ORIGINAL ARTICLE

THE CORRELATION BETWEEN DIFFERENT DOSES OF GLUTAMINE ON THE APOPTOSIS OF LYMPHOCYTES IN A MURINE MODEL OF SEPSIS

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Abstract

The aim of this study was to investigate the effects of glutamine in different doses on the apoptosis of lymphocytes in septic mice. 100 healthy mice were randomly divided into a control group (A), a sepsis model group (B), a high-dose glutamine sepsis group (C, 0.75 mg/kgbw), a medium-dose glutamine sepsis group (D, 0.5 mg/kgbw) and a low-dose glutamine sepsis group (E, 0.35 mg/kgbw), 20 animals per group. Mice from B, C, D and E groups underwent cecal ligation and puncture (CLP), and then they were transformed into sepsis models. 24 hours after injection, the respiration, heart rate and rectal temperature of the mice were recorded. The thymus and spleen specimens were taken and fixed. The apoptosis of lymphocytes was detected by means of in suit end-labelling. Compared to the mice in group A, the mice in group B, C, D and E had lower rectal temperature, increased respiratory frequency and increased heart rate, satisfying the diagnostic criteria for animal models of Systemic Inflammatory Response Syndrome (SIRS). There were few apoptotic thymus and liver cells in group A, while cell apoptosis was obvious in the other four groups, suggesting a significant difference compared to group A ($p < 0.05$). Comparing the groups of different doses of glutamine, the number of apoptotic cells in groups C, D and E was significantly lesser than that in group B ($p < 0.05$). Glutamine in different doses had a large impact on the apoptosis of lymphocytes. The supplementation with glutamine can enhance the anti-apoptosis mechanism of the organism.

Rezumat

Obiectivul studiului a fost evaluarea efectului diferitelor doze de glutamină asupra apoptozei limfocitelor într-un model experimental de sepsis. 100 de șoareci sănătoși au fost împărțiți în următoarele loturi: lotul control (A), lotul modelului de sepsis (B), lotul cu sepsis căruia i s-a administrat doză mare de glutamină (C, 0,75 mg/kgc), lotul cu sepsis căruia i s-a administrat doză medie de glutamină (D, 0,5 mg/kgc) și lotul cu sepsis căruia i s-a administrat doză mică de glutamină (E, 0,35 mg/kgc), fiecare lot având 20 de animale. La șoarecii din loturile B, C, D și E s-a realizat ligatură cecală și puncție (LPC), fiind transformați în modelul de sepsis. La 24 de ore după injectare, au fost determinate frecvența respiratorie, frecvența cardiacă și temperatura rectală. Au fost recoltate și fixate timusul și splina și a fost determinată rata de apoptoză a limfocitelor. În comparație cu șoarecii din grupul A, cei din grupurile B, C, D și E au prezentat temperatură rectală mai scăzută și frecvențe respiratorii și cardiace mai mari, ceea ce satisface criteriile de diagnostic pentru modelele de animale cu Sindrom de Răspuns Inflamator Sistemic (SIRS). La șoarecii din lotul A timusul și splina au prezentat puține celule apoptotice, comparativ cu celelalte grupuri, diferența fiind semnificativă statistic ($p < 0,05$). Comparând loturile la care s-au administrat diferite doze de glutamină, numărul celulelor apoptotice în loturile C, D și E au fost semnificativ mai scăzute decât în lotul B ($p < 0,05$). Glutamina în diferite doze are un efect protector, antiapoptotic limfocitar, deci administrarea glutaminei poate crește activitatea anti-apoptotică a organismului.

Keywords: glutamine, intraperitoneal anaesthesia, lymphocyte, apoptosis

Introduction

Lymphocytes are white cells produced by lymphoid organs. Lymphocyte, the cellular component of the immune system, mainly participates in specific immune response in immunologic processes, and its amount and differentiation capacity can produce direct effects in the response processes [5, 18]. The immune system may be disbalanced during specific treatments [6].
Glutamine is needed in the proliferation and differentiation of lymphocytes having a high utilization rate. Glutamine plays an important immunological enhancement role in the special growth stage or stress period of human body [8, 24]. Glutamine can not only rapidly split proliferative cells and lymphocytes, but also is effective in promoting the proliferation of lymphocytes. Moreover, glutamine is the important substrate of hepatic glycogen dysplasia, which has significant impacts in improving immunity and enhancing the anti-stress mechanisms [13]. Sepsis is a systemic inflammatory response induced by bacterial complications of some diseases such as pneumonia

associated with pleurisy [2], surgical site infections [3] or fungal infections with *Aspergillus* or *Fusarium genum* [20]. Septic shock is one of the most important factors for multisystem organ failure (MSOF). It develops rapidly and has a high mortality rate due to the complex onset, progress and difficulty to treat [22], especially if patients have comorbidities such as gastric adenocarcinomas [7] or colorectal cancer [25], severe coagulopathies [23] or metabolic liver diseases, especially non-alcoholic fatty liver disease [4].

With the deepening of researches concerning immunoreactions, new ideas come out for the treatment of sepsis. For cutaneous septic infections with *Staphylococcus aureus* new pharmaceutical forms have been assessed such as nanoparticles with cytotoxic effect [1] and anti-inflammatory effect [12].

Gao J. *et al.* [10] discussed the clinical effect and safety of Xuebijing® injection in the treatment of sepsis and MSOF. Forni L.G. *et al.* [9] proposed as treatment for acute sepsis patients with acute renal injury the *in vitro* renal replacement.

This study investigated the apoptosis of lymphocytes from the thymus and spleen under the effect of glutamine bringing in attention new alternatives for the treatment of sepsis that could be a starting point for a new relevant treatment.

Materials and Methods

Animals. 100 healthy male BALB/C mice were purchased from Nanjing Junke Bioengineering Co., Ltd., China and randomly divided into a control group (A), a sepsis model group (B), a high-dose glutamine group (C), a medium-dose glutamine group (D) and a low-dose glutamine group (E), 20 animals in each group. All the experiments involved in this study have been reviewed and approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University, Henan, China.

Reagents. Reagents used included chloral hydrate (Hubei Xinmingtai Chemical Co., Ltd., China), glutamine (Thermo Fisher Scientific Co., Ltd., USA), formaldehyde (Anhui Suzhou Boda Commerce and Trade Co., Ltd., China), phosphoric acid solution (PBS) (Jiangsu Nanjing Senbeiqie Biotechnology Co., Ltd., China), ethyl alcohol (Shanghai Shanyi Chemical Technology Co., Ltd., China), methyl-benzene (Shanghai Rongrong Chemical Engineering Co., Ltd., China), hydrogen peroxide (Guangzhou Bangrun Chemical Engineering Science and Technology Co., Ltd., China), DAB chromogen (Shanghai Weiao Biotechnology Co., Ltd., China), Terminal Deoxynucleotidyl Transferase (TdT) (Beijing Shengke Boyuan Biotechnology Co., Ltd., China), and digoxin (DIG)-d-uridine triphosphate (UTP) (Sigma-Aldrich Shanghai Head Office, China).

Model design. The mice were narcotized by chloral hydrate (10%) in a dose of 350 mg/kgbw by intra-peritoneal anaesthesia. After anaesthesia, a 5 cm long incision was cut in the middle of the abdomen of each mouse. Then the cecum was isolated and exposed. The middle part of the cecum of mice in group B, C, D and E was ligated and punctured by a syringe needle, and some of the content in the enteric cavity was sequenced out. After that, the abdominal cavity was sutured layer by layer, and each mouse was injected with normal saline to prevent shock. Two hours after test, mice in group B were intravenously injected with normal saline in the tail vein, while mice in group C, D and E were intravenously injected with glutamine in doses of 0.75 g/kgbw, 0.5 g/kgbw and 0.35 g/kgbw respectively. Mice in group A were not given cecal ligation and puncture; the other procedures were the same with the other four groups; they were intravenously injected with normal saline in the tail vein as well.

Preparation of thymus and spleen samples from mice. 24 hours after the model design, the mice were given intraperitoneal anaesthesia. The temperature, the heart rate and the respiratory frequency of the mice were recorded. An incision was performed in the middle of the abdomen, and the thymus and spleen were taken out and fixed with 10% formaldehyde solution. Then the thymus and spleen specimens were taken out and washed by PBS three times for removing the stationary liquid. After washing, the samples were dehydrated by ethyl alcohol in different concentrations for 2 h. The dehydrated specimens were processed by transparent processing using a mixture of absolute ethyl alcohol and methylbenzene. Then they were transferred to wax cups for wax dip. Other wax paraffin was melted and transferred to the embedding frame. The specimens were taken out after entire solidification and sliced.

The detection of lymphocyte apoptosis in the thymus and spleen. The specimen slices were dewaxed and hydrated using dimethylbenzene and ethyl alcohol solution and then 3% hydrogen peroxide solution was added. After that, the specimens were kept at 20°C. 0.01 M PBS was diluted according to the ratio of 1:200, and the diluents were added to the specimen slices. The specimens were kept in an incubator (37°C) for 10 minutes, then taken out, and washed with 0.01 M PBS for five times, 3 min each time. Each specimen slice was added with label buffer. Then 1 mL of DIG-d-UTP and 1 mL of TdT were added to the specimen, followed by 20 mL of label buffer and mixing. After the residual liquid was removed from the slices, 20 μL of label liquid was added. Then the specimens were put into a wet box and incubated for 3 h labelling. The labelled specimen slices were washed with 0.01 M PBS three times, 5 min each time. Then each specimen slice was treated with 50 μL of sealing solution.

Afterwards, they were put back to the incubator at 37°C, for 30 minutes. Biotinylated anti-digoxin and antibody diluents were diluted in a ratio of 1:100. Each specimen slice was added with 50 μL of diluent. They were put into the incubator (37°C) and washed by 0.01 M PBS for 5 times after thirty minutes, 3 minutes each time. HRP-Conjugated Streptavidin (SABC) and antibody diluents were diluted in a ratio of 1:100. After mixing, each sample slice was treated with 50 μL of diluents and then processed by the last procedures. The specimen slices were observed under a microscope after the addition of DAB chromogen. When brown yellow was observed on the cytomembrane, the slices were washed with distilled water to end colour development. Then the specimens were washed with 0.01 M PBS, differentiated using HCl-C_2H_5-OH for 10 s, washed with 0.01 M PBS, processed by ammonia water for 10 s, and finally washed with 0.01 M PBS again. The results were counted after dehydration, transparency disposal and mounting. The result of cell apoptosis was evaluated using the following equation:

$$P = \frac{Q_1}{Q} \times 100\%$$

where, P refers to cell apoptosis index, Q_1 refers to the total amount of apoptotic cells, and Q refers to the total amount of cells.

When the cells turned to be brown yellow, it represented the development of apoptosis. The total amount of cells in the thymus and spleen and the apoptotic cells were counted by using 10 high power lenses (x400). Smaller apoptosis indexes indicated less apoptotic cells.

Data statistics. The data obtained in this experiment were processed by SPSS ver. 18.0. Data were expressed as mean ± standard deviation. Difference was considered statistically significant if $p < 0.05$.

Results and Discussion

The conditions of models. As shown in Figure 1, the respiratory frequency and heart rate of group B, C, D and E were higher than those of group A, the rectal temperature decreased for more than 1°C, and all the indexes had significant differences ($p < 0.05$). No obvious differences were found between group C, D and E ($p > 0.05$). In addition, the ligation end was swollen, black and distributed with yellow fester on the surface, suggesting the successful modelling of sepsis mouse with cecal ligation and puncture.

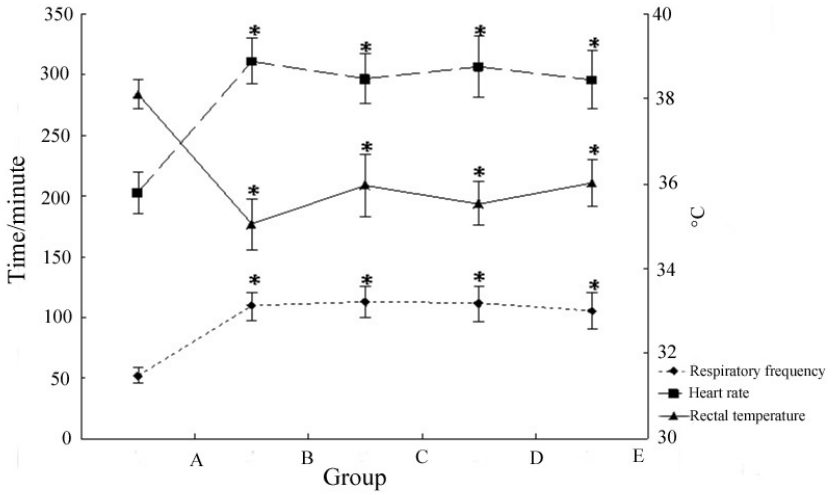

Figure 1.
The comparison of respiratory frequency, heart rate and rectal temperature between the five groups
*indicated $p < 0.05$ compared to group A

Apoptosis of lymphocyte in the thymus and spleen. As shown in Figure 2, the apoptosis index of lymphocyte in the thymus of groups B, C, D and E was significantly different compared to that of group A ($p < 0.05$), and groups C, D and E were also significantly different compared to group B ($p < 0.05$); among the three groups of glutamine in different doses, the cell apoptosis index from low to high were groups C, D and E. Groups C and D were significantly different from group E, and there was no remarkable difference between groups C and D.

According to Figure 3, the apoptosis index of lymphocytes in the spleen of groups B, C, D and E was significantly different from that of group A ($p < 0.05$); the index of groups C and D was remarkably different compared to group B ($p < 0.05$); but there was no obvious difference between groups E and B ($p > 0.05$). Considering the three groups of glutamine in different doses, the apoptosis index from low to high were groups C, D and E. There was no significant difference between groups C and E ($p < 0.05$), and there were no obvious differences between groups A, B and D.

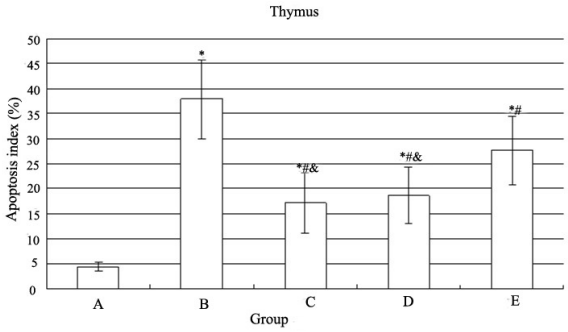

Figure 2.
The apoptosis index of the thymic cells
*p < 0.05, compared to group A; #p < 0.05 compared to group B; &p < 0.05 compared to group E.

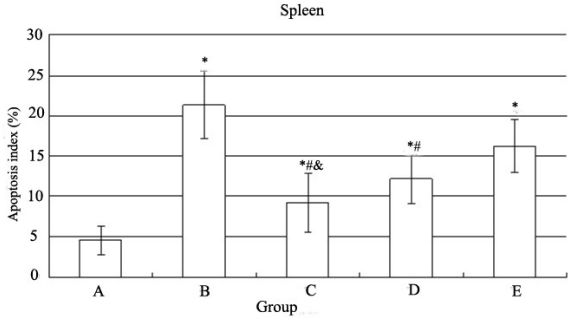

Figure 3.
The apoptosis index of the spleen cells
*p < 0.05 compared to group A; #p < 0.05 compared to group B; &p < 0.05 compared to group E.

Figure 4 demonstrates that the apoptosis of lymphocytes relieved under the effect of high-dose glutamine but apoptosis still existed.

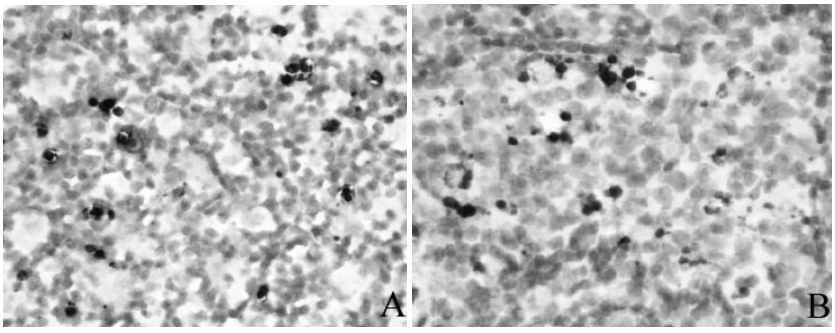

Figure 4.
Cell apoptosis in the thymoma (A) and spleen (B) of group C under a microscope (x400)

Abnormal apoptosis of lymphocyte is one of the manifestations of sepsis [17]. The progression of sepsis is accompanied by abnormal apoptosis of cells such as lymphocyte and gastrointestinal epithelial cells. In normal conditions, the abnormal apoptosis of lymphocytes can promote the growth and proliferation of immune cells and maintain the stability of immune process [16]. The establishment of cecal ligation and puncture is an effective research approach for the investigation of the pathological mechanism of sepsis. The model was established by inducing local infection through puncturing the cecum to let faeces penetrate the abdominal cavity. Systemic inflammatory reactions may be induced with the spread and deepening of infection [19]. The successful modelling of sepsis mice can be tested by the following standards: 2 times increase of respiratory frequency, increase or decrease of rectal temperature for more than 1°C and increase of heart rate for more than 50%; as to specific manifestations, the ligation end is swollen, black and distributed with yellow fester on the surface [21]. Li K. *et al.* [15] found that glutamine supplementation reduces cell apoptosis through oxidative-related mechanisms. Harnett C.C. *et al.* [11] found that B lymphoma at stage II experienced apoptosis in several minutes after glutamine deprivation. Other researchers found that, glutamine had an ignorable role in the apoptosis of lymphocyte; therefore the scientific literature is contradictory. Therefore, in our study we used glutamine in the doses of 0.75 g/kgbw, 0.5 g/kgbw and 0.35 g/kgbw in the sepsis models and found that the apoptosis index of the animals that received glutamine was significantly lower than that of models without glutamine, both in the spleen and thymus. This suggests that glutamine can strengthen the anti-apoptotic capacity of lymphocyte and reduces the apoptosis of lymphocytes. Moreover, the comparison between the groups administered with different doses suggested that the apoptosis index of lymphocyte in the thymus increased with the reduction of dose, meaning that a higher dose of glutamine could promote stronger anti-apoptosis capability and less apoptotic cells in the thymus; the higher dose of glutamine could determine lower cell apoptosis index, suggesting that glutamine in a high dose could effectively improve the anti-apoptosis capability of cells and reduces cells' apoptosis.

Conclusions

In conclusion, glutamine in a high dose is more effective in improving the anti-apoptotic capability of lymphocyte compared with low doses. High doses of glutamine determine the decrease of lymphocyte apoptosis index associated with a decrease of apoptotic lymphocytes in spleen and thymus.

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