Effects of enteral nutrition with parenteral glutamine supplementation on the immunological function in septic rats

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Abstract

The aim of the present study was to investigate the effects of enteral nutrition (EN) with parenteral glutamine (GLN) supplementation on inflammatory response, lymphatic organ apoptosis, immunological function and survival in septic rats by caecal ligation and puncture (CLP). Male rats were randomly assigned into two experimental groups and two sham CLP control groups (n 10 per group). After CLP or sham CLP model and nutrition programme were completed, the GLN concentrations of plasma and tissues and several indices of immunological function including serum Ig content, circulating lymphocyte number, the CD4:CD8 ratio, the neutrophil phagocytosis index (NPI), the organ index and apoptosis of thymus and spleen, and plasma cytokine levels were determined. Moreover, the survival in septic rats was observed. The results revealed that EN with parenteral GLN supplementation remarkably increased the GLN concentrations of plasma and tissues, serum Ig content, the circulating lymphocyte number, the CD4:CD8 ratio, the indexes of thymus and spleen, NPI and survival compared with the control group (P<0.05). In contrast, the apoptosis of thymus and spleen and the levels of TNF-α, IL-1β and IL-6 in plasma were obviously decreased compared with the control group (P<0.05). These results show that EN with parenteral GLN supplementation diminished the release of inflammatory cytokines, attenuated lymphatic organ apoptosis, enhanced the immunological function and improved survival in septic rats.

Key words: Enteral nutrition; Glutamine; Immunity; Cytokines; Sepsis

Severe sepsis is still one of the leading causes of death in critically ill patients(1–4). The precise pathophysiological mechanisms by which sepsis develops are not completely understood. However, experimental and clinical studies have revealed that immune dysfunction during sepsis plays a crucial role in the outcome(2). In the early phase of sepsis, the immune response may be characterised by the release of pro-inflammatory mediators, and most patients may survive. In the late phase, patients often demonstrate signs of anti-inflammatory immune suppression such as failing to eradicate invading pathogens and being susceptible to opportunistic organisms(1–4). Because of the mortality of severe sepsis, until now, great efforts have been undertaken to develop an immune-modulating therapy to improve the outcome of sepsis. A better therapeutic strategy in the modulation of immune dysfunction of sepsis might control non-pathogen-specific inflammatory response to avoid tissue damage as well as enhance anti-infectious immune response.

Since nutritional support may provide all kinds of macro- and micronutrients, including carbohydrate, fat, protein, vitamin and trace elements, for critically ill patients to meet the daily requirements and incremental expenditure due to stress-induced hypermetabolic and hypercatabolic states, it has become an integral and indispensable part of the treatment of critically ill patients(5). With the development of modern nutritional therapeutic theory and technique, it has been recognised that immunonutrition containing specific nutrients such as glutamine (GLN), arginine, RNA nucleotides, n-3 fish oil and others may attain the aim of

**Abbreviations:** AA, glutamine-free amino acid; CLP, caecal ligation and puncture; EN, enteral nutrition; GLN, glutamine; NPI, neutrophil phagocytosis index; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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Glutamine and immunity in septic rats

enhancing host immune function, modulating inflammatory response and improving protein synthesis (8). One of the more highly studied nutrients for this purpose is the amino acid GLN. GLN, traditionally considered to be a non-essential amino acid, is now considered as ‘conditionally essential’ amino acid during inflammatory and stress states (9). GLN is an important and unique amino acid, with intermediary metabolism roles and others (10). Numerous laboratory and clinical studies have demonstrated that GLN could modulate inflammatory response and stimulate immune function (11–25). It has been widely applied in critically ill patients. However, recently, a meta-analysis of randomised clinical trials has strongly supported that parenteral GLN supplementation in critically ill patients has no significant effects on infections, length of stay and mortality (26). Further, Heyland et al. (27) even reported that GLN supplementation has been associated with an increase in mortality among critically ill patients, whereas Wischmeyer et al. (28) strongly supported that parenteral GLN supplementation in critically ill patients could improve survival rate and decrease infectious complications, costs and hospital length-of-stay. These conflicting and confusing results may bring about the controversy over GLN supplementation in critically ill patients. Although the present data are contradictory, the potential benefit of parenteral GLN supplementation has been one of the most heavily investigated issues of nutritional interventions in critical care medicine. Further exploration of the mechanisms of GLN immunomodulating role appears warranted and necessary. The objective of the present study was to investigate the impact of EN with parenteral GLN supplementation on inflammatory response, lymphatic organ apoptosis, immunological function and survival in septic rats by caecal ligation and puncture (CLP).

Materials and methods

Animals preparation

Male Sprague–Dawley rats weighing 250–300 g were used in this experiment. All rats were housed in stainless steel cages maintained in a temperature- and humidity-controlled room. Rats were allowed free access to a rodent chow and water for a 7-d acclimatisation period. All procedures of the present study were in accordance with the guide for the care and the use of laboratory animals published by the Ministry of Science and Technology of China, and were approved by the Ethics Committee of our institute.

Surgical procedure and grouping

Male Sprague–Dawley rats were randomly assigned into four groups: a sham CLP+EN+GLN-free amino acid (AA) group (n = 10); a sham CLP+EN+GLN group (n = 10); a CLP+EN+AA group (n = 10); a CLP+EN+GLN group (n = 10). Sepsis was induced by CLP. Briefly, the rats were weighted and anaesthetised by intraperitoneal injection of 50 mg/kg body weight pentobarbital sodium, and were fixed in supine position, and the entire ventral surface was shaved, and the abdomen was sterilized opened through a 2 cm middle incision. The caecum was exposed. The distal 1 cm of the caecum was ligated with a 4-0 silk suture and perforated twice through-and-through with a 21-gauge needle. A small amount of faeces was extruded to ensure that the wound was patent. The caecum was placed back into the abdominal cavity, and the abdomen was closed with 4-0 silk suture in two layers. Immediately, the CLP rats received a 50 ml/kg body weight intraperitoneal injection of sterile 0.9% saline for fluid resuscitation. The rats in two sham CLP groups were treated in the same procedure as above. However, the caecum was exposed but not ligated or punctured, and then replaced. Animals were returned to their cages and allowed food and water ad libitum.

Nutrition programme and sample collection

Enteral feeding began 2 h after sham CLP or CLP and continued for 7 d (the enteral feedings were divided evenly two times per d). All rats in each of the four groups received an identical EN solution as their base formula. As described in Table 1, convention EN solution provided, per litre, 6279 kJ (1500 kcal), 60 g protein, 185 g carbohydrate and 58.4 g fat. The supplied energy ratio of the EN solution was 49% carbohydrate, 35% fat and 16% protein, and the proportion of energy to N was 133:1. Simultaneously, GLN was administered as alanyl-glutamine dipeptide (Dipeptiven; Fresenius Kabi). The rats in the sham CLP+EN+GLN and the CLP+EN+GLN groups were given 0.55 g GLN/kg body weight per d once via a tail vein injection for 7 d, whereas those in the sham CLP+EN+AA and the CLP+EN+AA groups were administered isoenergetic/isonitrogenous GLN-free amino acid solution for comparison. There was isonitrogenous, isovolumic and isoenergetic intake in four groups. All rats among four groups were given 1213 kJ (290 kcal)/kg body weight per d. A quarter of the daily requirement of energy was supplied within the first 24 h, and a half in the second 24 h. After the 2nd day, the full energy requirement was given. All rats were allowed to drink water freely. After nutrition programme was completed, all rats were weighted and anaesthetised. A middle abdominal incision was made, the abdominal cavity opened, and the inferior vena cava exposed. EDTA-anti-coagulated venous blood samples were collected in sterile vacuum collection tubes via inferior vena cava puncture for Ig quantification, lymphocyte count, flow

<table>
<thead>
<tr>
<th>Component</th>
<th>Enteral nutrition solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>1500</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>6279</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>60</td>
</tr>
<tr>
<td>N (g/l)</td>
<td>9.4</td>
</tr>
<tr>
<td>NPC:N</td>
<td>133:1</td>
</tr>
<tr>
<td>Fat (g/l)</td>
<td>58.4</td>
</tr>
<tr>
<td>Carbohydrate (g/l)</td>
<td>185</td>
</tr>
<tr>
<td>Composition of total energy (%)</td>
<td>16</td>
</tr>
<tr>
<td>Protein</td>
<td>35</td>
</tr>
<tr>
<td>Fat</td>
<td>49</td>
</tr>
</tbody>
</table>

NPC, non-protein calories.
cytometric analysis of T lymphocyte subsets, plasma GLN concentration and plasma cytokines assay, and the evaluation of neutrophil phagocytosis function. In addition, thymus, spleen and skeletal muscle were harvested for the analysis of GLN concentration, organ index and/or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL).

Ig quantification in serum
Serum IgA, IgM and IgG contents were quantified by ELISA. In brief, ninety-six-well polystyrene plates were coated with anti-rat IgA, IgM or IgG monoclonal antibodies (2 mg/ml in PBS; BD Biosciences) and, after blocking with 1% bovine serum albumin (Sigma-Aldrich) in PBS (1 h, room temperature), appropriate diluted samples and standard dilutions (purified IgA, IgM or IgG; BD Biosciences) were added. After washing, biotin-conjugated anti-rat IgA, IgM or IgG monoclonal antibodies (BD Biosciences) and subsequently peroxidase-conjugated ExtrAvidin (Sigma-Aldrich) were added, respectively. Ano-phenylenediamine dihydrochloride–H$_2$O$_2$ solution (Sigma-Aldrich) was used for the detection of bound peroxidase. 3 m-H$_2$SO$_4$ was added to stop the reaction. Optical density was measured on a microtitre microplate photometer (Labsystems Multiskan) at 492 nm. Data were interpolated by means of Ascent version 2.6 software (Thermo Fisher Scientific) into the standard curves, and expressed as $\mu$g/ml in serum.

Lymphocyte count in circulation
Lymphocyte count in circulation was determined by Coulter LH-750 Analyzer (A Beckman Coulter Company).

T lymphocyte subsets in circulation
The proportions of CD3, CD4 and CD8 T lymphocyte subsets in circulating blood were measured by flow cytometry. In brief, 100 $\mu$l of anti-coagulated blood were incubated with either isotypic control or monoclonal antibodies against fluorescein isothiocyanate-CD3, or allophycocyanin-CD4, or peridinin-chlorophyll proteins-CD8 (BD Pharmingen) for 30 min at room temperature in the dark. After haemolysis was added, stained cells were washed twice with cold PBS and assayed by flow cytometry (Becton-Dickinson, Inc.).

The glutamine concentration in plasma, thymus, spleen and skeletal muscle
The GLN concentration in plasma, thymus, spleen and skeletal muscle was determined by reversed-phase HPLC (Shimadzu LC-6A). The samples were prepared as described previously.$^{259}$ GLN was pre-column derivatised with o-phthalaldehyde and 3-mercaptopyrrolic acid under an alkaline condition and separated by HPLC on a LiChrosorb C18 column (Shimadzu). The mobile phase was a mixture of 50 mmol/l phosphate buffer (pH 7.0) and acetonitrile (94:6, v/v). The flow rate was 2.0 ml/min. A volume of 40 $\mu$l of the sample was vortex-mixed with 10 $\mu$l derivatisation reagent solution for 2 min. Then, a volume of 20 $\mu$l of the reaction mixture was injected into the HPLC system. The excited and the emitted wavelengths were selected at 230 and 389 nm, respectively. The result was expressed as $\mu$mol/l plasma or $\mu$mol/g tissues.

The indexes of thymus and spleen
Thymus and spleen were harvested and weighted. The index of thymus was obtained by thymus net weight divided by the rat body weight, likewise does the index of spleen. The results were expressed as mg (thymus or spleen net weight)/g (rat body weight).

Terminal deoxynucleotidyl transferase dUTP nick end labelling staining of thymus and spleen
Thymus and spleen were collected and fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 4 m thickness were affixed to slides, deparaffinised, rehydrated in graded alcohol (100, 95 and 85%) and stained with haematoxylin and eosin to evaluate morphological changes. For identification of apoptotic cells, TUNEL staining was conducted by commercially available kits (Wuhan Boster Biological Technology, Limited). All steps were performed according to the supplier’s instructions. In brief, paraffin-embedded sections were dewaxed, rehydrated and then incubated with proteinase K (20 $\mu$g/ml in 100 mM-Tris and 50 mM-EDTA) for 15 min at 25°C to digest proteins, and endogenous peroxidase activity was quenched with 2% H$_2$O$_2$ in PBS. After the slides were washed four times with distilled water, the sections were incubated in equilibration buffer for 5 min. The sections were then incubated with the labelling solution containing terminal deoxynucleotidyl transferase in a humidified chamber for 1 h at 37°C. The reactions were terminated by rinsing the sections in a stop/wash buffer. Next, the sections were incubated with anti-digoxigenin peroxidase for 30 min at room temperature. Sections were washed again, and diaminobenzidine–H$_2$O$_2$ was used for colour development. Sections were then counterstained with 2% haematoxylin and mounted for examination.

TUNEL analysis was conducted according to the method described previously.$^{259}$ Lymphocytes were identified by morphology, with cell sizes of approximately 10 m and low cytoplasm/nucleus ratios. Apoptotic cells were identified either as cells with brown-stained nuclei or as apoptotic bodies, which are fragments of apoptotic cells engulfed by neighbouring cells. The number of TUNEL-positive nuclei is enumerated for two random high power fields per tissue section by three reviewers blinded as to the sample origin. Averaging the number of TUNEL-positive nuclei for each field for all three reviewers resulted in an overall TUNEL-positive score for the tissue. The results were expressed as TUNEL-staining cells/high power fields.

Plasma cytokine assay
To clarify the change of inflammatory cytokine, the levels of TNF-$\alpha$, IL-1$\beta$, IL-6 and IL-10 in plasma were analysed by

The results were expressed as $\mu$mol/l plasma or $\mu$mol/g tissues.
ELISA. Briefly, the plasma was separated by centrifugation at 16 000 g for 15 min at 4°C, aliquoted, immediately frozen and stored (−84°C) until assayed. The levels of TNF-α, IL-1β, IL-6 and IL-10 were determined by commercially available ELISA kits (R&D Systems) according to the manufacturer’s recommendations. The results were expressed as pg/ml or ng/ml.

Neutrophil phagocytosis index

Neutrophil phagocytosis index (NPI) was determined according to the method as described previously (25). Briefly, about 2 ml of anti-coagulated blood was collected in a test-tube containing 0.4 ml of 6% dextran T500 (Pharmacia). The test-tube was placed upright for 45–60 min to allow the separation of red cells and plasma. The plasma layer was then removed to centrifuge tube, centrifuged at 2000 g for 5 min. After centrifugation, the mononuclear layer was removed to another centrifuge tube. Neutrophils contaminating erythrocytes was resuspended. The erythrocytes were removed by hypotonic lysis. The cells were washed two times with Hanks’ balanced salt solution and suspended in 1× Hanks’ balanced salt solution. Finally, cellular viability was evaluated using the trypan blue exclusion technique. The purity and viability of neutrophils for each assay were >97%. The neutrophils were counted and adjusted to a concentration of 1 × 10⁸ cells/ml.

Neutrophils (5 × 10⁶) were resuspended in Roswell Park Memorial Institute-1640 medium (Gibco) containing 10% (v/v) fetal bovine serum (Gibco) and subsequently incubated with 1 × 10⁷ Staphylococcus aureus in a final volume of 1 ml for 2 h at 37°C. The NPI was determined by microscopic observation after stained with Gram’s stain. Under a light microscope, NPI = cells of phagocytosis bacteria/total cells × 100%.

Survival studies

A separate set of forty male Sprague–Dawley rats was served as survival study. CLP or sham CLP model, grouping and nutrition programme were the same as described above. When nutrition programme began and went on, animals were observed at regular intervals for occurrence of mortality for 7 d.

Statistical analysis

Data were expressed as the means with standard deviations, and differences among groups were analysed by ANOVA using Newman–Keuls’ test. Fisher’s exact test was utilised to compare survival data. Values of P<0.05 were regarded as significant.

Results

Body weight change

As shown in Table 2, it was not observed that there was obvious difference in body weight at the beginning of the experiment among the four groups (P>0.05). However, at the end of the experiment, the rats lost more body weight in two experimental groups (the CLP+EN+AA group and the CLP+EN+GLN group) than that in two sham CLP control groups (the sham CLP+EN+AA group and the sham CLP+EN+GLN group; P<0.05). There was no difference in body weight between the CLP+EN+AA group and the CLP+EN+GLN group (P>0.05).

Neutrophil phagocytosis index

As shown in Table 2, NPI from the CLP rats were decreased when compared with the sham CLP rats (P<0.05). However, NPI from the CLP+EN+GLN group were significantly increased compared with the CLP+EN+AA group (P<0.05).

Ig quantification in serum

As shown in Table 3, the contents of IgA, IgG and IgM in serum in two experimental groups were significantly decreased compared with two sham CLP control groups (P<0.05). However, the contents of IgA, IgG and IgM in the CLP+EN+GLN group were higher than those in the CLP+EN+AA group (P<0.05).

Number of lymphocyte in circulation

As shown in Table 3, the numbers of circulating lymphocyte in two experimental groups were apparently small compared with two sham CLP control groups (P<0.05). However, the

Table 2. The changes of body weight and neutrophil phagocytosis index (NPI)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± sd</th>
<th>Mean ± sd</th>
<th>Mean ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Body weight before experiment (g)</td>
<td>Loss of body weight after experiment (g)</td>
<td>NPI (%)</td>
</tr>
<tr>
<td>Sham CLP+EN+AA</td>
<td>10</td>
<td>242.3 ± 24.2</td>
<td>−10.2 ± 0.8</td>
<td>43.8 ± 4.2</td>
</tr>
<tr>
<td>Sham CLP+EN+GLN</td>
<td>10</td>
<td>238.2 ± 26.4</td>
<td>−11.5 ± 1.0</td>
<td>45.2 ± 4.3</td>
</tr>
<tr>
<td>CLP+EN+AA</td>
<td>10</td>
<td>238.6 ± 24.6</td>
<td>−35.4* ± 2.8</td>
<td>24.7* ± 2.6</td>
</tr>
<tr>
<td>CLP+EN+GLN</td>
<td>10</td>
<td>240.5 ± 24.5</td>
<td>−36.3* ± 2.4</td>
<td>33.6*† ± 3.5</td>
</tr>
</tbody>
</table>

CLP, caecal ligation and puncture; EN, enteral nutrition; AA, glutamine-free amino acid; GLN, glutamine.

*Mean value was significantly different from that of the sham CLP+EN+AA group or the sham CLP+EN+GLN group (P<0.05).
†Mean value was significantly different from that of the CLP+EN+AA group (P<0.05).
The number of circulating lymphocyte in the CLP\(\text{+}\)EN\(\text{+}\)GLN group was bigger than that in the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P, 0.05)\).

As shown in Fig. 1 and Table 3, CLP led to changes in the percentage of T lymphocyte subsets in circulation in the CLP rats. The percentage of CD4 cells was remarkably decreased, whereas that of CD8 cells was correspondingly increased \((P, 0.05)\), which led to decreased CD4:CD8 ratio \((P, 0.05)\).

**Table 3.** The changes of Ig concentration in serum, circulating lymphocyte count and the percentage of circulating T lymphocyte subsets (Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IgA</th>
<th>SD</th>
<th>IgG</th>
<th>SD</th>
<th>IgM</th>
<th>SD</th>
<th>CD3</th>
<th>Mean</th>
<th>SD</th>
<th>CD4(^+)</th>
<th>Mean</th>
<th>SD</th>
<th>CD8(^+)</th>
<th>Mean</th>
<th>SD</th>
<th>CD4:CD8 ratio</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham CLP (\text{+})EN(\text{+})AA</td>
<td>10</td>
<td>0.58</td>
<td>0.05</td>
<td>3.8</td>
<td>0.3</td>
<td>0.41</td>
<td>0.03</td>
<td>12.6</td>
<td>1.2</td>
<td>66.5</td>
<td>4.3</td>
<td>44.2</td>
<td>4.3</td>
<td>25.2</td>
<td>2.8</td>
<td>1.9</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham CLP (\text{+})EN(\text{+})GLN</td>
<td>10</td>
<td>0.59</td>
<td>0.04</td>
<td>3.7</td>
<td>0.3</td>
<td>0.38</td>
<td>0.02</td>
<td>12.4</td>
<td>1.2</td>
<td>67.3</td>
<td>3.4</td>
<td>43.5</td>
<td>4.8</td>
<td>24.3</td>
<td>2.5</td>
<td>1.8</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLP (\text{+})EN(\text{+})AA</td>
<td>10</td>
<td>0.34*</td>
<td>0.02</td>
<td>2.4*</td>
<td>0.2</td>
<td>0.22*</td>
<td>0.02</td>
<td>6.3*</td>
<td>0.5</td>
<td>54.5</td>
<td>4.2</td>
<td>36.2</td>
<td>2.8</td>
<td>28.4*</td>
<td>2.6</td>
<td>1.6</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLP (\text{+})EN(\text{+})GLN</td>
<td>10</td>
<td>0.47†</td>
<td>0.04</td>
<td>3.2†</td>
<td>0.3</td>
<td>0.30†</td>
<td>0.03</td>
<td>8.8†</td>
<td>0.6</td>
<td>65.4</td>
<td>5.7</td>
<td>42.7</td>
<td>2.6</td>
<td>24.6</td>
<td>2.3</td>
<td>1.9</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CLP, caecal ligation and puncture; EN, enteral nutrition; AA, glutamine-free amino acid; GLN, glutamine.

* Mean value was significantly different from that of the sham CLP \(\text{+}\)EN\(\text{+}\)AA group or the sham CLP \(\text{+}\)EN\(\text{+}\)GLN group \((P<0.05)\).

† Mean value was significantly different from that of the CLP \(\text{+}\)EN\(\text{+}\)AA group \((P<0.05)\).

As shown in Table 4, the GLN concentrations of plasma, thymus, spleen and skeletal muscle in two experimental groups were apparently decreased compared with the two sham CLP control groups \((P, 0.05)\). However, there was a notable increment in the GLN concentrations of plasma, thymus, spleen and skeletal muscle in the CLP\(\text{+}\)EN\(\text{+}\)GLN group compared with the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P, 0.05)\).

**Cytokines in plasma**

As shown in Table 4, levels of TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-10 in plasma from the CLP rats were increased when compared with the sham CLP rats \((P, 0.05)\). The present results revealed that CLP markedly increased the release of pro- and anti-inflammatory mediators. However, levels of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in plasma in the CLP\(\text{+}\)EN\(\text{+}\)GLN group were significantly decreased compared with the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P, 0.05)\). There was no obvious difference in level of IL-10 between the CLP\(\text{+}\)EN\(\text{+}\)GLN group and the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P>0.05)\).

The indexes of thymus and spleen

As shown in Table 5, CLP caused a significant atrophy in the size and weight of thymus and spleen, which was perfectly consistent with evidently decreased indexes of thymus and spleen. However, the indexes of thymus and spleen were markedly enlarged in the CLP\(\text{+}\)EN\(\text{+}\)GLN group compared with the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P, 0.05)\).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling analysis of thymus and spleen apoptosis**

To determine whether the observed increase in the indexes of thymus and spleen conferred by EN combined with parenteral GLN supplementation was associated with a reduction in thymus and spleen apoptotic death, apoptosis in thymus and spleen was analysed by TUNEL staining. As shown in Figs. 2, 3 and Table 5, TUNEL-staining cells/high power fields in thymus and spleen from CLP rats were dramatically increased when compared with the sham CLP rats \((P, 0.05)\). Yet, TUNEL-staining cells/high power fields in thymus and spleen from the CLP\(\text{+}\)EN\(\text{+}\)GLN group were dramatically reduced when compared with the CLP\(\text{+}\)EN\(\text{+}\)AA group \((P, 0.05)\).
spleen in the CLP+EN+GLN group had a marked decrease compared with the CLP+EN+AA group ($P<0.05$).

**Survival rate**

Animals were observed for 7 d for survival rate. No mortality occurred in the two sham CLP control groups. The survival rate in the CLP+EN+GLN group was 60%, whereas that in the CLP+EN+AA group was 40%. The survival rate of the CLP rats was significantly decreased in comparison to the sham CLP rats ($P<0.05$). Nevertheless, EN with parenteral GLN supplementation improved survival of the CLP rats. The survival rate in the CLP+EN+GLN group was significantly increased when compared with the CLP+EN+AA group ($P<0.05$).

**Discussion**

The CLP model of sepsis in rodent is the most commonly used for studying the process of septic peritonitis and can be used as a preclinical model to test the efficacy of pharmacological agents for the treatment of sepsis$^{[31]}$. We attempted to explore whether EN with parenteral GLN supplementation could improve humoral and cellular immunity as well as modulate the imbalance of inflammatory response by a CLP rat model.

With respect to the dosage of GLN, the dosage of 0.35 g/kg per d GLN was chosen in accordance with the clinical relevance and the results of our preliminary experiment. Some studies have reported that there is a trend to an increased mortality in those patients treated with a high dose of GLN (1 g/kg per d GLN)$^{[32]}$. In our preliminary experiment, we found that the dosage of 0.35 g/kg body weight per d GLN was safe, effective and appropriate, and had good clinical relevance.

The most important finding in the present study confirmed that parenteral GLN supplementation could effectively attenuate thymus and spleen apoptosis, subsequently boosting cellular and humoral immunity in septic rats.

In the present study, we observed that CLP led to an obvious decrease in plasma Ig and circulating lymphocyte count. Meanwhile, the percentage of CD4 cells was declined and that of CD8 cells was increased, which resulted in an obviously decreased CD4:CD8 ratio. Additionally, NPI significantly decreased. Neutrophils are critical components of the innate immune response to infectious challenges$^{[40]}$. The present results confirmed that humoral- and cellular immune pathways...
function was suppressed after CLP. Clinical data also documented that there was a significant decrease in circulating lymphocytes from septic patients. Other data have reported that phagocytic and bactericidal function of neutrophils was impaired after major operation, severe burn and sepsis. The principal outcome of such immune suppression is the increased susceptibility of injured host to opportunistic pathogens causing high risk of death. However, this negative situation might be partially reversed by EN combined with intravenous GLN supplementation. The present study disclosed that there was an apparent amelioration in humoral immune function such as Ig content and cellular immune function including lymphocyte count, the CD4:CD8 ratio and NPI in the CLP+EN+GLN group, which would be beneficial to the improvement of anti-infectious immune function.

The present study also measured the changes of the GLN concentrations of plasma, thymus, spleen and skeletal muscle by HPLC in the CLP rats. The results showed that the stress, injury and infection owing to CLP contributed to a rapid decrease in the GLN concentrations of plasma, thymus, spleen and skeletal muscle. Lymphocyte is very sensitive to alterations in nutritional substrate supply, especially in GLN supply. Indeed, rapidly dividing immune cells deeply depend on the availability of GLN as an energy (C and N) source. GLN functions as a N shuttle, carrying N for purine and pyrimidine synthesis, which are essential for DNA and RNA synthesis. Further, immune cells require more GLN during inflammation and stress in which cell proliferation is dramatically increased. Sepsis frequently causes hypercatabolic response, which is characterised by a significant increase in energy requirements, BMR, tissue V_{O_2}, N and body weight loss. Because of the impairment of the ability to use glucose and fat, there is an increased dependency on muscle breakdown, providing essential substrates for acute phase protein synthesis and energy production. Skeletal muscle, as the major repository of GLN, can obviously increase GLN release during stress and severe infection. Despite an increase in GLN release from skeletal muscle, the intracellular GLN pool becomes depleted, indicating that release rates exceed rates of synthesis. Simultaneously, the circulating pool of GLN does not increase, indicating accelerated uptake by other organs such as intestine, liver and the immune system. GLN requirements increase markedly, and its consumption in visceral organs frequently exceeds its release from muscle. Thus, it may cause protein–energy malnutrition and specific nutrient deficiency such as GLN. GLN depletion decreases proliferation of lymphocytes, affects expression of surface activation markers on lymphocytes and the production of cytokines and induces apoptosis. GLN supplementation may partially reverse the changes. The present results displayed that EN combined with intravenous GLN supplementation remarkably increased GLN concentrations of plasma, thymus, spleen and skeletal muscle in the CLP rats, which improved GLN metabolism and exerted beneficial effects on the improvement of immunological function during the inflammation and stress.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean (Mean)</th>
<th>SD</th>
<th>Mean (Mean)</th>
<th>SD</th>
<th>Mean (Mean)</th>
<th>SD</th>
<th>Mean (Mean)</th>
<th>SD</th>
<th>Mean (Mean)</th>
<th>SD</th>
<th>Mean (Mean)</th>
<th>SD</th>
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<td>566.7</td>
<td>56.5</td>
<td>8.4</td>
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<td>6.0</td>
<td>0.5</td>
<td>7.6</td>
<td>1.1</td>
<td>10.4</td>
<td>0.5</td>
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<td>62.2</td>
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<td>0.7</td>
<td>6.7</td>
<td>0.7</td>
<td>7.8</td>
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<td>21.2</td>
<td>1.2</td>
<td>10.4</td>
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<tr>
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<td>28.5</td>
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<td>0.5</td>
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<td>0.2</td>
<td>2.7</td>
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<td>0.3</td>
<td>5.8</td>
<td>0.5</td>
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<tr>
<td>CLP+EN+GLN</td>
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<td>48.4</td>
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<td>6.0</td>
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<td>7.6</td>
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<td>4.0</td>
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Table 4. The changes of glutamine (GLN) concentration and levels of plasma inflammatory mediator (Mean values and standard deviations)
In the present study, quantified analysis of TUNEL staining disclosed that apoptosis in thymus and spleen from the CLP rats was dramatically increased, which was closely correlated with an obvious atrophy in the size and weight of thymus and spleen, and decreased circulating lymphocytes. In fact, appropriate lymphocyte apoptosis may be beneficial to septic patients by down-regulating the excessive inflammatory response. However, exaggerated apoptotic cell death may decrease immune cells available to combat pathogens. It is proposed that in the condition of stress and severe injury, apoptosis-induced extensive loss of lymphocytes in the central and peripheral immune system may lead to immunocompromising, leaving the patients vulnerable to subsequent infections or unable to mount an appropriate host response to invading pathogens. Extensive experimental and clinical data support the hypothesis that one of the major pathophysiological mechanisms in sepsis is uncontrolled, extensive and accelerated lymphocyte apoptosis. Therefore, modulating the imbalance of apoptosis response of immune cell might present another attractive therapy target for sepsis. The present study indicated that EN with parenteral GLN supplementation decreased apoptosis in thymus and spleen. Other studies have reported that GLN supplementation could attenuate

Table 5. The changes of organ index and apoptosis in thymus and spleen
(Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Organ index (mg/g)</th>
<th>TUNEL-staining cells/hpf</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Sham CLP+EN+AA</td>
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<td>2·1</td>
<td>0·2</td>
</tr>
<tr>
<td>Sham CLP+EN+GLN</td>
<td>10</td>
<td>2·2</td>
<td>0·2</td>
</tr>
<tr>
<td>CLP+EN+AA</td>
<td>10</td>
<td>0·9*</td>
<td>0·1</td>
</tr>
<tr>
<td>CLP+EN+GLN</td>
<td>10</td>
<td>1·4*†</td>
<td>0·1</td>
</tr>
</tbody>
</table>

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; hpf, high power fields; CLP, caecal ligation and puncture; EN, enteral nutrition; AA, glutamine-free amino acid; GLN, glutamine.

* Mean value was significantly different from that of the sham CLP+EN+AA group or the sham CLP+EN+GLN group (P<0·05).

† Mean value was significantly different from that of the CLP+EN+AA group (P<0·05).

Fig. 2. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) analysis of thymus lymphocyte apoptosis from rats. (a–d) Representative photomicrographs of TUNEL-stained thymus sections in the sham caecal ligation and puncture (CLP)+enteral nutrition (EN)+glutamine (GLN)-free amino acid (AA), sham CLP+EN+GLN, CLP+EN+AA and CLP+EN+GLN group, respectively. (a, b) Sporadic apoptotic lymphocytes without apparent clustering of apoptotic cells are scattered throughout thymus in a random distribution. (c) Apparent clustering of apoptotic cells are scattered throughout thymus in a random distribution in the CLP+EN+AA group. (d) Apparently decreased apoptotic cells are scattered throughout thymus in a random distribution in the CLP+EN+GLN group. TUNEL-positive (apoptotic) nuclei appear in crown ×400. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn
apoptosis occurring in heart, lung, liver, kidney and intestine during severe stress and inflammation (49–53). For instance, Yin et al. (49) demonstrated that GLN reduced myocardial apoptotic cell death by decreasing the gene and protein expression of caspase-3. Also, de Oliveira et al. (50) certified that early therapy with intravenous GLN reduced inflammation, fibrosis and apoptosis, minimising lung and distal organ injury. Moreover, Hu et al. (21) found that GLN prevented apoptosis of T cells, which may partly be responsible for ameliorating acute lung injury induced by sepsis. Kim et al. (51) showed that GLN attenuated tubular cell apoptosis in acute kidney injury via inhibition of the c-Jun N-terminal kinase phosphorylation of 14-3-3. In addition, Uehara et al. (52) proved that GLN pretreatment significantly ameliorated intestinal injury by preventing apoptosis. Furthermore, Margaritis et al. (53) documented that GLN in the presence of obstructive jaundice ameliorated bacterial translocation, endotoxemia and apoptosis and improved the ileal and liver histology. Therefore, it is possible that GLN becomes a clinically relevant nutrient that modulates the imbalance of apoptotic response.

The present study also evaluated the impact of EN with parenteral GLN supplementation on survival in the CLP rats. The result showed that CLP resulted in significantly increased mortality in the CLP rats, that EN with parenteral GLN supplementation significantly improved survival in the CLP rats, which was consistent with another investigation(56). It is probably for the reasons that parenteral GLN supplementation increases the GLN concentrations in plasma and tissues, attenuates immune organ apoptosis, enhances humoral- and cellular immunity and down-regulates the levels of TNF-α, IL-1β and IL-6 in plasma responsible for the change.

In conclusion, the present findings show that EN with parenteral GLN supplementation may decrease the release of
pro-inflammatory cytokines, attenuate lymphatic organ apoptosis, enhance immune function and improve survival in septic rats. Clinically, these results suggest that immunomodulating therapy by GLN supplementation may affect favourably on outcome of septic patients. In addition, more research is needed to explore the mechanisms by which GLN may prevent apoptosis. They would be further studied in the future research.

Acknowledgements

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The authors declare that they have no conflict of interest.

References