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Effect of glutamine on intestinal barrier function following liver transplantation in rats

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Abstract. – OBJECTIVES: Glutamine is an important fuel for intestinal mucosal epithelial cells, and it promotes intestinal mucosal cell differentiation and proliferation. Most liver transplantation (LT) patients suffer from intestinal barrier dysfunction. Whether enteral glutamine supplementation has beneficial effects on intestinal barrier function following LT is not known. We investigated the effect of glutamine (Gln) supplementation on NF-κB and on the intestinal barrier in rats after an allogenic LT with concomitant immunosuppressive therapy.

MATERIALS AND METHODS: Inbred Sprague-Dawley rats (n=40) receiving allogenic LT were randomly divided into Gln and control groups (n=20, each). Gln group rats were administered Gln (0.4 g/kg·day) by gastric infusion for 6 days, while control rats received saline. Ten rats from each group were sampled for basal parameters on the 3rd day, prior to LT. The remaining 10 from each group were sampled after receiving LT. Twenty inbred Sprague-Dawley rats were selected as donors. The 20 recipients underwent orthotopic LT after 3 days of treatment and were given immunosuppressive therapy for 6 days post-operation. They were euthanized for sample collection on the 7th day. NF-κB protein in the intestinal mucosa, portal plasma Gln, endotoxin and TNF-α levels, ileocecal sIgA content, bacterial translocation and mucosal ultrastructure were assessed.

RESULTS: On the postoperative day 6, the Gln group had increased plasma Gln and ileocecal sIgA (secretory IgA). Gln group also showed improvement in mucosal microvilli structure and had reduced levels of intestinal mucosal NF-κB, portal endotoxin and TNF-α and decreased bacterial translocation as compared to the control group.

CONCLUSIONS: Parenteral supplementation of glutamine ameliorated mucosal injury during allogenic LT, and improved intestinal barrier function. These findings suggest that glutamine supplementation may be an effective therapy to ensure successful recovery from liver transplantation.

Key Words:
Glutamine, NF-κB, TNF-α, Intestinal barrier function, Liver transplantation, Malnutrition.

Introduction

Patients who have undergone liver transplantation (LT) often experience various degrees of malnutrition during end-stage liver disease[1]. The recipients inevitably suffer from an anhepatic phase, and from and gastrointestinal congestion lasting 45-60 minutes or longer during the LT surgical operation[2]. Additionally, the liver graft itself is also be subjected to ischemia-reperfusion injury, and the epithelial cells of the gastrointestinal mucosa become susceptible to apoptosis and necrosis by hypoxia-ischemia[3]. Inflammatory cascade reactions are activated after the operation, thus, aggravating intestinal and hepatic injury[4]. These factors may lead to many postoperative complications, such as intestinal endogenous infection, intestinal barrier dysfunction, and increased postoperative mortality rate. Therefore, protection of intestinal barrier function and improvement of malnutrition in LT recipients are essential during the peri-operative period.

Glutamine is preferentially metabolized by rapidly proliferating enterocytes, and is also a critical substrate for general metabolism[5]. As a supplement, glutamine has the advantage of being highly water-soluble and stable, and can be sterilized at a high temperature, making it easy to utilize in the clinic[6]. Moreover, a large number of dipeptide carriers are expressed on the cell surface of the mucosal epithelium, which can effectively transport glutamine even if the intestinal barrier is impaired[7]. It has not been previously reported that enteral Gln supplementation affects intestinal barrier function in recipients of LT. In the current study, we have investigated the effects of Gln supplementation on parameters of intestinal barrier function in order to determine its potential as a therapy to prevent post-operative complications following liver transplantation.

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Materials and Methods

Study Animals

Male, inbred, specific pathogen-free (SPF) Sprague-Dawley SD rats (purchased from Beijing Vital River Laboratories) were housed in an SPF room in the Laboratory Animal Center of Tong Ji University, China. The rats weighed 252 ± 36 g, and were caged at 25°C on a 12-hour light/dark cycle at a humidity of 60-70%. Animals were provided sterilized, deionized water and standard rat chow *ad libitum*. All animals received human care and the study was conducted according to the Guide for the Care and Use of Laboratory Animals.

Experimental Protocol and Operation Methods

Animals were randomly divided into the Gln group (n=20) and the control group (n=20), Both groups were administered enteral nutrition with equal heat and nitrogen (heat 125.4 kJ/kg·day, nitrogen 0.2 g/kg·day). In the present experiment, we used rats with slight malnutrition as the research model, which more closely represents the malnutrition seen in patients in a clinical setting. At the beginning of the study, rats were given 2/3 their normal daily food intake, but were provided water ad libitum (3 days pre-operation). The weight of all rats decreased by about 15% after 3 days and the rats showed slight malnutrition before receiving LT. The Gln group was treated with Gln (0.4 g/kg·day) by gastric perfusion, and control rats received 0.9% saline (0.4 g/kg·day) by gastric perfusion. Ten SD rats from each group were euthanized and sampled for normal parameters on preoperative day 3. The remaining 10 rats in each group received LT from 20 inbred SD rats that were previously selected as donors (n=20). Orthotopic LT was performed in the 20 recipients under aseptic conditions after a 3-day fast and they were subcutaneously injected with 2 mg/kg cyclosporin A daily after the operation. The 20 recipients underwent orthotopic LT and were given immunosuppressive therapy under sterile conditions for 3 days after surgery. These animals were euthanized for sample collection the 7th day of the experiment (4 days post-operation).

Surgical Procedures

The orthotopic LT was performed using a modified two-cuff technique without anastomosis of the hepatic artery. Both donor and recipient were anesthetized by intraperitoneal injection of ketamine (100 mg/kg). After the donor liver was segregated, the graft was perfused via the portal vein with chilled saline (0-4°C) containing 25 U/mL heparin. The graft was submerged in 0-4°C Ringer's lactate until being placed in the recipient abdomen. After anastomosis of the supra-hepatic vein cava and portal vein was completed, the liver was re-perfused. The common bile duct was reconstructed by tying the duct over a stent, and the anhepatic phase lasted for 25-30 minutes. Normal saline (2-3 mL) was injected through the penile vein of the recipient after the operation. The rats were placed on an electric platen for re-warming for 30 min, and were given a continuous supply of oxygen. Upon awakening from anesthesia, rats were given free access to sterilized water and standard rodent chow. No serious complications of blood vessels or the bile duct occurred during surgery. The recipients in each group were still alive on postoperative day 6.

Sample Collection

All rats were sampled under strictly sterile conditions. The portal vein was punctured and a blood sample was collected for measurement of plasma Gln, endotoxin and TNF-α. About 20 cm of the terminal ileum was obtained in order to test for intestinal mucosal NF-κB content, and ileocecal secretory IgA (sIgA) levels. Tissue samples from the left lobe of the liver were harvested for the study of bacterial translocation. Ileal mucosal samples were biopsied about 1.5-2.0 cm from the ileocecal valve, and fixed in 2.5% glutaraldehyde for histological study by electron microscopy.

Portal Plasma Gln, Endotoxin and TNF-α

The portal blood sample (100 μL) was placed in a pyrogen-free heparin-containing tube and centrifuged at 3000xg for 15 minutes at 4°C. Plasma Gln was measured by high performance liquid chromatography (HPLC; Waters Corporation, Milford, MA, USA). Plasma endotoxin was measured using a quantitative, chromogenic Limulus amebocyte lysate assay (Eihua Medical, Shanghai, China) according to the manufacturer's instructions. Plasma TNF-α was assessed using an enzyme-linked immunosorbent assay (ELISA) (Groundwork Biotechnology Diagnosticate Ltd., San Diego, CA, USA) in accordance with the manufacturer's protocol. All plasma levels are expressed in ng/L.

Intestinal Mucosal NF-κB Content

About 10 cm of the terminal ileum was acquired and washed with normal saline. Sections were taken for hemotoxylin-eosin staining and for tissue microarray using methods as previously described[3]. Rabbit anti-human NF-κB polyclonal antibody (Bioworld, Dublin, OH, USA), immunohistochemical SP method broad spectrum kit SP-9000, and DAB chromogenic kit (Golden Bridge Company, Zhongshan, China), were used for immunohistochemical staining following manufacturer's protocols. A known positive biopsy was used as a positive control, and treatment of sample with phosphate buffered saline (PBS) instead of primary antibody was used as a negative control. NF-κB positive staining was observed as nuclei that were stained yellow/brown. A semi-quantitative scoring method was used to categorize staining: first, the number of positive cells < 5%, 5-25%, 26-50%, and > 50% are denoted as 0, 1, 2, 3 respectively. Second, for each section observed, positive cells staining intensity was ranked: uncolored, yellow, brown and tan were assigned 0, 1, 2, 3 resepctively. The final result was judged according to the sum of the two ratings with a score < 3 indicating a sample negative for NF-κB expression, and a score ε 3 denoting a sample positive for expression.

Ileocecal sIgA Content

Ileocecum samples (1.0 g) were homogenized in 1 mL PBS (pH 7.4) and centrifuged at 12000 × g for 20 minutes. The supernatant was taken for the measurement of sIgA by ELISA (R&D Ltd., Minneapolis, MN, USA) following the manufacturer's instructions. The sIgA content is presented as micrograms per g of tissue (sIgA μg/g).

Bacterial Culture and Identification

Liver samples were weighed and placed in a sterile glass homogenizer containing a nine-fold volume of anaerobic buffer 100 mL phosphate buffered saline (PBS) (0.2 M, pH 7.4) with 0.5 g cysteine-HCl, 0.5 mL Tween 80, and 0.5 g agar/L), and homogenized. A 50 μL aliquot of 10% diluted homogenate was streaked on a Colombia culture plate within 30 minutes of sample collection and incubated for 48 hours at 37°C. Bacterial colonies were counted, and results are expressed as bacterial colony forming units (CFU) per g tissue (log10 CFU/g). Additionally, specific species of the bacteria from the liver samples were identified by an automatic analyzer of bacteria (Model Viger 60, Saint-Pierre-Le-Viger, France).

Investigation of Intestinal Mucosal Ultrastructure

Ileal mucosal specimens were collected and prepared for electron microscopy using previously described, standard technical procedures[8]. Briefly, samples were fixed in 2.5% glutaraldehyde (4°C, pH 7.4), post-fixed in 1% osmium tetroxide, and embedded in an epon-araldite mixture. Ultra-thin sections were prepared, placed on mesh copper grids, and stained with uranyl acetate and lead citrate. The ultrastructure of the mucosa was analyzed on a Tecnai 10 electron microscope (Philips, Eindhoven, The Netherlands). Particular attention was paid to the ultrastructure of microvilli.

Statistical Analysis

All analyses were performed using the statistical software SPSS13.0 (SPSS Inc., Chicago, IL, USA). All data are mean ± standard deviation. Analysis of variance was used to compare groups. A $p < 0.05$ was considered statistically significant.

Results

Portal Plasma Gln, TNF-α, Endotoxin and sIgA

Compared to day 3 pre-operation, plasma Gln was reduced in both recipients groups on day 6 post-operation (both $p < 0.05$). Plasma Gln concentration in the Gln group (156.4 ± 26.8 ng/L) was significantly higher than that of the control group (121.8 ± 17.5 ng/L) on post-operative day 6 (Figure 1). The plasma levels of TNFα in both groups on postoperative day 6 were much higher than those on pre-operative day 3 (Figure 2).

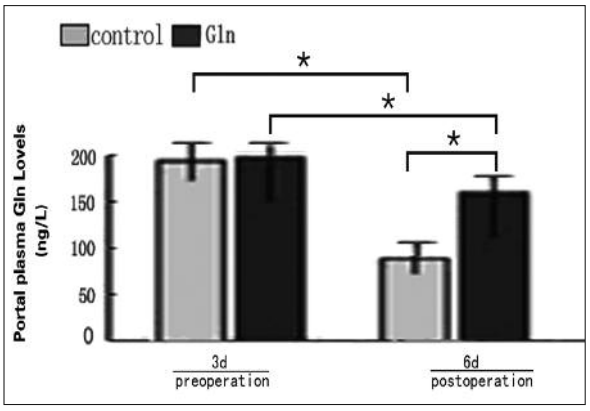

Figure 1. The changes of glutamine in rat portal plasma in control and Gln group. Data are mean ± SEM, *p value < 0.05.

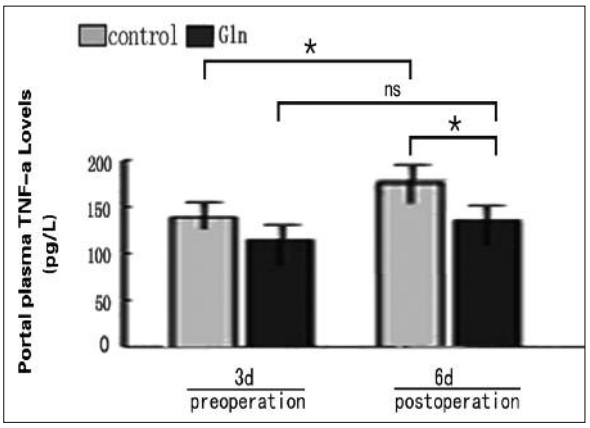

Figure 2. Glutamine affect the leveles of TNF-α in rat portal plasma in control and Gln group. Data are mean ± SEM, *p value < 0.05, ns: p value > 0.05.

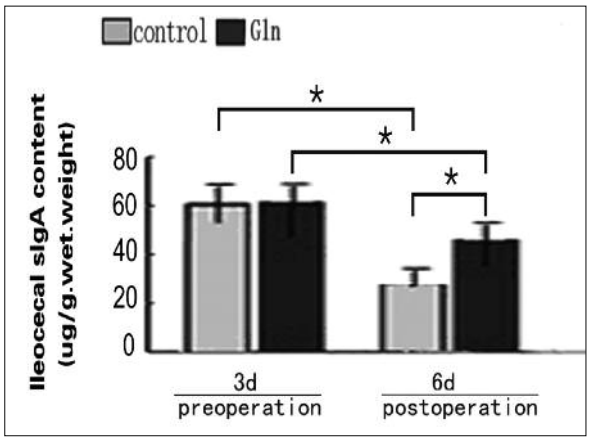

Figure 4. The levels of sIgA in rat portal plasma in control and Gln group. Data are mean ± SEM,*p value < 0.05.

However, the plasma level of TNF-α in the Gln group (102.6 ± 12.5 pg/L) was lower than that in the control group (137.7 ± 15.6 pg/L) on postoperative day 6. Compared to levels on preoperative day 3, plasma endotoxin was increased in both recipient groups on postoperative day 6, though there was a clear reduction in endotoxin levels in the Gln group (107.3 ± 11.5 ng/L) as compared to the control group (142.7 ± 23.8 ng/L) on postoperative day 6 (Figuere 3). The plasma levels of sIgA in both groups on postoperative day 6 were much lower than on pre-operative day 3. However, the plasma level of sIgA in the Gln group (43.2 ± 8.4 ng/L) was higher than that in the control group (24.6 ± 6.1 ng/L) on postoperative day 6 (Figure 4).

Bacterial Culture and Identification

Bacteria in samples from the liver were cultured, and the results indicated very low counts of bacterial colonies from samples taken on preoperative day 3. Bacterial counts on postoperative day 6 were significantly increased in both groups. Compared to the control group (4.37 ± 0.35 log10 CFU/g), however, bacterial counts were markedly decreased in the Gln group (3.56 ± 0.28 log10 CFU/g) on postoperative day 6 suggesting that there was a remarkable inhibition of bacterial translocation to the liver (Figure 5). Identification of bacterial species from the hepatic samples on postoperative day 6 revealed that the presence of *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae,*

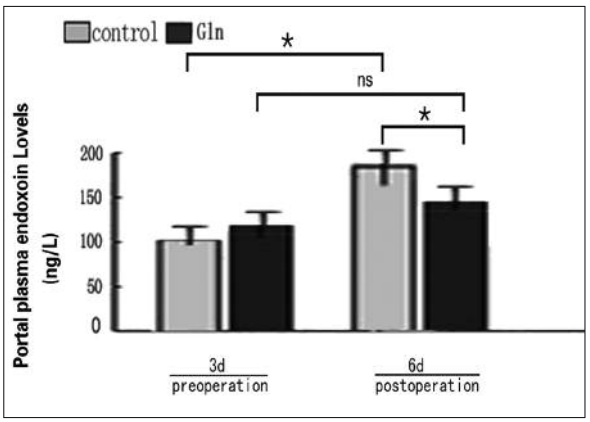

Figure 3. The portal plasma levels of endotoxin in rat in control and Gln group. Data are mean ± SEM, *p value < 0.05, ns: p value > 0.05.

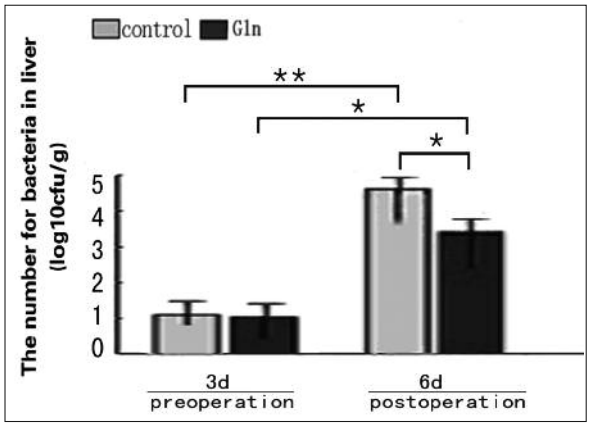

Figure 5. Bacterial translocation to rat liver in control and Gln group. Data are mean ± SEM, **p value < 0.001, *p value < 0.05, ns: p value > 0.05.

Table I. The number of bacteria in liver (log10 cfu/g).

Experimental	Control group			Gln group		
	POD3d	POD6d	*p* value	POD3d	POD6d	*p* value
Escherichia coli	0.37 ± 0.01	2.56 ± 0.85	0.002	0.37 ± 0.01	2.08 ± 0.51*	0.006
Enterobacter cloacae	0	1.37 ± 0.47	0.000	0	0.85 ± 0.26*	0.000
Klebsiella pneumoniae	0	1.12 ± 0.36	0.000	0	0.73 ± 0.23*	0.000
Proteus vulgaris	0	0.51 ± 0.17	0.000	0	0.48 ± 0.17	0.000
Streptococcus agalactiae	0	0.33 ± 0.11	0.000	0	0.21 ± 0.10	0.000

Proteus vulgaris, and *Streptococcus agalactiae* in the colonies found translocating to the liver (Table I).

NF-κB Content

The intestinal mucosal levels of NF-kB in both groups on postoperative day 3 and day 6 were much higher than those on pre-operative. However, the intestinal mucosal levels of NF-kB in the Gln group were much lower than those in the control group on postoperative day 6 (Table II).

Ultrastructure of Intestinal Mucosa

On postoperative day 6 the villus height and surface area were significantly lower than what was observed in samples from preoperative day 3 in both groups. In the Gln group, villus height and surface area were significantly greater than in the control group on postoperative day 6 (Table III).

Discussion

Malnutrition of patients in the perioperative period greatly increased the occurrence of complication and mortality. Patients with end-stage liver disease undergoing LT very frequently suffer from malnutrition, which is associated with increased morbidity and mortality. Therefore, it is essential to carefully assess patient nutrition along with treatment by conducting nutritional risk screening and identifying the appropriate nutritional deficiencies that must be corrected. Provision of adequate nutritional support would significantly improve surgery success rate in LT patients.

During LT surgery graft recipients have to undergo ischemia-reperfusion injury of the intestine and liver[9], which can lead to intestinal barrier damage. The destruction of intestinal barrier function might cause translocation of bacteria and endotoxin to the liver, and may trigger hepat-

Table II. Immunohistochemical examination of nuclear NF-κB expression in intestinal mucosal epithelial cells.

Group	n	pre-operation	POD3d POD6d
Control	24 0 (0.0)	16 (66.7)*	15 (62.5)*
Gln	24 0 (0.0)	14 (58.3)*	9 (37.5)*,$^{\Delta}$

Compared with pre-operative, *$p < 0.05$; Compared with control group, $^{\Delta}p < 0.05$.

Table III. The changes of rat intestinal villus height villus surface area in control group and in Gln group.

Group	Index	POD3d POD6d	*p* value
Control villus height (μm)	417.5 ± 62.8	325.2 ± 52.4	0.027
Gln	417.5 ± 62.8	362.5 ± 51.4*	0.041
Control villus surface area (mm^2)	0.28 ± 0.02	0.12 ± 0.01	0.032
Gln	0.28 ± 0.02	0.17 ± 0.01*	0.045

Data are mean ± SEM, *p value < 0.05, vs control group on postoperative day 6.

ic and peritoneal macrophage activation, increasing production of inflammatory mediators and cytokines which can induce systemic inflammatory response syndrome[10].

Gln is a conditionally essential amino acid, most abundant in the blood and skeletal muscle[11], and also important to the maintenance of intestinal barrier function[12]. Though Gln deficiency does not occur normally, Gln concentration can decrease significantly in blood and tissues during ischemia reperfusion injury[11]. Our results show that increasing plasma Gln concentration by enteral supplementation of Gln protects intestinal barrier function and significantly reduces bacterial translocation and inflammation in LT. Inflammation plays a major role in contributing to the dysfunction of the intestinal barrier[4]. TNF-α is an important pro-inflammatory factor that is produced from monocyte-macrophage cells and endothelial cells. Increased TNF-α levels during LT can trigger inflammatory cascades, aggravate intestinal injury, and even induce systemic inflammatory response syndrome[13,14]. Our measurements confirmed that post-operation, TNF-α and endotoxin levels are increased. However, enteral Gln supplementation significantly reduced endotoxin and TNF-α levels in portal vein blood, indicating that provision of Gln can effectively reduce inflammation during LT. Gln supplementation also inhibited the post-operative increase in bacterial counts, mainly of aerobic bacteria such as *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Proteus vulgaris* and *Streptococcus agalactiae* being lower in the Gln group.

NF-κB is a key transcription factor that controls inflammation. In the normal state, NF-κB and IκB inhibitor are present in a non-active complex in the cytoplasm. When phosphorylated, IκB is degraded, allowing the release and activation of NF-κB. NF-κB then enters the nucleus and initiates transcription of pro-inflammatory genes such as cytokines including TNF-α and IL-6. Buron et al[15] found that the degradation of IκB increased in intestinal epithelial cells during Gln deficiency, and that Gln supplementation inhibited this degradation, preventing NF-κB activation and decreasing the secretion of inflammatory cytokines. Our data show that intestinal mucosal NF-κB expression was significantly up-regulated post-operation. However, NF-κB expression was significantly lowered by Gln supplementation. While the exact mechanism by which Gln reduces degradation of IκB and activation of NF-κB is not clear, we have shown that Gln supplementation markedly improves markers of inflammation, providing strong evidence for the use of Gln treatment to increase the success rate of LT.

In terms of histological morphology, rats after LT had intestinal mucosal destruction, manifested by intestinal villus epithelial cell necrosis, loss of ultrastructure, shortened mucosal villi length, increased gap between epithelial cells, accompanied by capillary congestion, interstitial edema and inflammatory cell infiltration. Previous research[16] revealed that enteral supplementation with Gln enhances Na^+-K^+-ATP enzyme activity and promotes the absorptive function of the small intestine. Another study[17] indicated that long-term parenteral Gln supplementation improves intestinal structure and function during heterotopic small-bowel autotransplantation in pigs. Furthermore, it has been shown that oral glutamine ameliorates chemotherapy-induced changes in intestinal permeability in patients with breast cancer[18]. We have also observed in our study that supplementation with Gln leads to significant improvement of intestinal mucosal ultrastructure during LT.

The intestinal immune barrier plays a pivotal role in defense. sIgA is an important component (secretory IgA) of the intestinal immune barrier. We measured sIgA levels in the ileocecum as a marker of altered immune barrier function. We found that ileocecal sIgA was significantly reduced after LT in the two groups, which might be due to the atrophy of gut-associated lymphoid tissue and reduction of intestinal sIgA secretion following intestinal ischemia-reperfusion injury[19]. However, enteral supplementation with Gln increased intestinal sIgA levels. These results are consistent with the report that glutamine-enriched enteral nutrition maintains small intestine gut-associated lymphoid tissue and protects against intestinal mucosal barrier injury[20].

Conclusions

Our study has provided strong evidence that enteral supplementation with Gln reduces bacterial and endotoxin translocation, decreases inflammation, and improves immune barrier function. These findings suggest that enteral supplementation with Gln is an effective mean to improve outcomes of liver transplantation, and may have a vital, positive impact on the recovery of patients in liver transplantation.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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