ATP content in in testine was decreased from days 1 to 7 postburn. A significant recovery was observed after glutamine supplementation, in particular, the ATP content in intestine was restored to normal level on postburn 3 days, ATP content was significantly higher in group Gln than in group B on days 3, 5, and 7 postburn ($P < 0.01$, **Figure 3D**).

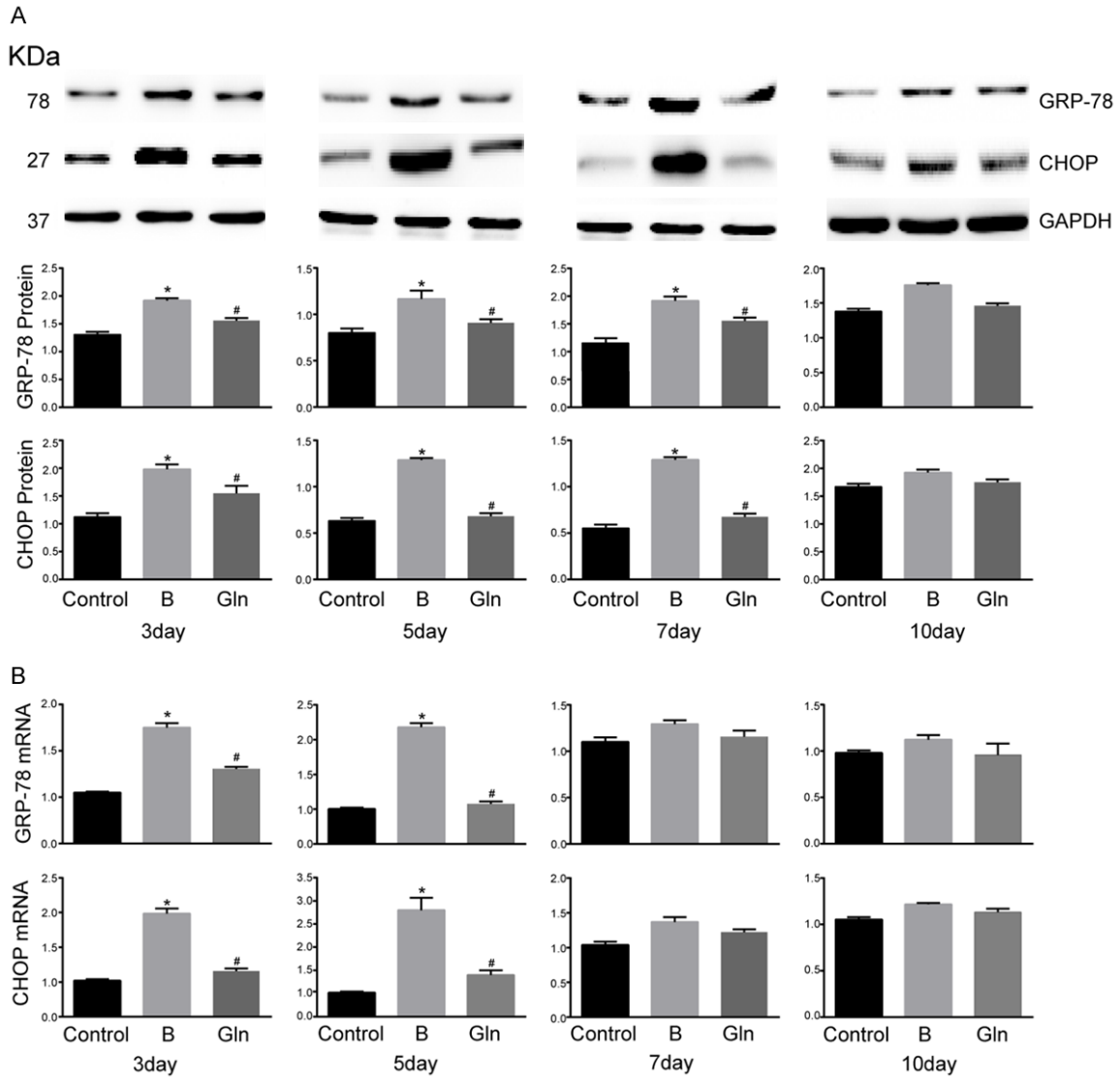

Figure 4. Effects of glutamine on intestinal mucosa GRP-78 and CHOP. A. The protein levels of GRP-78 and CHOP were detected by Western blot, which showed a increase after burns and an decrease after glutamine treatment. B. mRNA levels of GRP-78 and CHOP were detected by Real-time PCR. Data are expressed as mean ± SD, and there were n = 8 animals per group. *P < 0.05 versus C groups; #P < 0.05 versus B groups.

Effects of glutamine on GRP-78 and CHOP in intestinal mucosa

Protein levels of GRP-78 and CHOP in intestinal mucosa were markedly decreased on days 3 and 7 postburn, compared with normal levels. Treatment with glutamine could enhance the protein expression of GRP-78 and CHOP significantly on days 3 and 7 postburn (P < 0.05, **Figure 4A**). The general change trend of GRP-78 and CHOP mRNA was consistent with its protein change. Glutamine administration could promote gene expression of GRP-78 and CHOP, there was significant difference compared with group B on days 3 through 5 postburn (P < 0.05, **Figure 4B**).

Changes in ITF and MUC2

Protein level of ITF and MUC2 in intestinal mucosa was decreased on days 5 through 10 postburn. Comparison with group B showed that glutamine supplementation may increase protein content of ITF and MUC2 on days 5 and 10 postburn (P < 0.05, **Figure 5A**). The changes of ITF and MUC2 mRNA were earlier than its

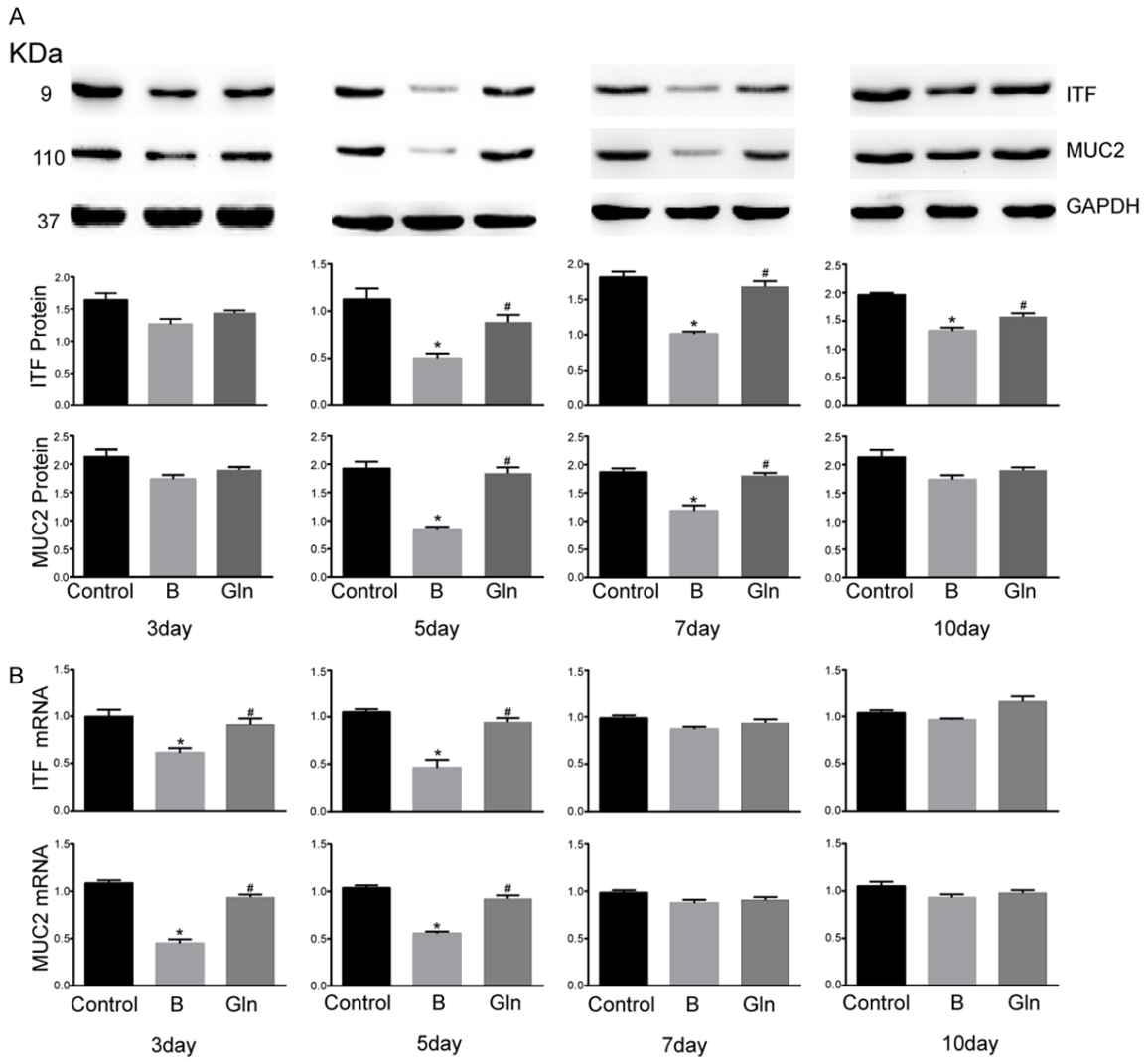

Figure 5. Effects of glutamine on ITF and MUC2. A. The protein levels of ITF and MUC2 were detected by western blot, which showed a decrease after burns and an increase after glutamine treatment. B. The mRNA levels of ITF and MUC2 were detected by Real-time PCR. Data are expressed as mean ± SD, and there were n = 8 animals per group. *P < 0.05 versus C groups; #P < 0.05 versus B groups.

protein changes, significant difference was found on days 3 through 5 postburn. The effect of glutamine on these gene expression was consistent with its protein synthesis (P < 0.05, **Figure 5B**).

Morphological effects on intestinal mucosa

To observe the morphological changes of intestinal tissue structure and the characteristics of goblet cells secreting mucus after burn injury, this study using light microscope with HE and three types of mucus histochemical stains. These three kinds of dyeing are HID-AB, PAS, and AF, for observing the change of sulfated mucus, carboxylated mucus, sialic acid, and alkaline mucus respectively. The results revealed that there was obvious damage in the intestinal mucosa after burn injury, it was characterized by tissue hyperaemia, edema, inflammatory cell infiltration, lamina propria exfoliation, villi disordered and atrophy and even formation superficial ulcer. Compare with group B, the degree of mucosal damage was significantly reduced by supplement exogenous glutamine, the main pathological changes were congestion and edema, no ulcer formation. These findings indicate that glutamine could

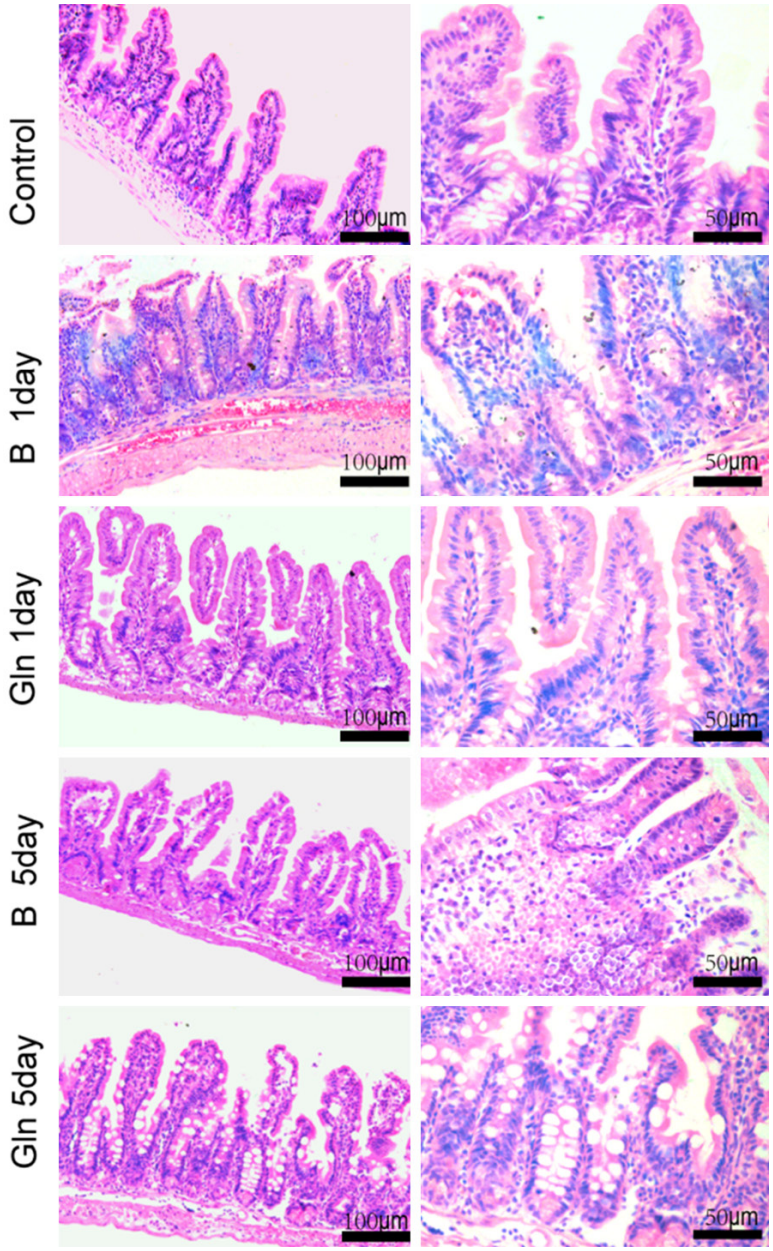

Figure 6. Intestinal tissue in the burn and gln groups on days 3 and 7 after burn injury, compared with control. HE staining, × 40 magnification.

decrease mucosal damage and maintain the structure of the intestinal mucosa after burn injury (**Figure 6**). Further studies found that goblet cells damaged and mucus synthesis ability was reduced postburn. Mucus histochemical staining showed that the chemical composition of mucus is obviously changed after burn. The degree of the three mucus staining was significantly reduced whether it was intracellular (goblet cells) or extracellular (enteric cavity), especially, the goblet cell number with strong positive staining by HID-AB, PAS, and AF was decreased significantly. Glutamine administration could obviously reverse this trend, there was significant difference between group B and group Gln from 3 days to 7 days after medication (**Figures 7-9**).

Discussion

A series of basic and clinical studies had proven that the large, deep burn causes intense stress and persistent inflammation, resulting in vascular endothelial leak, hypovolemia and poor peripheral perfusion [28, 29]. It is now recognized that organ damage after burn injury may largely be ascribed to ischemia/hypoxia [30]. In this study, we found that IMBF decreased significantly after burn injury, with lowest levels observed one day postburn. Blood flow subsequently recovered but remained significantly lower than baseline levels at days 10 postburn. Inadequate tissue perfusion may lead to obvious organ structural damage and dysfunction. The results of this study showed clear pathologic changes in intestinal mucosa after burn injury (**Figure 6**). Some indicators related to tissue damage (including intestinal mucosal damage index, DAO activity, and intestinal mucosa permeability) were remarkably higher in mice subjected to burn injury than in the control group (**Figure 1B-D**). Tissue ischemia may cause cell damage, interfere with cellular metabolism and slow tissue repair. The significant decrease in ATP levels in intestinal epithelial cells indicates impaired energy synthesis in enterocytes after burn injury. Insufficient energy exacerbates cell damage, delays repair, and interferes with mucus synthesis in goblet cells. The

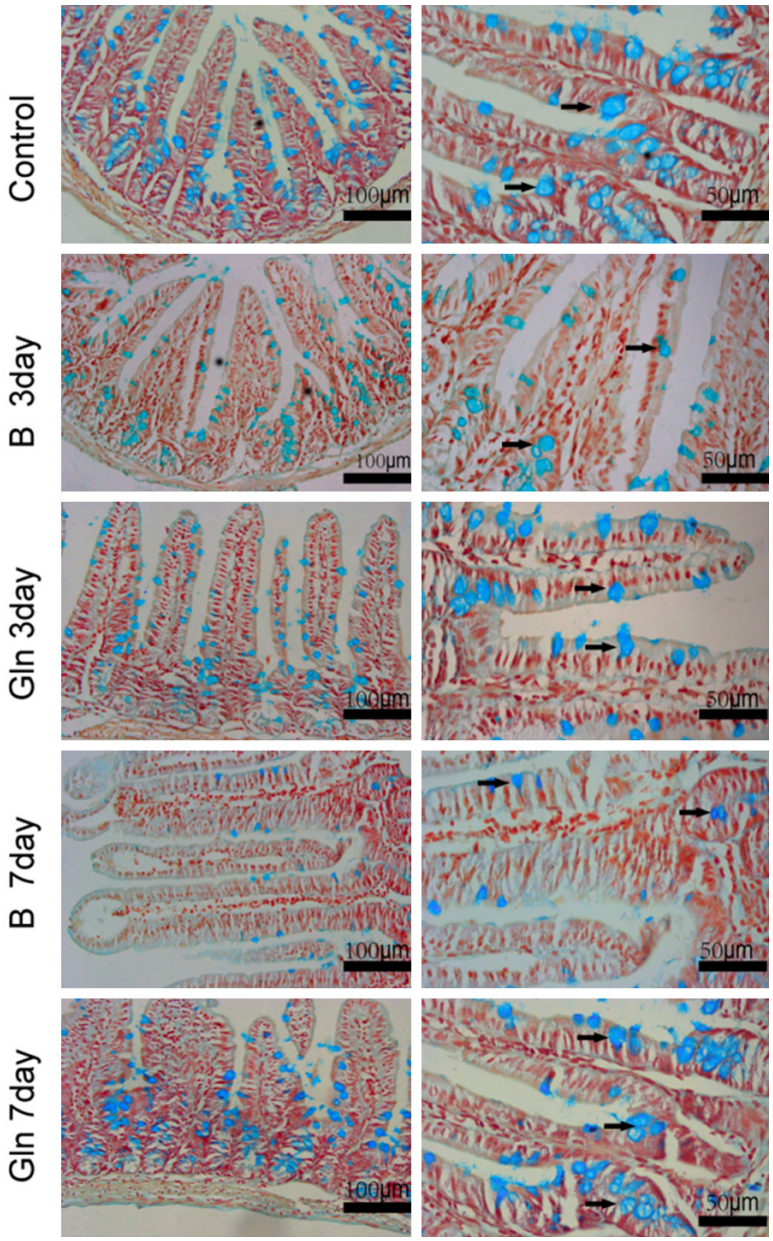

Figure 7. Intestinal tissue in the burn and gln groups on days 3 and 7 after burn injury, compared with control. HID-AB staining, × 40 magnification.

results presented above include significantly decreased levels of protein, hexose, and sialic acid, as well as decreased mucosal thickness in group B, compared with levels in normal controls (**Figure 2**). Our study have obtained similar results through histochemical staining of mucosal tissue (**Figures 7-9**). These results demonstrate that burn injury resulted in significant damage to intestinal mucosa, decreased secretion of mucus by goblet cells decreased, as well as altered mucus composition. These findings indicate that the intestinal mucosal barrier was remarkably impaired after burn injury.

Glutamine is important for energy synthesis in enterocytes and plays a key role in maintaining the integrity of intestinal mucosa [3]. However, whether glutamine may protect the mucus barrier has remained unclear. A variety of research methods were used in this study to investigate the effects of glutamine on the intestinal mucus barrier. The results clearly reflected the value of glutamine in maintaining the integrity of the intestinal mucus barrier. Mucosal levels of protein, hexose, and sialic acid, as well as mucosal thickness, were significantly higher after treatment with glutamine, compared with group B (**Figure 2**). These results support the findings obtained through histochemical analysis. The number of goblet cells with histochemical positive staining for HID-AB, PAS and AF increased (**Figures 7-9**), indicating improved mucus processing and modification in goblet cells.

Supplementation with glutamine improved hemoperfusion, suggesting glutamine contributes to maintenance of the intestinal mucus barrier through alleviation of damage to goblet cells after burn injury. In our previous studies, glutamine supplementation was shown to lessen the stress response to burn injury, decrease the secretion of adrenergic neurotransmitters, decrease intestinal vascular systolic state, and improve intestinal perfusion [11]. Another study showed that through transamination, glutamine may be converted to arginine, the substrate of nitric oxide (NO), which promotes NO synthesis, and eventually improves intestinal

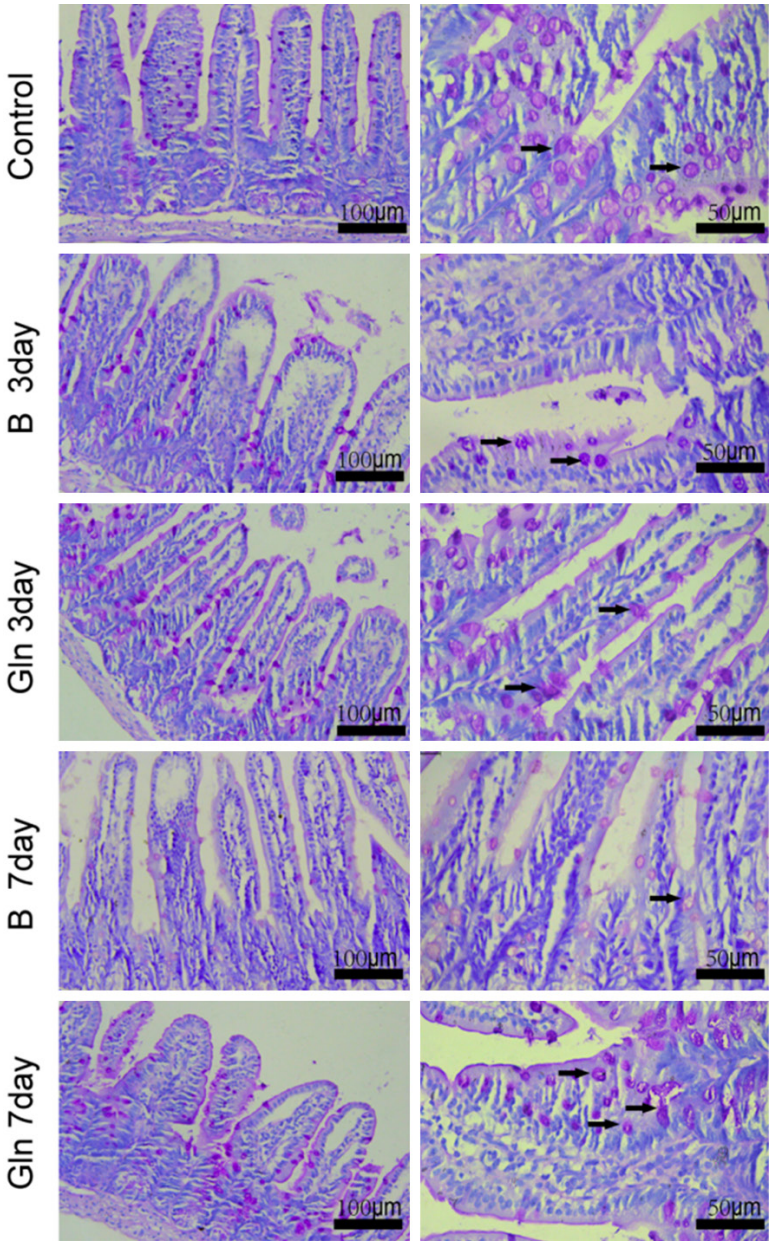

Figure 8. Intestinal tissue in the burn and gln groups on days 3 and 7 after burn injury, compared with control. PAS staining, × 40 magnification.

blood-flow [31]. It will ultimately improve intestinal mucosal perfusion and facilitates cells energy metabolism. The results presented above showed that the ATP content of intestinal epithelial cells was significantly increased after supplementation with glutamine (**Figure 3D**). Moreover, glutamine is regarded as an important part of energy synthesis in the small intestine, it provides a source of carbon for intestinal epithelial cells and participates in the Krebs cycle, and generates energy [32]. One previous study showed that glutamine could protect intestinal epithelial cells against cellular oxidative stress [14]. In addition, the level of reduced GSH in mucosa was remarkably elevated after supplementation with glutamine (**Figure 3C**). Observed changes in levels of GSH were consistent with the observed changes in mucus content and composition, all of which were negatively correlated with the degree of tissue damage and mucosal permeability.

The experiments conducted also showed that levels of MUC2, a core component of intestinal mucus, were significantly reduced postburn. This effect was reversed by treatment with glutamine (**Figure 5**). The family of mucin glycoproteins includes 12 members, MUC2 is primarily expressed in intestine [33]. MUC2 is glycosylated and polymerizated in the endoplasmic reticulum, then released to mediate the synthesis of synthesis [34].

Levels of CHOP and GRP-78, a marker of endoplasmic reticulum stress (ERS), were increased after burn injury. The administration of glutamine may inhibit this increase in ERS (**Figure 4**). Glutamine may effectively inhibit ERS induced by burn injury. One previous study found that the degree of ERS in burned rats was decreased after treatment with glutamine [14]. Ischemia/hypoxia, energy deficiency and oxidative stress are the main factors leading to ERS. Glutamine appears to inhibit ERS by increasing blood flow to the intestinal mucosa, improving energy metabolism, and enhancing GSH synthesis. These effects alleviate ERS and allow for the modification of proteins.

Our study also showed that glutamine may promote the secretion of ITF by goblet cells. ITF,

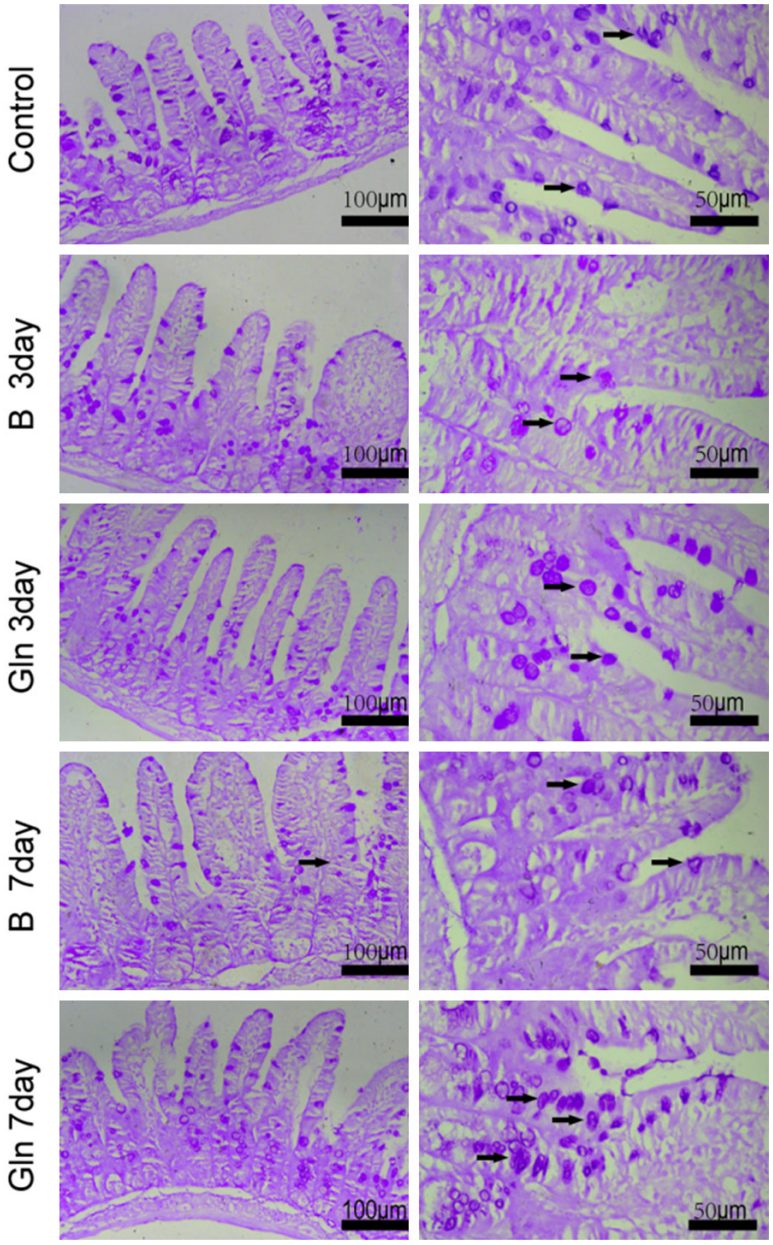

Figure 9. Intestinal tissue in the burn and gln groups on days 3 and 7 after burn injury, compared with control. AF staining, × 40 magnification.

initially known as mucus-binding peptide, is a polypeptide growth factor secreted by goblet cells [34]. The molecule's structure allows it to associate with the sugar chains on mucin, resulting in the stabilization of intestinal mucus [35]. The synthesis and modification of ITF require effective endoplasmic reticulum function. After burn injury, intestinal ERS caused by ischemia/hypoxia may interfere with the synthesis and modification of ITF, which decreases the stability of the mucosal layer and accelerates degradation. The administration of glutamine may significantly promote ITF secretion, with associated improvements in mucosal thickness and composition. We therefore believe that maintenance of the intestinal mucus barrier is mediated by the effects of glutamine in promoting ITF synthesis by goblet cells.

The results presented above show that glutamine may protect the intestinal mucosa. After burn injury, glutamine not only reduces the severity of mechanical damage to the intestinal mucosa but also significantly improves barrier function. Glutamine treatment improves blood perfusion and increases energy generation by enterocytes. Glutamine also decreases ERS and increases the synthesis of mucin and ITF.

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Disclosure of conflict of interest

None.

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Supplementary Data

Index of intestinal mucosal damage

Histopathologic examination of jejunum was performed by HE staining; the slides were observed and photographed with an Olympus microscope. The system for scoring mucosal injury was defined as follows: normal villi, 0 points; presence of a cystic space on 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 villous tip, 3 points; degeneration of the epithelial cell layer, necrosis, shedding, partial villi abscission, nude lamina propria, capillary dilation, hyperemia, 4 points; villi shedding, disintegration of lamina propria, bleeding, ulceration, 5 points.

Measurement of intestinal mucous membrane permeability

1480 KBq/kg of [^{99m}Tc] DTPA-HAS was injected into each mouse via the caudal vein, the mice were decapitated 30 min later. Scintillation counting of intestinal lavage solution was performed. Permeability of the intestinal mucous membrane at various phrases after burn injury was normalized to the level of flux across the intestinal mucous membrane in control mice. A decay control was set for count adjustment.

GSH levels in intestinal mucosa

Levels of GSH in intestinal mucosa were measured with commercially available kits (Nangjing Jiancheng Biochemical Ltd, China), according to the manufacturer's instructions.

Levels of ATP in intestinal mucosa

A mortar and pestle were used to convert intestinal tissue to powder form. Powder was then transferred to a test tube containing 0.6 N perchloric acid. Extracted metabolites were neutralized with a mixture of KOH and K_2CO_3, then centrifuged at 8000 rpm for 15 min at 4°C. Ten milliliters of supernatant was subjected to HPLC with UV/VIS-152 (Gilson, France). Results are expressed as micromole ATP per gram protein.

Hematoxylin and eosin (HE) staining

Intestinal tissue specimens were removed from 10% formaldehyde solution after 24 hr and rinsed with saline. Filter paper (Yiyuan Machine Tool Accessory Co., Ltd, Yantai, China) was used to gently and carefully wipe surface liquid. Specimens were dehydrated overnight in 95-100% alcohol in an automatic hydro-extractor (TP1020, Leica, Wetzlar, Germany). Intestinal tissue was sectioned into 3-μm slices with a pathologic tissue embedding machine (HistoCore Arcadia, Leica, Wetzlar, Germany). Slices were gently placed onto glass slides using forceps, and any folds were stretched. Slices were then dewaxed with xylene (twice, each time for 10 min). Sections were rinsed with saline, stained for 5 min with hematoxylin, and rinsed, then stained for 30 s with 0.5% eosin. Sections were then dehydrated with alcohol and dealcoholized with xylene. Neutral gum and coverslips were applied. Sections were examined using light microscopy with × 400 magnification (Leica DM1000, Leica, Wetzlar, Germany) and photographed.