Effects of Glutamine-Containing Total Parenteral Nutrition in Rats Undergoing Gastrectomy

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Running title: Effect of glutamine in rats with gastrectomy

Precis

TPN supplemented with GLN improved nitrogen balance and peritoneal macrophage phagocytic activity was enhanced after gastrectomy. GLN had no effect on phagocytic cell in the systemic circulation, and growth hormone and insulin like growth factor-1 may not responsible for attenuating nitrogen losses in rats with gastrectomy.

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ABSTRACT

Background: Surgery is known to impair immune response and increase nitrogen loss. Previous reports have shown that glutamine (GLN) increase nitrogen retention and enhance immune functions after surgery. However, no study examined GLN on phagocytic activity after gastrectomy, and the role of GLN on the secretion of growth hormone (GH) and nitrogen retention is not clear. ***Methods***: Rats with internal jugular catheter, were divided into 2 experimental groups and received total parenteral nutrition (TPN). The TPN solutions were isonitrogenous and identical in nutrients composition except for differences in amino acid content. One group received conventional TPN (control), the other group replaced 25% of the total amino acid nitrogen as GLN (GLN). After receiving TPN for 3 days, one third of the rats in each experimental group were sacrificed as baseline group. The remaining rats underwent partial gastrectomy and were sacrificed 1 and 3d, respectively, after surgery. Plasma, peritoneal lavage fluid (PLF) and urine samples were collected for further analysis. ***Results***: The GLN group had lower nitrogen loss at 1^{st} and 2^{nd} d after surgery. There were no differences in plasma GH and insulin like growth factor-1 levels between the 2 groups before or after surgery. The phagocytic activity of peritoneal macrophage was higher in the GLN group than the control group 1 d after surgery. There were no differences in the phagocytic activities of blood polymorphonuclear neutrophils between the 2 groups at baseline and postoperative days. No significant differences of IL-1β and IL-6 concentrations in PLF were observed between the two groups. However, TNF-α levels in PLF was significantly lower in the GLN group than the control group on postoperative d 3. ***Conclusions:*** TPN supplemented with GLN improved nitrogen balance, enhanced macrophage phagocytic activity at the site of injury. However,

GLN supplementation had no effect on phagocytic cell in the systemic circulation, and GH and insulin like growth factor-1 may not responsible for attenuating nitrogen losses in rats with partial gastrectomy.

INTRODUCTION

Gastrectomy is an abdominal surgery, which usually causes metabolic stress to patients. An altered protein metabolism that is characterized by negative nitrogen balance and changes in plasma free amino acid pattern was often observed in surgical trauma.[1,2] For most gastrectomized patients for gastric diseases, preoperative protein-energy malnutrition is often present, and adequate oral intake after surgery is achieved late.[3,4] It is known that the visceral organ exposure and manipulation that occur with an abdominal operation result in an intestinal hypodynamic state and dyssynchrony of pancreatic enzyme supply, which may prevent immediate resumption of oral alimentation by the patient.[5,6] Artificial nutritional support is essential for these patients. Most surgeons use the parenteral route to administer nutrients before and after gastrectomy. However, the optimal formulation of TPN for patients with gastrectomy is still unknown.

In recent years, glutamine (GLN) has elicited great attention for its therapeutic use in treating diseases, because it has been demonstrated to have several desirable biological properties. GLN is the most abundant free amino acid in plasma and the tissue pool.[7] It has traditionally been thought of as a nonessential amino acid, but laboratory and clinical data suggest that it may be essential during certain catabolic conditions,[8,9] because studies have shown that hypercatabolic states are associated with profound GLN deprivation.[10-12] A number of studies have demonstrated the

beneficial effects of supplying GLN for metabolic-stressed conditions; these effects include increasing nitrogen retention, preserving the integrity of the intestinal mucosa and intestinal permeability, maintaining immunologic function, and reducing infections.[8,9,13,14] Parry-Billings et al.[9] reported that the depressed GLN concentrations were associated with reduced proliferation of lymphocytes from healthy volunteers and depressed phagocytosis by peritoneal macrophages from normal mice. Ogle et al.[15] also reported that GLN improved the bactericidal ability of abnormal neutrophils from pediatric patients after burns. An in vitro study by Wallace and Keast[16] showed that GLN is required for phagocytosis of opsonized sheep erythrocytes in macrophage culture. Furukawa et al.[17]revealed that supplemental GLN enhances phagocytosis by neutrophils from postoperative patients in vitro. Although Parry-Billings et al.[9] and Ogle et al.[15] suggested the efficacy of GLN supplementation, they did not supply GLN to their patients. In in vitro studies GLN was added to the culture medium, and the environment was different from the actual physiologic conditions. The beneficial effect of GLN on phagocytosis observed in those studies may not reflect in vivo situations. To our knowledge, there has been no study, so far, investigating the effect of GLN supplementation on phagocytic activity in surgery. Therefore, in this study, we infused GLN-containing parenteral nutrition before and after gastrectomy to investigate the effect of GLN on phagocytic activity at the site of injury and systemic circulation. Growth hormone (GH) is a anabolic hormone that can reduce whole body nitrogen loss after surgery.[18,19] A study showed that a low dose GLN supplementation is capable of elevating plasma GH.[20] We analyzed plasma GH and insulin like growth factor (IGF)-1 to elucidate the possible role of GLN on the secretion of anabolic hormone and nitrogen balance after gastrectomy.

CLINICAL RELEVANCY STATEMENT

In this study, we administered GLN-containing parenteral nutrition before and after gatrectomy to investigate the effect of GLN on phagocytic activity and to elucidate the possible role of GLN on the secretion of anabolic hormone and nitrogen balance in rats undergoing gastrectomy. The results demonstrate that TPN supplemented with GLN improved nitrogen balance, enhanced macrophage phagocytic activity at the site of injury. However, GLN had no effect on phagocytic cell in the systemic circulation, and growth hormone and insulin like growth factor-1 may not responsible for attenuating nitrogen losses in rats with gastrectomy.

MATERIALS AND METHODS

Animals

Male 7-week-old Wistar rats with body weights of 170 to 210 g at the beginning of the experiment were used. All rats were housed in temperature- and humidity-controlled rooms, and were allowed free access to a standard rat chow for 7 days prior to the experiment. The care of the animals followed standard experimental animal care procedures. This study was approved by the Taipei Medical University Animal Care Committee.

Study protocol and operation procedures

Rats were assigned to 2 experimental groups, with each group 30 rats, according to the weight of each animal in order to make average weights between the groups as similar as possible. After overnight fasting, the rats were anesthetized with

intraperitoneal pentobarbital (50 mg/kg), and the right internal jugular vein was cannulated with a Silastic catheter (Dow Corning, Midland, MI, USA) under sterile conditions. The catheter was tunneled subcutaneously to the back of neck and exited through a coil spring that was attached to a swivel, allowing free mobility of animals inside individual metabolic cages. Two milliliters per hour was administered on the first day, and the full-strength TPN solution was given thereafter. The infusion rate was maintained with a Terufusion pump (model STC-503, Terumo, Tokyo, Japan). All animals were allowed to drink water during the experimental period.

TPN provided 270 kcal/kg body weight, and the kcal/nitrogen ratio in the TPN solution was 145:1. The TPN solutions were isonitrogenous (6.84 mg/mL) and identical in nutrient composition except for the difference in amino acid content. One group received conventional TPN (control), the other group replaced 25% of the total amino acid nitrogen as GLN (GLN). The energy distribution of the TPN solutions in the experimental groups was glucose 72%, protein 18%, and fat 10% (Table I). GLN was dissolved and sterilized by passage through a 0.2 um Minisart NML filter (Sartorius Inc., Goettingen, Germany), and stored at 4^{o}C until used. GLN solution was stable at room temperature for at least 2 days as previously described.[14] The TPN solution was refilled daily and infused for 24 hr at room temperature. The rats received 46-57 kcal/d according to their body weight. TPN solution without fat was prepared every other day in a laminar flow hood, and the fat emulsion was added daily just before use. After receiving TPN for 3 days, one third of the rats in each experimental group were sacrificed as baseline group. The remaining rats underwent partial gastrectomy on the 4^{th} day of TPN, and were sacrificed 1 and 3d, respectively, after surgery. A partial gastrectomy was performed using the same method as in our previous study.[21,22] TPN was maintained for 3, 5 or

7 days according to the sacrifice schedule of the rats.

Measurements and analytical procedure

Rats in respective groups were sacrificed before or 1 or 3 d after surgery. The rats were anesthetized and a middle abdominal incision was made, and 10 mL of phosphate-buffered saline (PBS) was intra-peritoneally injected to elute the peritoneal cells. After harvesting the peritoneal lavage fluid (PLF), the rats were exsanguinated by drawing arterial blood from the aorta. The blood samples were collected in tubes containing heparin and were immediately centrifuged. Plasma amino acid was analyzed by standard ninhydrin technology (Beckman Instrument, model 6300, Palo Alto, CA), after deprotienization of the plasma with 5% salicylic acid.[23] Plasma growth hormone (GH) (Cayman Chemical, Ann arbor, MI) and insulin-like growth factor (IGF)-1 (Diagnostic Systems, Webster, TX) were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Interleukin(IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels in plasma and peritoneal lavage fluid (PLF) were measured using commercially ELISA microtiter plates, with antibodies specific for rat IL-1β, IL-6, and TNF-α having been coated onto the wells of the microtiter strips provided (Amersham Pharmacia Biotech Inc., UK).

A flow cytometric phagocytosis test was used to evaluate the phagocytic activity of blood polymorphonuclear neutrophils.[24,25] One hundred microliters of heparinized whole blood was aliquoted on the bottom of a 12 x 75-mm Falcon polystyrene tube (Becton Dickinson) and placed in an ice water bath. Twenty microliters of precooled opsonized FITC-labeled *E. coli* (Molecular Probes, Eugene, Oregon, USA.) was added to each tube. Control tubes remained on ice, and assay

samples were incubated for precisely 10 min at 37 ℃ in a shaking water bath. After incubation, samples were immediately placed in ice water, and 100 μL of a precooled trypan blue (Sigma, St. Louis, MO, USA) solution (0.25 mg/ml in citrate salt buffer; pH 4.4) was added to quench the fluorescence of the bacteria merely adhering to the surface of the phagocytizing cells. Cells were washed twice in Hanks buffered saline (HBSS), and erythrocytes were lysed by the addition of FACS lysing solution (Becton Dickinson). After an additional wash in HBSS, 100 μL of propidium iodide (PI) solution (1μg/mL in HBSS) was added to stain the nuclear DNA 10 min before the flow cytometric analysis. Flow cytometry was performed on a FACSCalibur^{TM} flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. A live gate was set on the red (PI) fluorescence histogram during acquisition to include only those cells with a DNA content at least equal to human diploid cells. The number of cells with phagocytic activity did not exceed 6% at 0 ^{o}C.

A Vybrant ^{TM} Phagocytosis Assay kit (Molecular Probes) was used to evaluate the phagocytic activity of peritoneal macrophages. After washing the peritoneal macrophages 3 times with HBSS, the cell concentration was counted, and the cell number was adjusted to 10^6 cells/ml with RPMI-1640 supplemented with 5% fetal bovine serum and an adequate amount of antibiotics. After distributing 100 μl of diluted solutions into each well on a 96-well microplates it was transferred to a 37 ℃ CO_2 incubator for 1 h to allow the cells to adhere to the microplate surface. The RPMI solution was removed from all microplate wells by vacuum aspiration, and then 100 μl of the prepared FITC-labeled *E. coli* was added to each well for 2 h. Labeled bacteria were removed by vacuum aspiration, and 100 μl of trypan blue suspension was added to all wells within 1 min. The excess trypan blue was immediately aspirated, and the experimental and control wells (without peritoneal macrophages) were read in the fluorescence plate reader using ~480 nm for excitation and ~520 nm

for emission.

Twenty-four-hour urine specimens were collected during the 3 infusion days after surgery for determination of nitrogen balance. Nonprotein nitrogen in urine was measured by a colorimetric method (Randox, Antrim, Ireland)

Statistics

Data are expressed as the mean ± SD. Differences among groups were analyzed by ANOVA using Duncan's test. A p value of less than 0.05 was considered statistically significant.

RESULTS

There were no differences in initial body weights between the two experimental groups at the beginning of TPN administration. All rats gained weight after TPN infusion, and weights were maintained postoperatively. No difference in body weight was seen between the two groups on postoperative day 1 and 3 (Fig 1). The GLN group had higher plasma GLN levels and on postoperative day 1. No significant difference were observed before surgery and 3 days postoperatively (Fig 2).

Compared with control group, GLN group had lower nitrogen loss at 1st and 2nd d after surgery (Fig 3 A). Significant better cumulative nitrogen balance was observed in the GLN group on postoperative days (Fig 3 B). Plasma GH concentrations were significantly lower after surgery in the control group, whereas no difference in GH levels were observed before and after surgery in the GLN group. There were no differences in GH and IGF-1 levels between the 2 groups before or

after surgery (Fig 4A,B). The phagocytic activity of peritoneal macrophage is higher in the GLN group than the control group on postoperative day 1 (Fig 5A). The phagocytic activities of blood PMNs were significantly higher after surgery, despite GLN was supplemented or not. There were no significant differences in the phagocytic activities of blood PMNs between the 2 groups at various time points (Fig 5B). No significant differences in concentrations of IL-1β and IL-6 in PLF were observed between the 2 groups at various time points. However, TNF-α levels in PLF is significantly lower in the GLN group than the control group on postoperative day 3 (Table 2).

DISCUSSION

In this study, 25% of total nitrogen in the TPN solution was supplied by GLN. This amount of GLN was found to enhance the immune response in rodents.[26,27] We administered TPN before and after gastrectomy, because patients undergoing gastrectomy are frequently malnourished, and TPN is essential for adequate nutrition support. Since human study may have wide variations owing to the age of the patients, severity of the diseases, infected area of the stomach and complication of other diseases, these variables may cause the interpretation of the data difficult. In this study we used an animal model with partial gastrectomy to investigate the effect of GLN on the catabolic and immune response in abdominal surgery.

Injury to the body results in a generalized catabolic response. Negative nitrogen balance together with a progressive loss of body protein is often observed,[28,29] possibly resulting from hormonal changes and cytokine secretion.[30,31] Many studies have shown that GLN supplementation enhances skeletal muscle synthesis which may

consequently improve nitrogen balance after elective surgery.[8,32,33] GH is known to exert many metabolic effects. Amongst them are nitrogen retention and preservation of muscle protein mass.[18,19] IGF-1 is one of the major effectors of GH action. The effects of GH are mediated in part by IGF-1 that is produced in the liver and locally in GH target tissues.[34] Study by Welbourne et al.[20] reported that oral GLN load is capable of elevating plasma GH in healthy adults. Hammarqvist et al.[35] demonstrated that GH together with GLN-containing TPN reduced nitrogen losses compared with Gln alone. The nitrogen retention data in the present study are in good agreement with previous reports.[8,32,33] In order to understand whether the nitrogen sparing effect of GLN is associated with GH and IGF-1, we analyzed these anabolic hormones and found that there were no differences in plasma GH and IGF-1 levels between the 2 group before and after operation. This finding suggests that GH and IGF-1 may not responsible for attenuating nitrogen losses under the present experimental condition.

Previous reports have shown that parenterally or enterally administered GLN lowered the incidence of infection in patients with bone marrow transplantation and multiple trauma.[36,37] Supplemental GLN improved the survival in experimentally *Escherichia coli*-induced peritonitis in rodents.[38,39] Nevertheless, the mechanisms underlying the enhancing effect of GLN on bactericidal capacity have not been fully elucidated. GLN is an important fuel for immune cells.[9] Macrophages use GLN at a very high rate.[40] Some in vitro studies have shown that GLN is required for macrophage phagocytosis.[9,15-17] In this study we found that the phagocytic activity of peritoneal macrophages was much higher in the GLN group after surgery compared to the control group, whereas no differences in the phagocytic activities of blood PMNs between the 2 groups was found. These findings indicate that GLN

supplementation enhances the macrophage phagocytic activity at the site of injury. The effect of GLN on phagocytic cells in the systemic circulation was not obvious. Since the relationship between higher plasma GLN concentrations and increased phagocytic activity in peritoneal macrophage remained consistent, high body GLN pool may favor the activation of macrophage at the location of injurious stimulus. In this study, we did not observed a reduced plasma GLN levels after surgery, this might mean the extent of tissue damage as partial gastrectomy performed here was not severe enough to evoke systemic response. This result was consistent with the report by Parry-Billings et al.[41] that plasma GLN levels did not change under minor surgery.

Cytokines are peptides produced by cells of the immune system that act as mediator of immune response and the response of tissues to injury. TNF-α, IL-1β, and IL-6 are considered to be important mediators of the integrated host response.[30,31] Studies showed that alterations in TNF-α and IL-6 have been proposed as biochemical markers of the stress response.[30,31,42] High plasma concentrations of IL-1 and TNF-α are associated with increased severity of inflammatory diseases.[42] Also, previous studies revealed that endotoxin is a potent stimulator of IL-6, and IL-6 is thought to be the most consistently identified cytokine mediator of postinjury infections.[43] These cytokines in plasma were not detectable at the time we took measurements. However, the cytokines in PLF were measured. Compared with baseline, IL-1β, IL-6 levels did not change after surgery. This result may indicate that postinjury infection was not obvious in this study. We observed that TNF-α was lower in the GLN group than the control group on postoperative day 3. This might mean that TPN with GLN reduces the production of inflammatory mediators at the site of injury. An in vitro study by Rohde et al.[44] showed that Gln had no effect on the production of IL-1β, IL-6, or TNF-α. Since the result of in vitro studies may not

actually reflect the in vivo situations; and samples used for evaluating the effect of Gln on cytokine production were derived from healthy volunteers, this may differ from the stressed metabolic condition observed in this study, and may consequently lead to different immune responses.

In summary, this study showed that parenterally infused GLN significantly enhances peritoneal macrophage phagocytic activity, and nitrogen balance was improved. However, GLN supplementation had no effect on phagocytic cell in the systemic circulation, and GH and IGF-1 may not responsible for attenuating nitrogen losses in rats with partial gastrectomy.

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Figure legends

Fig 1. Body weights of the experimental groups at beginning of TPN administration and before sacrifice were performed. No significant differences in initial body weight and weights after 1 and 3 d after surgery were seen between the 2 groups.

Fig 2. Plasma glutamine (GLN) levels of the 2 groups before and after surgery. Different letters indicate significant difference among the groups.

Fig 3. A) Nitrogen balance and B) Cumulative nitrogen balance between two groups after operation. Different letters indicate significant difference among the groups.

Fig 4. A) Plasma growth hormone (GH) and B) insulin-like growth factor-I (IGF-I) concentrations between two groups before and after operation. Different letters indicate significant difference among the groups

Fig 5. Phagocytic activity of A) peritoneal macrophage and B) peripheral blood neutrophils by flow cytometry between two groups before and after operation. Different letters indicate significant difference among the groups

Table 1. Formulation of the TPN solution

	Gln	Control
50% glucose	420	420
20% lipofudin	50	50
*Moriamine 10%	345	450
$NaCl_3$ 3%	35	35
K_3PO_4 8.7%	10	10
KCL 7%	10	10
Calcium gluconate 10%	10	10
$MgSO_4$ 10%	4	4
$ZnSO_4$ 0.6%	2	2
**Infuvita	8	8
Choline chloride (g)	1	1
GLN (g)	8.4	---
H_2O	105	---
Total volume	998	998
Total kcal	986	994

*From Chinese Pharmaceuticals, Taipei, Taiwan. Per deciliter contains: Leu 1250 mg, Ile 560 mg, Lys acetate 1240 mg, Met 350 mg, Phe 935 mg, Thr 650 mg, Trp 130 mg, Val 450 mg, Ala 620 mg, Arg 790 mg, Asp 380 mg, Cys 100 mg, Glu 650 mg, His 600 mg, Pro 330 mg, Ser 220 mg, Tyr 35 mg, aminoacetic acid (Gly) 1570 mg.

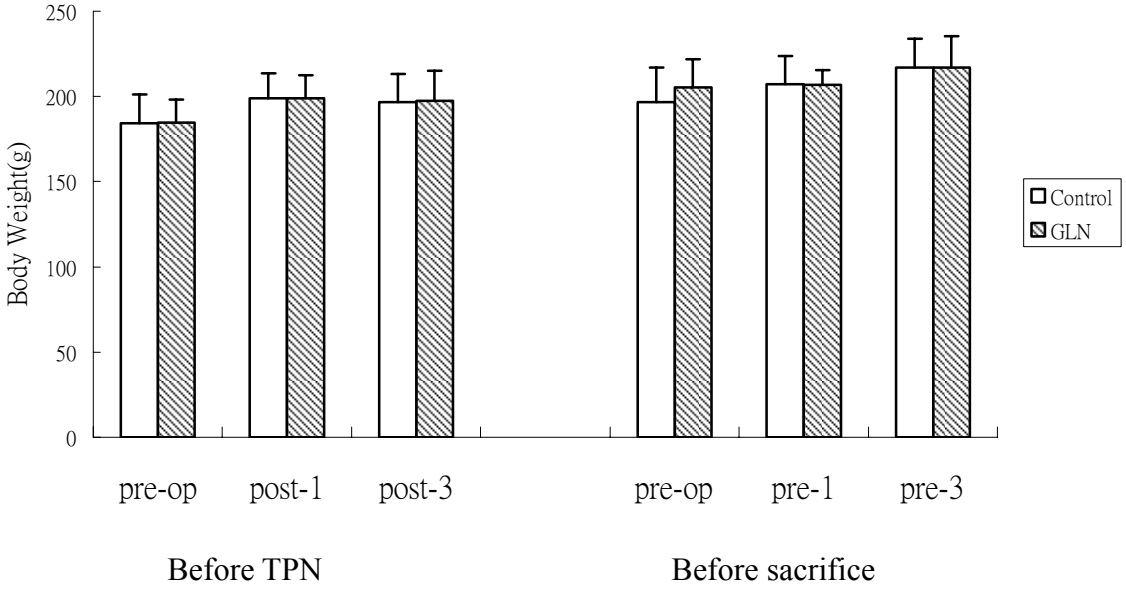
**From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Each milliliter contains: ascorbic acid 20 mg, Vitamin A 660 IU, ergocalciferol 40 IU, thiamine HCl 0.6 mg, riboflavin 0.72 mg, niacinamide 8 mg, pyridoxine HCl 0.8 mg, d-panthenol 3 mg, dl-alpha-tocopheryl acetate 2 mg.

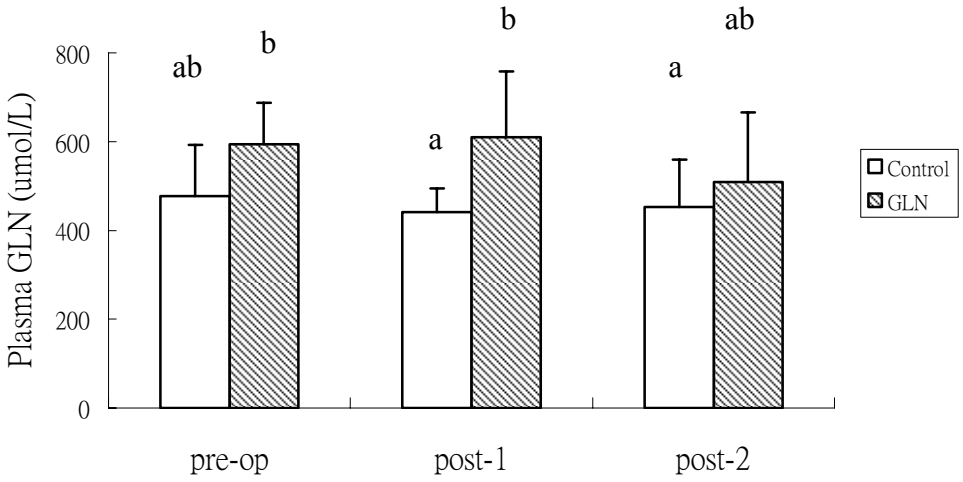
Table 2. Peritoneal lavage fluid (PLF) interleukin (IL)-1β IL-6 tumor necrosis factor (TNF)-α concentrations between two groups before and after operation.

	pre-op	post-1	post-3
		pg/mL	
IL-1β			
Control	10.1 ± 6.8	13.6 ± 13.6	17.9 ± 22.6
GLN	8.7 ± 9.3	13.2 ± 5.6	5.9 ± 6.1
IL-6			
Control	88.9 ± 46.1	130.0 ± 21.7	131.5 ± 50.8
GLN	94.0 ± 10.4	144.5 ± 51.7	118.0 ± 64.3
TNF-α			
Control	24.0 ± 16.6	10.2 ± 8.3	54.7 ± 28.5*
GLN	12.7 ± 5.3	22.1 ± 24.9	27.1 ± 21.5#

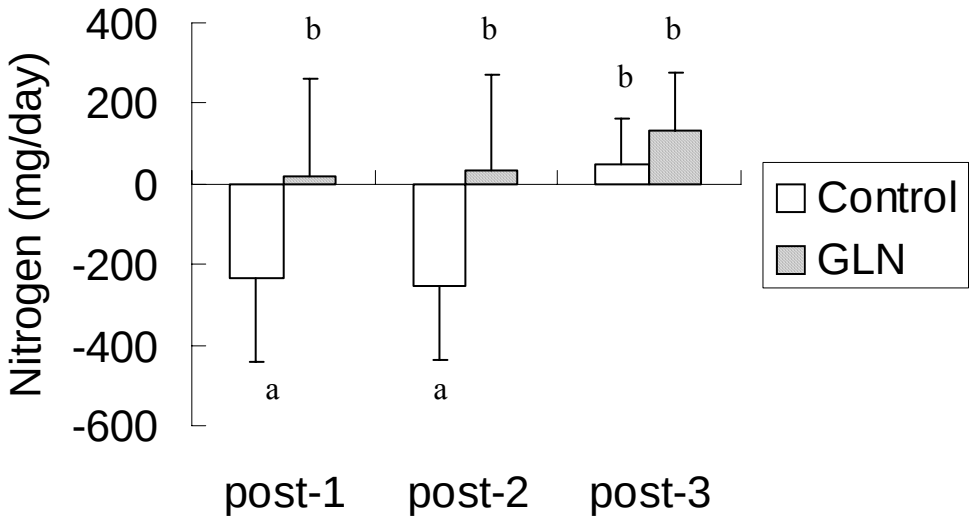
* Significant difference from pre-op and post-1 groups in the same line at $p < 0.05$

Significant difference from control group at $p < 0.05$

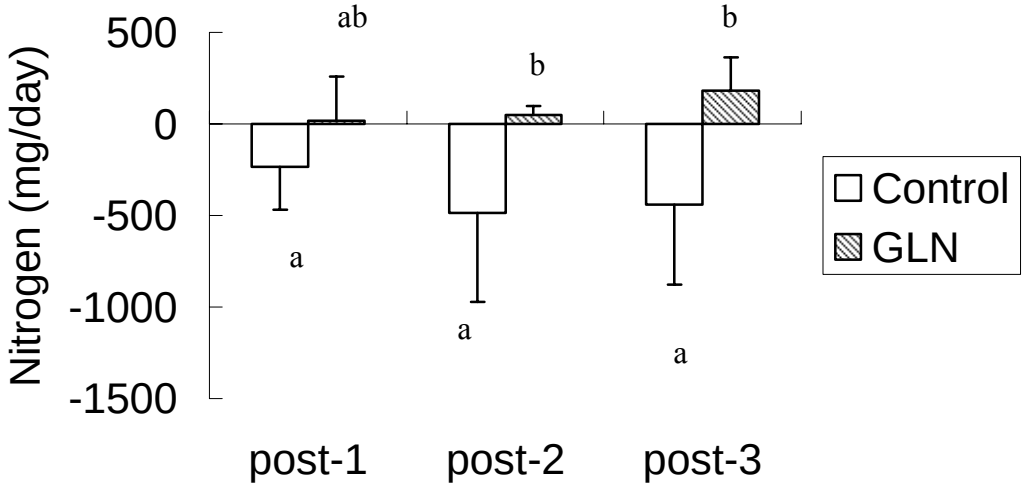

250
200
150
100
50
0
Body Weight(g)
Control
GLN
pre-op
post-1
post-3
pre-op
pre-1
pre-3
Before TPN
Before sacrifice


Plasma GLN (umol/L)
800
600
400
200
0
ab
b
a
b
a
ab
pre-op
post-1
post-2
Control
GLN

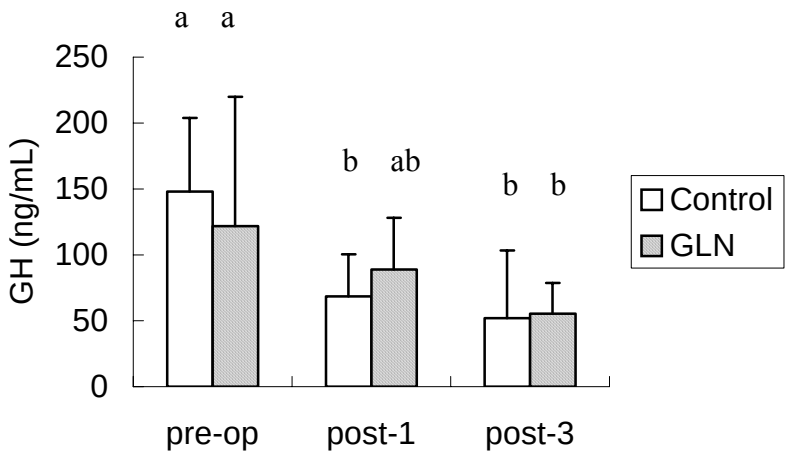
A)


400
200
0
-200
-400
-600
Nitrogen (mg/day)
b
b
b
b
a
a
post-1
post-2
post-3
Control
GLN

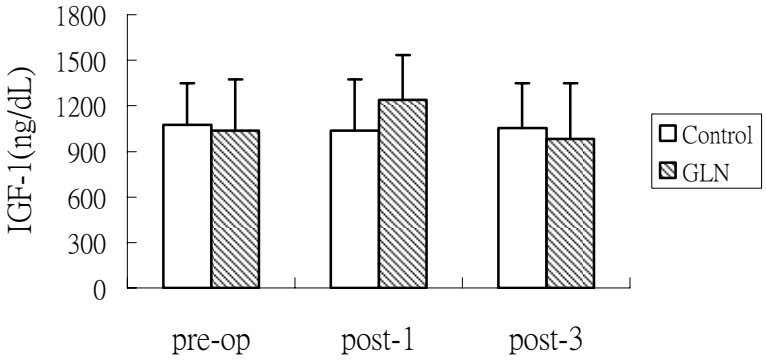
B)


500
0
-500
-1000
-1500
Nitrogen (mg/day)
ab
b
b
a
a
a
post-1
post-2
post-3
Control
GLN

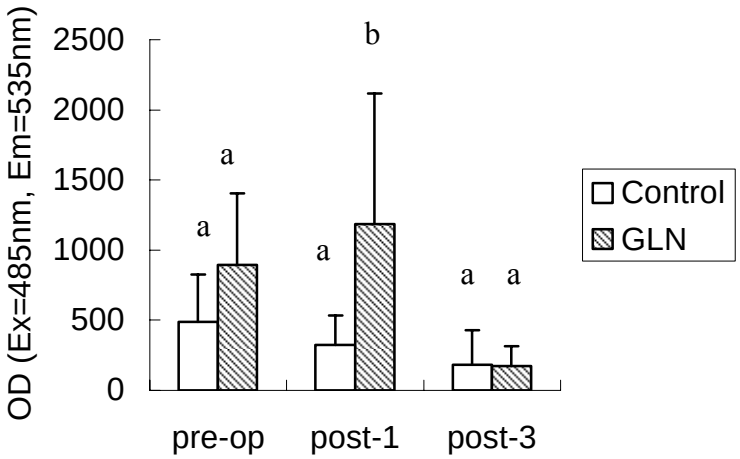
A)


a a
b ab
b b
250
200
150
100
50
0
GH (ng/mL)
pre-op
post-1
post-3
Control
GLN

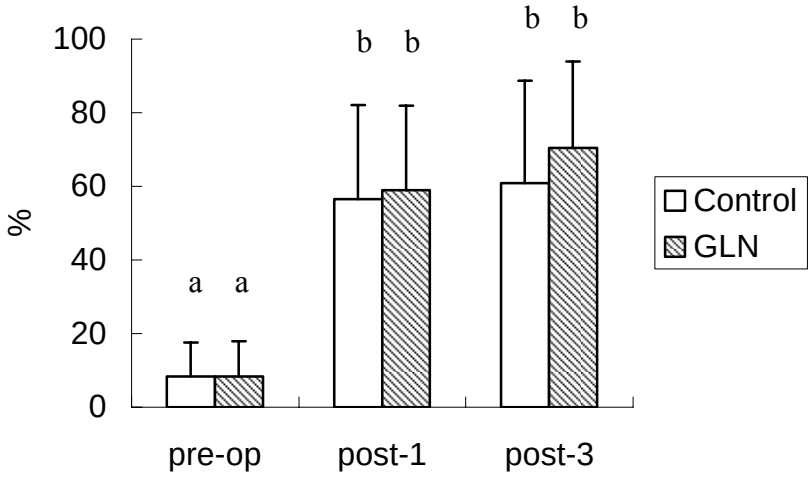
B)


1800
1500
1200
900
600
300
0
IGF-1(ng/dL)
pre-op
post-1
post-3
Control
GLN

A)


OD (Ex=485nm, Em=535nm)
2500
2000
1500
1000
500
0
a
a
b
a
a
a
pre-op
post-1
post-3
Control
GLN

B)


%
100
80
60
40
20
0
a a
b b
b b
pre-op
post-1
post-3
Control
GLN