Effect of Glutamine on Caspase-3 mRNA and Protein Expression in the Myocardium of Rats With Sepsis

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Abstract: Apoptosis and caspase-3 play an important role in the pathogenesis of sepsis. In this study, the authors monitored myocardial apoptosis and investigated caspase-3 protein expression change in rats with sepsis. In addition, we investigated the protective effect of glutamine (Gln) on myocardial injury in septic rats. A rat model of sepsis was established by intraperitoneal injection of lipopolysaccharide (LPS). Rats were divided into control group, endotoxin (LPS) group and LPS + Gln group, which were further divided into 4 subset groups (0, 6, 12 and 24 hour subgroups; n = 6). The rate of myocardial apoptosis, caspase-3 mRNA expression and caspase-3 protein expression were examined. Data were analyzed using the F-test or linear correlation test. The results revealed that the rate of myocardial apoptosis in the LPS group was significantly higher than that in the control group ($P < 0.05$). Compared with the control group, LPS group has an upregulated caspase-3 mRNA expression level. However, the caspase-3 protein was low expressed ($P < 0.05$). The LPS + Gln group has significant lower myocardial apoptosis rate compared with the LPS group ($P < 0.05$). In addition, caspase-3 mRNA expression levels and caspase-3 protein expression levels were lower in the LPS + Gln group ($P < 0.05$). We found that Gln reduces the extent of myocardial apoptotic cell death by decreasing the gene and protein expression of caspase-3. Therefore, Gln may be used to prevent the onset of sepsis at an early stage.

Key Indexing Terms: Sepsis; Glutamine; Myocardial apoptosis; Caspase-3. **[Am J Med Sci 2014;348(4):315–318.]**

Researches confirmed that cardiac myocyte apoptosis occurs during sepsis both in animal trial and clinical condition.[1,2] Apoptosis is a complex process mediated by caspase family proteases, wherein caspase-3 plays an important role in initiating cell death.[3] In this study, lipopolysaccharide (LPS) was used to induce a model of sepsis in rats. Myocardial cell apoptosis, caspase-3 gene and protein expression were monitored. In addition, the effect of glutamine (Gln) on apoptotic cell death was investigated.

MATERIALS AND METHODS

Experimental Reagents

LPS and Gln were purchased from Sigma-Aldrich (St. Louis, MO); the diaminobenzidine kit and caspase-3 antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); the Hoechst staining kit was purchased from Jiangsu Biyuntian Biotechnology Research Institute (Jiangsu, China); the Triton X-100 was purchased from BBI Company; the Takara RNA PCR kit (AMV) ver2.1 and RNAiso Plus were purchased from Takara Biotechnology Co, Ltd and the DNA Marker was purchased from Dongsheng Biotechnology Co, Ltd.

Preparation and Grouping of Experimental Animals

Healthy Sprague-Dawley rats (250 ± 50 g) were provided by the Experimental Animal Center of Sun Yat-Sen University. All animal care and experiments were performed under institutional protocols approved by the Institutional Animal Care and Use Committee at Jinan University, Guangzhou, China. Sprague-Dawley rats (250 ± 50 g) were randomly divided into 3 groups: control group (CON group), LPS group and LPS + Gln group. LPS was intraperitoneally injected to induce sepsis. The CON group was injected with 1 mL of phosphate-buffered saline (PBS) solution as blank control; The LPS group was injected with LPS (4 mg/kg) dissolved in 1 mL PBS; The LPS + Gln group was injected with LPS (4 mg/kg) and Gln (0.3 g/kg) dissolved in 1 mL PBS. Each group was divided into 0, 6, 12 and 24 hour subgroups. Each subgroup contains 6 rats.

Specimen Collection and Handling

Animals from each subgroup were anesthetized with 0.1% (vol/vol) pentobarbital sodium (4 mL/kg). The rat heart was harvested, and 2 pieces of myocardial tissue were selected from the front wall of the left ventricle after being flushed with ice-cold salt water. Each tissue sample was fixed with 10% (vol/vol) formaldehyde, which was then embedded and sliced for immunohistochemical staining. The remaining myocardial tissue was stored at −80°C.

Examination of Myocardial Apoptosis by Electron Microscopy

Myocardial tissue was fixed with glutaraldehyde, then rinsed with PBS and fixed with 1% (wt/vol) osmic acid. Tissue was dehydrated with alcohol gradients after being rinsed with PBS, infiltrated and embedded into epoxy resin, followed by polymerization at 60°C for 3 days. Tissue was sliced and stained with uranium and lead, and myocardial apoptosis was observed under an electron microscopy.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

The left ventricle cardiac muscle of rats was selected for paraffin freezing and slicing. TUNEL labeling was performed according to the TUNEL labeling kit instructions. Sections were permeabilized for 5 minutes with 0.1% (vol/vol) Triton X-100 after washing with PBS, and tissue was blocked with 3% (vol/vol) normal goat serum (containing 0.25% [vol/vol] Triton X-100) for 1 hour. The caspase-3 monoclonal antibody was added and incubated overnight at 4°C. After the sections were washed with PBS, the horseradish peroxidase–labeled anti-digoxigenin secondary antibody was added to slices, incubated at room temperature and kept away from light for 30 minutes. Hoechst 33258 staining was added dropwise (approximately 0.5 mL, 3–6 minutes), the

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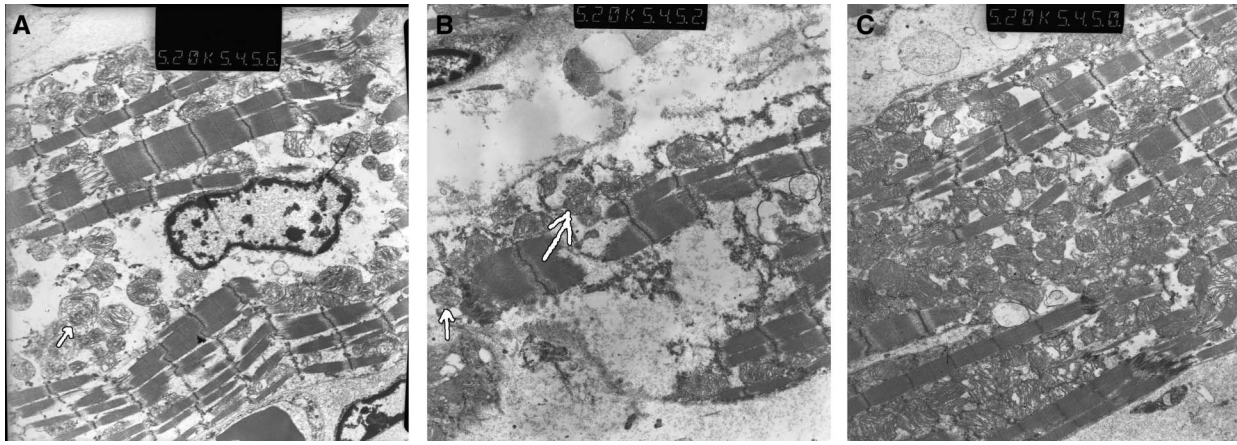

FIGURE 1. The myocardial pathology in rat by electron microscope at 12 hours. (A) CON group, (B) LPS group, (C) LPS + Gln group. Comparing with the myocardial in the CON group, the LPS group has more density under the electron microscopy, which reveals the apoptosis (as indicated by arrows).

sections were washed with PBS. The sections were placed on a slide add antifade mounting medium and cover glass slides were loaded. Staining was observed with a fluorescence microscope. Section without the addition of primary antibody and secondary antibody was used as a control. Fluorescence staining appearing inside the cytoplasm and membrane indicated positive labeling, and cells with dense blue nuclei were considered apoptotic. Ten sections were observed for each group (each rat), 10 views were randomly selected for each slice under light microscopy (×400), and the MICRO-COSMOS MiVnt image analysis system was applied for image analysis to measure the average optical density of positive cell nuclei and cytoplasm (Mean Optical Density [MOD] value). The cell apoptotic index (AI) was calculated using the equation: AI% = MOD × percentage of positive cells × 100.

RT-PCR for Caspase-3 mRNA Expression

Total RNA was isolated from myocardial tissue using RNAiso Plus kit following the manufacture's instructions. The primers used were: caspase-3, 5′-GGACCTGTGGACCT-GAAAAA-3′ and 5′-GCATGCCATATCATCGTCAG-3′; β-actin, 5′-TCAGGTCATCACTATCGGCAA-3′ and 5′-AAA-GAAAGGGTGTAAAACGCA-3′.

RT-PCR was performed under the following conditions: denaturation for 2 minutes at 94°C, followed by 35 cycles, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 1 minute at 72°C; the last extension was at 72°C for 5 minutes. The PE GeneAmp 5700 Fluorescence PCR detector was used for automatic analysis. β-actin was used as an internal reference to calculate the ratio between caspase-3 and β-actin. The ratio was the quantitative relative value of the PCR product.

Caspase-3 Protein Immunohistochemistry

Cardiac slices (5 μm) were processed with acetaldehyde dewaxing and antigen retrieval. Slices were first digested with 0.1% (wt/vol) trypsin, washed with distilled water and then incubated with 3% (vol/vol) hydrogen peroxide followed by PBS wash. Slices were blocked for 10 minutes with goat normal serum. The caspase-3 primary antibody was added to the slices and incubated overnight at 4°C. The sections were incubated at 37°C for 30 minutes with a goat anti-mouse secondary antibody after being washed with PBS. Slices were washed with PBS, and diaminobenzidine reagent was added. The sections were restained with hematoxylin after being washed with clean water. The slice was sealed with neutral resin after being washed. Staining was observed under a light microscope (×200 magnification), and 10 views were randomly selected. The average optical density of positive staining and the positive expression ratio (the percentage of the positive total area in all cells viewed) was detected (MOD), and the protein positive expression index (PEI) was calculated (%): PEI = MOD × expression area ratio × 100%.

Statistical Analysis

All data were analyzed using SPSS 11.5 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Electron Microscopy of Myocardial Apoptosis

In the LPS group, some myofilaments were observed disrupted. The number of mitochondria appeared to be increased; however, their arrangement were disordered. Mitochondrial cristae were partially broken and dissolved. In addition, some

TABLE 1. Apoptotic index in septic rat myocardial cells ($\bar{x} \pm s$)

Group	n	0 hr	6 hr	12 hr	24 hr	*F*	*P*
CON	24	26.68 ± 5.48	27.33 ± 6.12	26.62 ± 5.98	27.48 ± 7.23	186.78	0.000
LPS	24	26.33 ± 5.87	169.13 ± 27.31	309.40 ± 42.68	248.65 ± 47.93	6	
LPS + Gln	24	26.33 ± 6.85	147.08 ± 36.30	155.83 ± 49.39	126.68 ± 34.59		
F			75.187				
P			0.000				

SNK test: $P < 0.05$, compared between groups; $P < 0.05$, compared between time points.