Effects of Glutamine-Containing Total Parenteral Nutrition on Phagocytic Activity and Anabolic Hormone Response in Rats Undergoing a Gastrectomy

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Running title: Effect of glutamine in rats with a gastrectomy

Precis

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

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ABSTRACT

Background: Surgery is known to impair the immune response and increase nitrogen loss. Previous reports have shown that glutamine (Gln) increases nitrogen retention and enhances immune functions after surgery. However, no study has examined the effect of Gln on phagocytic activity after a gastrectomy, and the roles of Gln in the secretion of growth hormone (GH) and nitrogen retention are not clear. Methods: Rats with an internal jugular catheter were divided into 2 experimental groups and received total parenteral nutrition (TPN). The TPN solutions were isonitrogenous and identical in nutrient compositions except for differences in amino acid content. One group received conventional TPN (control), and in the other group, 25% of the total amino acid nitrogen was replaced with Gln (GLN). After receiving TPN for 3 d, one-third of the rats in each experimental group were sacrificed as the baseline group. The remaining rats underwent a partial gastrectomy and were sacrificed 1 and 3 d, respectively, after surgery. Plasma, peritoneal lavage fluid (PLF), and urine samples were collected for further analysis. Results: The GLN group had lower nitrogen losses 1 and 2 d after surgery (p < 0.05). 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 in the control group 1 d after surgery (p < 0.05). There were no
differences in the phagocytic activities of blood polymorphonuclear neutrophils between the 2 groups at the baseline or on the postoperative days. No significant differences in IL-1β or IL-6 concentrations in PLF were observed between the 2 groups. However, TNF-α levels in PLF was significantly lower in the GLN group than in the control group on postoperative day 3 (p < 0.05). **Conclusions:** TPN supplemented with Gln improved the nitrogen balance, and enhanced macrophage phagocytic activity at the site of injury. However, Gln supplementation had no effect on phagocytic cell activity in the systemic circulation, and GH and insulin-like growth factor-1 might not be responsible for attenuating nitrogen losses in rats with a partial gastrectomy.

**INTRODUCTION**

Surgeries of the upper gastrointestinal tract usually produce a moderate degree of metabolic stress. Altered protein metabolism characterized by a negative nitrogen balance and changes in plasma free amino acid pattern is often observed in surgical trauma.\(^1,2\) For most gastrectomized patients with gastric diseases, preoperative protein-energy malnutrition is often present, and adequate oral intake after surgery is achieved late.\(^3,4\) Artificial nutritional support is essential for these patients. Most surgeons use the parenteral route to administer nutrients before and after a
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 role in the treatment of diseases. Gln has traditionally been thought of as a nonessential amino acid, but laboratory and clinical data suggest that it may become essential during certain catabolic conditions,\(^5,6\) because studies have shown that hypercatabolic states are associated with profound depressed plasma Gln levels.\(^7-9\) A number of studies have demonstrated the beneficial effects of supplying exogenous Gln in the diet 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.\(^5,6,10,11\) Parry-Billings et al.\(^6\) demonstrated that depressed Gln concentrations were associated with depressed phagocytosis by peritoneal macrophages in normal mice. Ogle et al.\(^12\) also reported that Gln improved the bactericidal ability of abnormal neutrophils from pediatric patients after burns. Furukawa et al.\(^13\) revealed that supplemental Gln enhances phagocytosis by neutrophils from postoperative patients in vitro. Although Parry-Billings et al.\(^6\) and Ogle et al.\(^12\) suggested the efficacy of Gln supplementation, they did not supply Gln to their patients. The beneficial effect of Gln on phagocytosis in in vitro study might not reflect in vivo situations. To our knowledge,
there has been no study, to date investigating the effect of Gln supplementation on phagocytic activity after gastrectomy. Therefore, in this study, we infused Gln-containing parenteral nutrition before and after a gastrectomy to investigate the effect of Gln on phagocytic activity at the site of injury and systemic circulation. Growth hormone (GH) is an anabolic hormone that can reduce whole-body nitrogen loss after surgery.\textsuperscript{14,15} A study showed that low-dose Gln supplementation is also capable of elevating plasma GH.\textsuperscript{16} We analyzed plasma GH and insulin-like growth factor (IGF)-1 to elucidate whether Gln supplementation enhances the secretion of anabolic hormones and thus attenuates the nitrogen losses after a gastrectomy.

**CLINICAL RELEVANCY STATEMENT**

In this study, we administered Gln-containing parenteral nutrition before and after a gastrectomy to investigate the effect of Gln on phagocytic activity and to elucidate the possible roles of Gln in the secretion of anabolic hormones and nitrogen balance in rats undergoing a gastrectomy. The results demonstrate that TPN supplemented with Gln improved nitrogen balance and enhanced macrophage phagocytic activity at the site of injury. However, Gln had no effect on phagocytic cells in the systemic circulation, and growth hormone and insulin-like growth factor-1 might not be responsible for attenuating nitrogen losses in rats with a 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 d 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 randomly assigned to 2 experimental groups, with each group containing 30 rats. The average weights between the groups were adjusted as similar as possible. After overnight fasting, 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. All animals were allowed to drink water during the experimental period. TPN provided 270 kcal/kg body weight, the energy was proved to be adequate for weight maintenance for normal TPN rats.17 The
kcal/nitrogen ratio in the TPN solution was 145:1. The calorie density is almost 1 kcal/ml. 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). Although the amount of essential amino acids (EAA) was lower in the GLN group than the control group, the EAA was expected to be adequate for maintenance according to the reported EAA requirements for rats.\textsuperscript{18} The energy distribution of the TPN solutions in the experimental groups was 72% from glucose, 18% from protein, and 10% from fat (Table I). GLN was dissolved and sterilized by passage through a 0.2-um Minisart NML filter (Sartorius, Goettingen, Germany); it was stored at 4 °C until being used. Gln solution was stable at room temperature for at least 2 d as previously described.\textsuperscript{17} The TPN solution was refilled daily and infused for 24 h at room temperature. Two milliliters per hour was administered on the first day, and then the rats received 48-57 kcal/d according to their body weight. The infusion rate was maintained with a Terufusion pump (model STC-503, Terumo, Tokyo, Japan). The 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 d, one-third of the rats (n = 10) in each experimental group were sacrificed as the baseline group. The remaining rats underwent a partial
gastrectomy on the 4\textsuperscript{th} day of TPN, and were sacrificed 1 or 3 d, respectively, after surgery. A partial gastrectomy was performed using the same method as in previous study.\textsuperscript{19,20} TPN was maintained for 3, 5, or 7 days according to the sacrifice schedule of the rats.

\textit{Measurements and analytical procedure}

Rats in the respective groups were sacrificed before or 1 or 3 d after surgery. The animals were anesthetized with intraperitoneal pentobarbital (50mg/kg BW). A middle abdominal incision was made, and 10 mL of phosphate-buffered saline (PBS) was intraperitoneally injected to elute the peritoneal cells. After harvesting the peritoneal lavage fluid (PLF), rats were exsanguinated by drawing arterial blood from the aorta. Blood samples were collected in tubes containing heparin and were immediately centrifuged. Plasma amino acids were analyzed by standard ninhydrin technology (Beckman Instruments, model 6300, Palo Alto, CA, USA), after deproteinization of the plasma with 5\% salicylic acid.\textsuperscript{21} Plasma GH (Cayman Chemical, Ann Arbor, MI, USA) and insulin-like growth factor (IGF)-1 (Diagnostic Systems, Webster, TX, USA) 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 commercial ELISA microtiter plates, with antibodies specific
for rat IL-1β, IL-6, and TNF-α having been coated onto wells of the microtiter strips provided (Amersham Pharmacia Biotech, Buckinghamshire, UK).

A flow cytometric phagocytosis test was used to evaluate the phagocytic activity of blood polymorphonuclear neutrophils.22,23 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, OR, USA.) was added to each tube. Control tubes remained on ice, and assay samples were incubated for precisely 10 min at 37 °C 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 Hank’s 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 (1 μg/mL in HBSS) was added to stain the nuclear DNA 10 min before the flow cytometric analysis. Flow cytometry was performed on a FACS Calibur™ 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 °C.

A Vybrant™ 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 96-well microplates, it was transferred to a 37 °C CO₂ 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 the 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 2 experimental groups at the beginning of TPN administration. All rats gained weight after the TPN infusion, and weights were maintained postoperatively. No differences in body weights were seen between the 2 groups on postoperative days 1 and 3 (Fig. 1). The GLN group had higher plasma Gln levels on postoperative day 1. No significant differences were observed before surgery and 3 days postoperatively (Fig. 2).

Compared with the control group, the GLN group had lower nitrogen loss at 1 and 2 d after surgery (Fig. 3A). A significantly better cumulative nitrogen balance was observed in the GLN group on postoperative days (Fig. 3B). Compared with levels before surgery, plasma GH concentrations were significantly lower after surgery in the control group on both postoperative day 1 and 3, whereas there was only a difference on day 3 postoperatively in the GLN group. There were no differences in GH and IGF-1 levels between the 2 groups before or after surgery (Fig.
The phagocytic activity of peritoneal macrophages was 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 than the baseline, regardless of whether or not Gln was given. There were no significant differences in the phagocytic activities of blood PMNs between the 2 groups at various time points (Fig. 5B). Plasma IL-1β, IL-6, and TNF-α levels were undetectable. No significant differences in concentrations of IL-1β and IL-6 in PLF were observed between the 2 groups at the time we took the measurements. However, TNF-α levels in PLF were significantly lower in the GLN group than the control group on postoperative day 3 (Table II).

**DISCUSSION**

In this study, 25% of total nitrogen in the TPN solution was supplied by Gln. This amount of Gln was previously found to enhance the immune response in rodents. We administered TPN before and after a gastrectomy, to mimic the usual treatment for patients who are scheduled to undergo gastrectomy. These patients are frequently malnourished, and perioperative TPN is essential for adequate nutritional support. Since human studies may have wide variations owing to the age of patients, severity of disease, infected area of the stomach, and complications of
other diseases, these variables may make interpretation of the data difficult. We used an animal model with a partial gastrectomy to investigate the effect of Gln on the catabolic and immune response after abdominal surgery.

Injury to the body results in a negative nitrogen balance together with a progressive loss of body protein, possibly resulting from hormonal changes and cytokine secretion. Many studies have shown that Gln supplementation enhances skeletal muscle synthesis which may consequently improve nitrogen balance after elective surgery. GH is known to exert many metabolic effects. Among them are nitrogen retention and preservation of muscle protein mass. 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. A study by Welbourne et al. reported that oral Gln load is capable of elevating plasma GH in healthy adults. Hammarqvist et al. 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 those of previous reports. However, we did not find an association between plasma GH, IGF-1 levels and Gln supplementation before or after the operation. This finding suggests that GH and IGF-1 might not be responsible for attenuating nitrogen losses under the present experimental conditions.
Previous reports have shown that parenterally or enterally administered Gln lowered the incidence of infection in patients with bone marrow transplantation and multiple trauma. Supplemental Gln improved the survival in experimentally *Escherichia coli*-induced peritonitis in rodents. 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. Macrophages use Gln at a very high rate. Some in vitro studies have shown that Gln is required for macrophage phagocytosis. 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 were 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. In this study, we did not observe reduced plasma Gln levels after surgery. This result was consistent with the report by Parry-Billings et al. that plasma Gln levels did not change after minor surgery. It is possible that partial gastrectomy performed in this study resulted in minor metabolic stress. The rats were free of infection or other stress that would cause a systemic response. Therefore, the effects of a tissue or organ specific nutrient like Gln exert its effects locally but not systemically.
Cytokines are peptides produced by cells of the immune system that act as a mediator of the immune response and the response of tissues to injury. Studies have proposed that alterations in TNF-α and IL-6 can be used as biochemical markers of the stress response. IL-6 is thought to be the most consistently identified cytokine mediator of postinjury infections. High plasma concentrations of IL-1 and TNF-α are associated with increased severity of inflammatory diseases. These cytokines in plasma were not detectable at the time we took measurements. However, cytokines in PLF were measurable. Compared with the baseline, IL-1β, and 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. showed that Gln had no effect on the production of IL-1β, IL-6, or TNF-α. Since it is an in vitro study, and samples used for evaluating were derived from healthy volunteers, response to the stressed metabolic condition observed in this study may differ, and may consequently lead to different immune responses.

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

ACKNOWLEDGMENTS

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REFERENCES


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33. Houdijk AP, Rijinsburger ER, Jansen J, et al: Randomized trial of


Figure legends

Fig 1. Body weights of experimental groups at the beginning of TPN administration and before sacrifice. No significant differences in initial body weight and weights at 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.
*Significant between 2 groups (p < 0.05).

Fig 3. A) Nitrogen balance and B) cumulative nitrogen balance between the 2 groups after the operation. *Significant between 2 groups (p< 0.05).

Fig 4. A) Plasma growth hormone (GH) and B) insulin-like growth factor-I (IGF-I) concentrations between the 2 groups before and after the operation. *Significant difference from the corresponding group on post-op (p < 0.05). No significant differences in plasma IGF-1 levels were observed between the 2 groups on pre- or postoperative days (p > 0.05)

Fig 5. Phagocytic activity of A) peritoneal macrophages measured by phagocytosis assay and read in the fluorescence plate reader using 480 nm for excitation and 520 nm for emission. *Significant difference from the control group on post-1 and groups of pre-op and post-3 (p < 0.05). B) peripheral blood neutrophils measured by flow cytometry. †Significant difference from the corresponding groups on post-op days (p < 0.05).
<table>
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<tr>
<th></th>
<th>GLN (g)</th>
<th>Control (g)</th>
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<tbody>
<tr>
<td>50% glucose</td>
<td>420</td>
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</tr>
<tr>
<td>20% lipofudin</td>
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<td>50</td>
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<tr>
<td>*Moriamine 10%</td>
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<td>450</td>
</tr>
<tr>
<td>NaCl$_3$ 3%</td>
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<td>35</td>
</tr>
<tr>
<td>K$_3$PO$_4$ 8.7%</td>
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<td>10</td>
</tr>
<tr>
<td>KCL 7%</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Calcium gluconate 10%</td>
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<tr>
<td>MgSO$_4$ 10%</td>
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<td>ZnSO$_4$ 0.6%</td>
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<td>**Infuvita</td>
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<td>Choline chloride (g)</td>
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</tr>
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<td>GLN (g)</td>
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<tr>
<td>H$_2$O</td>
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<tr>
<td>Total kcal</td>
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*From Chinese Pharmaceuticals, Taipei, Taiwan. Each 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, and aminoacetic acid (Gly) 1570 mg.

**From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Each milliliter contains: vitamin A 660 IU, 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, and dl-alpha-tocopheryl acetate 2 mg.
Table 2. Peritoneal lavage fluid (PLF) interleukin (IL)-1β IL-6 tumor necrosis factor (TNF)-α concentrations between the 2 groups before and after the operation.

<table>
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<th>post-1 (n =10)</th>
<th>post-3 (n =10)</th>
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<tr>
<td></td>
<td>pg/mL</td>
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<tr>
<td>IL-1β</td>
<td></td>
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<tr>
<td>Control</td>
<td>10.1 ± 6.8</td>
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<td>IL-6</td>
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<tr>
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<td>88.9 ± 46.1</td>
<td>130.0 ± 21.7</td>
<td>131.5 ± 50.8</td>
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<td>94.0 ± 10.4</td>
<td>144.5 ± 51.7</td>
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<tr>
<td>TNF-α</td>
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<tr>
<td>Control</td>
<td>24.0 ± 16.6</td>
<td>10.2 ± 8.3</td>
<td>54.7 ± 28.5*</td>
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<tr>
<td>GLN</td>
<td>12.7 ± 5.3</td>
<td>22.1 ± 24.9</td>
<td>27.1 ± 21.5#</td>
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</table>

Values are presented as Mean ± SD.
* Significant difference from the pre-op and post-1 groups in the same line at p < 0.05
# Significant difference from the control group at p < 0.05
Body weight (g)

Before TPN

Before sacrifice

pre-op  post-1  post-3

pre-op  post-1  post-3

Control  GLN
Plasma Glc (umol/L)

<table>
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<tr>
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<tr>
<td>post-2</td>
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</tbody>
</table>

* indicates significant difference.
A) 

OD (Ex = 485nm, Em = 535nm)

- Control
- GLN

B) 

% of phagocytizing cells

- Control
- GLN