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Carbachol alleviates myocardial injury in septic rats through PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of carbachol on myocardial injury in septic rats, and to further study its influence on the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway.

MATERIALS AND METHODS: A total of 48 healthy male Sprague-Dawley rats were randomly divided into sham group (n=16), model group (n=16), and carbachol group (n=16). The rat model of sepsis was established *via* cecal ligation and puncture. Carbachol was intraperitoneally injected (10 μg/kg) immediately after operation in carbachol group, and no cecal ligation was performed in sham group. At 48 h after operation, the survival rate of rats in each group was recorded, the activity of plasma creatine kinase-MB (CK-MB) was detected, and the cardiac function in each group was determined. Moreover, the heart was isolated, and the myocardial tissues were taken to detect the apoptosis level using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit. The content of inflammatory factors in myocardial tissues was determined using enzyme-linked immunosorbent assay (ELISA) kits, and the expression levels of apoptosis-related proteins and the PI3K/AKT signaling pathway-related proteins were detected *via* Western blotting.

RESULTS: Carbachol could significantly raise the survival rate of septic rats ($p<0.01$), remarkably decrease the activity of CK-MB ($p<0.01$), markedly reduce the left ventricular internal diameter at end-systole (LVIDs), and markedly increase the left ventricular ejection fraction (LVEF, %) and left ventricular fractional shortening (LVFS, %). Besides, carbachol could evidently lower the apoptosis level of myocardial cells of septic rats ($p<0.01$), reduce the content of inflammatory factors including tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß) and IL-6 ($p<0.01$), notably decrease the expression of Caspase-3 in myocardial tissues ($p<0.01$), remarkably increase the expression of Bcl-2/Bax ($p<0.01$), and distinctly inhibit the expressions of phosphorylated (p-)PI3K, p-AKT, Nod-like receptor protein 3 (NLRP3), and Caspase-1 ($p<0.01$).

CONCLUSIONS: Carbachol can reduce the release of inflammatory factors in myocardial cells, the expression of apoptotic proteins and the apoptosis of myocardial cells, and improve the cardiac function and survival rate of septic rats by inhibiting the PI3K/AKT signaling pathway.

Key Words:
Sepsis, Carbachol, Myocardial injury, PI3K/AKT signaling pathway, Inflammation.

Introduction

Sepsis refers to a kind of severe systemic inflammatory response syndrome caused by infection, which often causes damage to multiple organs in the body and even threatens the life[1]. Sepsis has acute onset, and it frequently occurs in the elderly and immunodeficiency people, with a high mortality rate, which is one of the main factors leading to the death of critical patients[2]. The pathogenesis of sepsis is complex, mainly involving systemic inflammatory response, immune dysfunction, and tissue damage, and it is closely related to pathological and physiological changes in multiple organs in the body[3]. Sepsis can lead to hemodynamic dysfunction and further result in cardiac insufficiency, dysfunction of ventricular dilatation, relaxation, and contraction, cardiac function involvement, and damage to myocardial cells[4]. Kim et al[5] found that about 50% of septic patients suffer from cardiac insufficiency in different degrees, and reducing the inflammatory response in the body can effectively improve the cardiac function. "Cholinergic

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anti-inflammatory pathway" is a neuro-immune regulatory mechanism that exerts a rapid and direct anti-inflammatory effect through cholinergic nerves, which provides a new idea for the treatment of systemic inflammatory response[6]. Carbachol, a cholinergic receptor agonist, is able to dilate blood vessels, facilitate glandular secretion and promote gastrointestinal motility. Lee et al[7] found that carbachol reduces the cerebral infarction area and improves the neurological function in ischemia/reperfusion rats by resisting oxidation and reducing the release of inflammatory factors. Phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) are closely related to inflammatory response, and activating the PI3K/AKT signaling pathway can significantly increase the expression of Nod-like receptor protein 3 (NLRP3), and promote the release of inflammatory factors[8,9]. Currently, there are few studies on the association between carbachol and the PI3K/AKT signaling pathway. In the present research, therefore, the rat model of sepsis was established to evaluate the effect of carbachol on the PI3K/AKT signaling pathway and its protective effect against myocardial injury in septic rats.

Materials and Methods

Laboratory Animals and Grouping

Sprague-Dawley (SD) rats aged 28 weeks old and weighing 220-250 g were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine. All rats were adaptively fed for 7 d under the humidity of (50±3)%, temperature of (25±1)°C, and 12/12 h light/dark cycle, and had free access to food and water. 48 rats were randomly divided into sham group (n=16), model group (n=16), and carbachol group (n=16). The rat model of sepsis was established *via* cecal ligation and puncture in carbachol group and model group. Carbachol (Sigma-Aldrich; St. Louis, MO, USA) was intraperitoneally injected (10 μg/kg) immediately after operation in carbachol group, while the same amount of normal saline was injected as the control in model group. In sham group, the abdominal wall was opened without cecal ligation, and the remaining operations were the same as those in model group.

All experimental operations were performed in accordance with the Guide for the Use of Laboratory Animals of the National Institutes of Health, and the experimental scheme was reviewed and approved by the Laboratory Animal Ethics Committee.

Modeling

At 12 h before operation, the rats were deprived of food, not water, and the rat model of sepsis was established *via* cecal ligation and puncture[10], as follows: after anesthesia with 1% pentobarbital sodium, the abdomen was depilated and the rats were fixed on the operating table, followed by abdominal disinfection and draping with sterile sheet. Then, the mid-lower abdomen was cut open along the midline of the abdomen to expose the cecum, the content of cecum was squeezed to the distal end, and the cecum was ligated at 3/4 of the inferior margin of cecum (avoid ligating the mesenteric vessels of ileum and cecum). The 18-gauge needle was used to perforate the dorsal cecum at the distal and proximal ends, and a small amount of content was squeezed out. Then, the cecum was placed back into the abdominal cavity, and the abdominal wall incision was sutured layer by layer. After operation, the rats were placed on the warming blanket. After resuscitation, the rats were fed in separate cages and had free access to food and water.

Survival Study

At 48 h after operation, the survival condition of rats in each group was recorded, and the survival rate was calculated. The survival condition of rats was evaluated using the rating scale[11], including the appearance, autonomic activity, respiratory rate, respiratory quality, independence consciousness, response to stimuli and secretions. There are 5 grades (0-4) in each index, the total score is the sum of the 7 indexes, and death of rats is recorded as the highest score.

Determination of Cardiac Function

At 48 h after operation, the cardiac function of rats in each group was detected using the Vevo2100 high-resolution small animal ultrasound imaging system (VisualSonics, Toronto, ON, Canada; transducer frequency: 250 MHz). M-mode echocardiography was performed as follows: after anesthesia, the rats were fixed on a heating plate in the supine position, and the limbs were connected to the electrocardiograph electrodes. After depilation, the ultrasonic guiding agent was added onto the skin for ultrasonic detection. The left ventricular internal diameter at end-systole (LVIDs), left ventricular ejection fraction (LVEF, %), and

left ventricular fractional shortening (LVFS, %) were recorded.

Determination of Creatine Kinase-MB (CK-MB) Activity and Content of Inflammatory Factors

At 48 h after operation, 2 mL of arterial blood was drawn from rats, in which the activity of plasma CK-MB in each group was detected using the HITACHI7170 full-automatic analyzer (Hitachi, Tokyo, Japan).

After the determination of cardiac function, the rats were immediately sacrificed, and the myocardial tissues were taken to detect the content of inflammatory factors [tumor necrosis factor-α (TNF-α), interleukin-1b (IL-1b) and IL-6] using the enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) in strict accordance with the instructions. The optical density (OD) value of each well was measured at 450 nm using a microplate reader (BioTek, Biotek Winooski, VT, USA), and the standard curves were plotted, based on which the levels of TNF-α, IL-1b, and IL-6 in each group were detected.

Detection of Apoptosis Level of Myocardial Cells

At 48 h after operation, the rats in each group were sacrificed, and the myocardial tissues were taken to detect the apoptosis level of myocardial cells using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining kit (Wuhan Beyotime Biotechnology Co., Ltd., Wuhan, China) in strict accordance with the instructions. The myocardial tissues were observed and photographed under a confocal fluorescence microscope (Nikon, Tokyo, Japan), and the apoptosis level of myocardial cells was calculated (the apoptotic cells showed yellowish green fluorescence, while normal cells showed no fluorescence).

Detection of Protein Expression Levels Via Western Blotting

At 48 h after operation, the rats in each group were sacrificed, and the myocardial tissues were taken and added with radioimmunoprecipitation assay (RIPA) lysis buffer (1 mg: 1 mL; Yeasen, Shanghai, China), followed by homogenization and centrifugation to extract the total protein. The protein was quantified using the bicinchoninic acid (BCA) protein quantification kit (Invitrogen, Carlsbad, CA, USA). After the loading buffer at an equal concentration was prepared, the protein was subjected to electrophoresis, transferred onto a membrane and sealed. Then, the target band was cut according to the protein molecular weight, and incubated with the monoclonal antibodies of Caspase-3, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), phosphorylated (p-)PI3K, PI3K, p-AKT, AKT, NLRP3, Caspase-1 and GAPDH (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After the band was washed with Tris-Buffered Saline and Tween-20 (TBST), it was incubated again with the secondary antibodies (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) at room temperature for 1 h, and washed again with TBST. Then, an appropriate amount of enhanced chemiluminescence (ECL) solution (solution A and solution B were mixed evenly at 1:1) was added in a darkroom for image development and fixation. After scanning, the gray value was analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The data in this study were expressed as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the data processing. Analysis of variance (ANOVA) was performed for the comparison among groups. Bonferroni's method was adopted for pairwise comparison in the case of homogeneity of variance, while Welch's method was adopted in the case of heterogeneity of variance. The survival curves were plotted using the Kaplan-Meier method. $p<0.05$ suggested that the difference was statistically significant.

Results

Survival Rate and Behavior Score of Rats

The survival rate in each group was recorded at 48 h after operation. As shown in Figure 1, there was no death in sham group within 48 h, and sepsis could significantly reduce the survival rate of rats ($p<0.01$), while carbachol could remarkably raise the survival rate of septic rats ($p<0.01$). At 48 h after operation, the clinical behavior score was notably higher in model group than that in sham group ($p<0.01$), while it was markedly lower in carbachol group than that in model group ($p<0.01$).

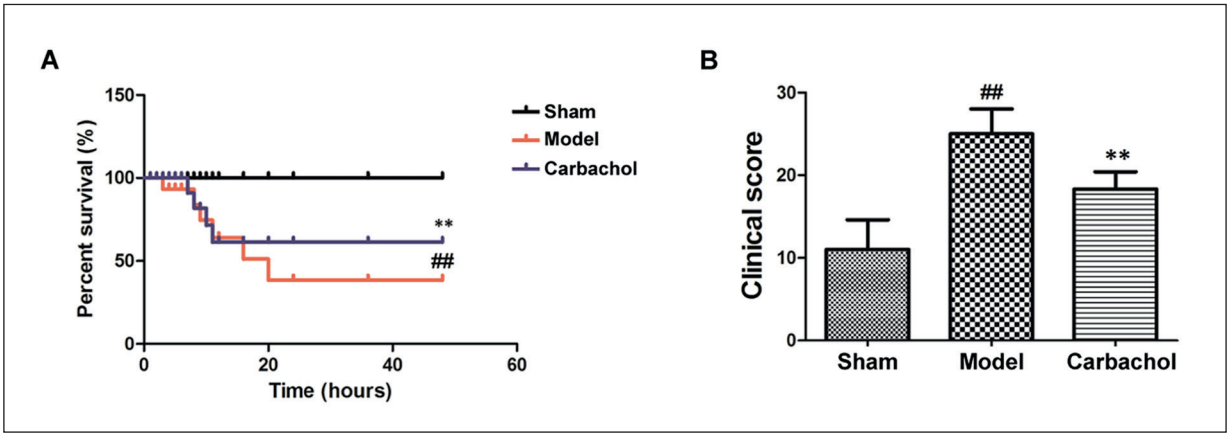

Figure 1. A, Survival rate of rats. **B,** Behavior score of rats. The survival rate is significantly lower in model group than that in sham group, while it is higher in carbachol group than that in model group. The clinical behavior score is notably higher in model group than that in sham group and carbachol group. $^{**}p<0.01$ *vs.* model group, $^{\#\#}p<0.01$ *vs.* sham group.

Changes in Cardiac Function of Rats

The cardiac function of rats in each group was recorded at 48 h after operation. As shown in Figure 2, model group had significantly increased LVIDs ($p<0.01$), and distinctly decreased LVEF (%) and LVFS (%) compared with sham group. Carbachol could markedly reduce LVIDs ($p<0.01$) and prominently increase LVEF (%) and LVFS (%) ($p<0.01$).

Changes in CK-MB Activity

At 48 h after operation, the activity of plasma CK-MB in each group was detected. It was found that the activity of plasma CK-MB evidently rose in model group compared with that in sham group ($p<0.01$), while carbachol could overtly reduce the activity of plasma CK-MB in septic rats ($p<0.01$) (Figure 3).

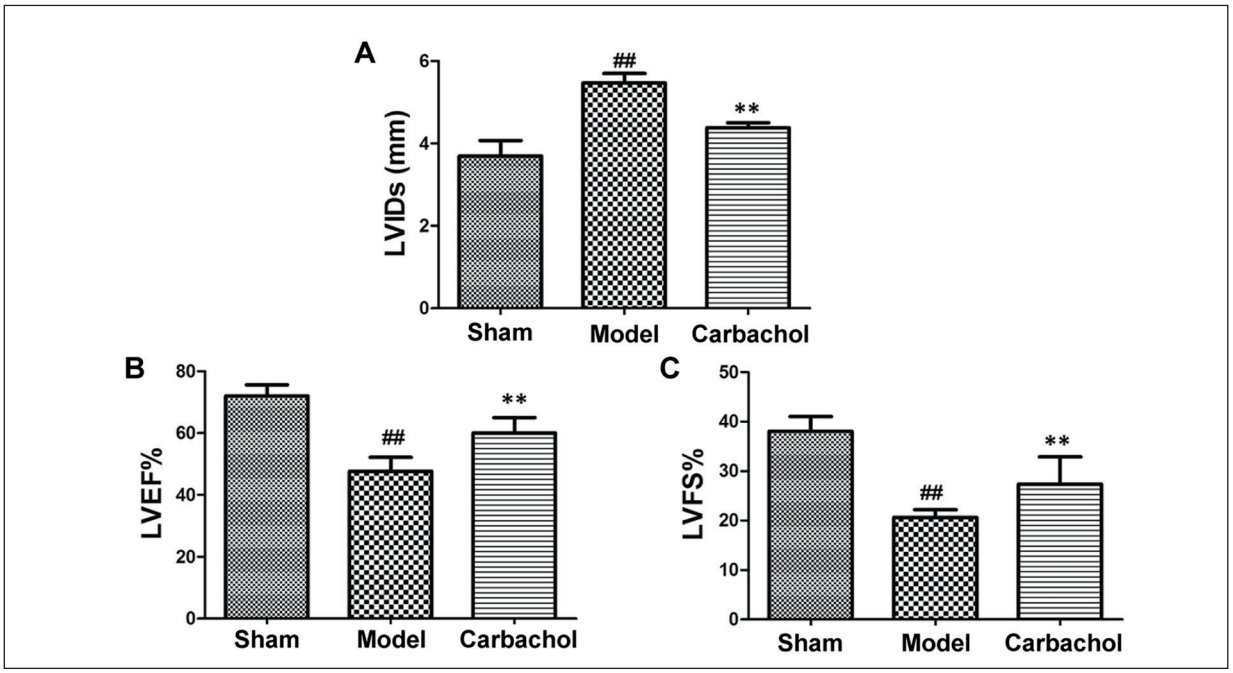

Figure 2. Cardiac function of rats detected *via* echocardiography. **A,** LVIDs. **B,** LVEF (%). **C,** LVFS (%). Model group has a significantly larger LVIDs and prominently lower LVEF (%) and LVFS (%) than sham group and model group. $^{**}p<0.01$ *vs.* model group, $^{\#\#}p<0.01$ *vs.* sham group.

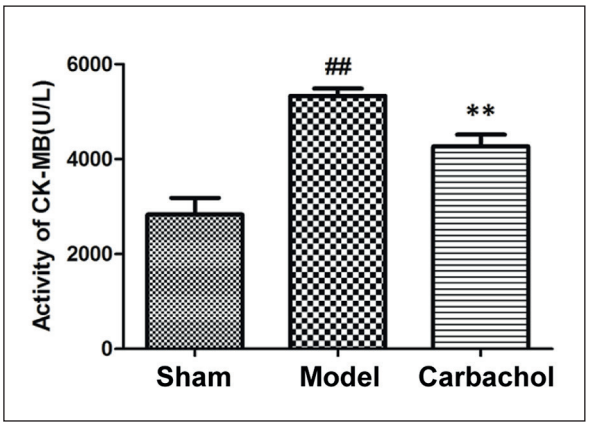

Figure 3. CK-MB activity. The activity of CK-MB in model group is evidently higher than that in sham group and carbachol group. **p<0.01 *vs.* model group, $^{##}p$<0.01 vs. sham group.

Changes in Apoptosis Level of Myocardial Cells

At 48 h after operation, the apoptosis level of myocardial cells in each group was determined using the TUNEL staining kit. The results showed that the number of apoptotic cells in myocardial tissue was remarkably increased in model group compared with that in sham group (p<0.01), while carbachol could clearly lower the number of apoptotic cells in septic rats (p<0.01; Figure 4).

Changes in Content of Inflammatory Factors in Myocardial Tissues

At 48 h after operation, the content of inflammatory factors in myocardial tissues was detected in each group. It was found that the content of TNF-α, IL-1b, and IL-6 in myocardial tissues was markedly higher in model group than that in sham group (p<0.01), while it was markedly lower in carbachol group than that in model group (p<0.01; Figure 5).

Expression Levels of Apoptosis-Related Proteins Detected Using Western Blotting

At 48 h after operation, the rats in each group were sacrificed and the myocardial tissues were isolated to detect the expression levels of apoptosis-related proteins in myocardial tissues in each group through Western blotting. As shown in Figure 6, the expression of Caspase-3 in myocardial tissues was significantly increased (p<0.01) and that of Bcl-2/Bax notably declined in mod-

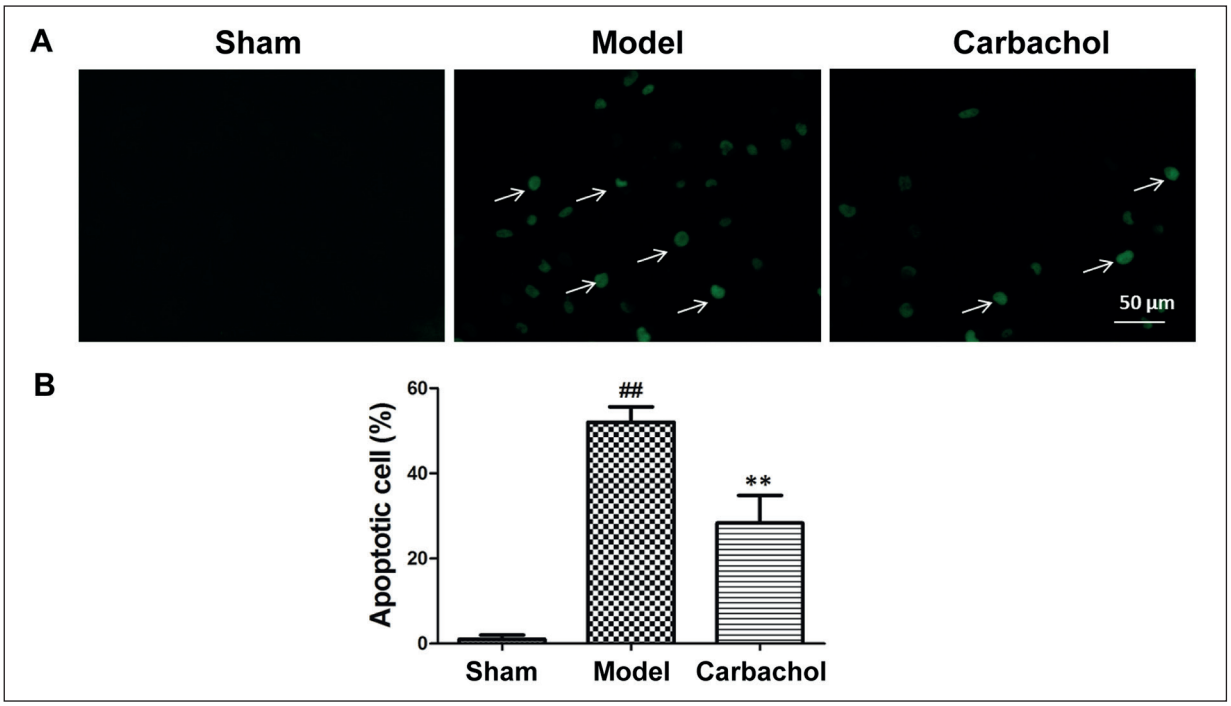

Figure 4. Apoptosis level of myocardial cells detected *via* TUNEL staining (magnification: 100×). The apoptosis level of myocardial tissues in model group is remarkably higher than that in sham group and carbachol group. **p<0.01 *vs.* model group, $^{##}p$<0.01 *vs.* sham group.

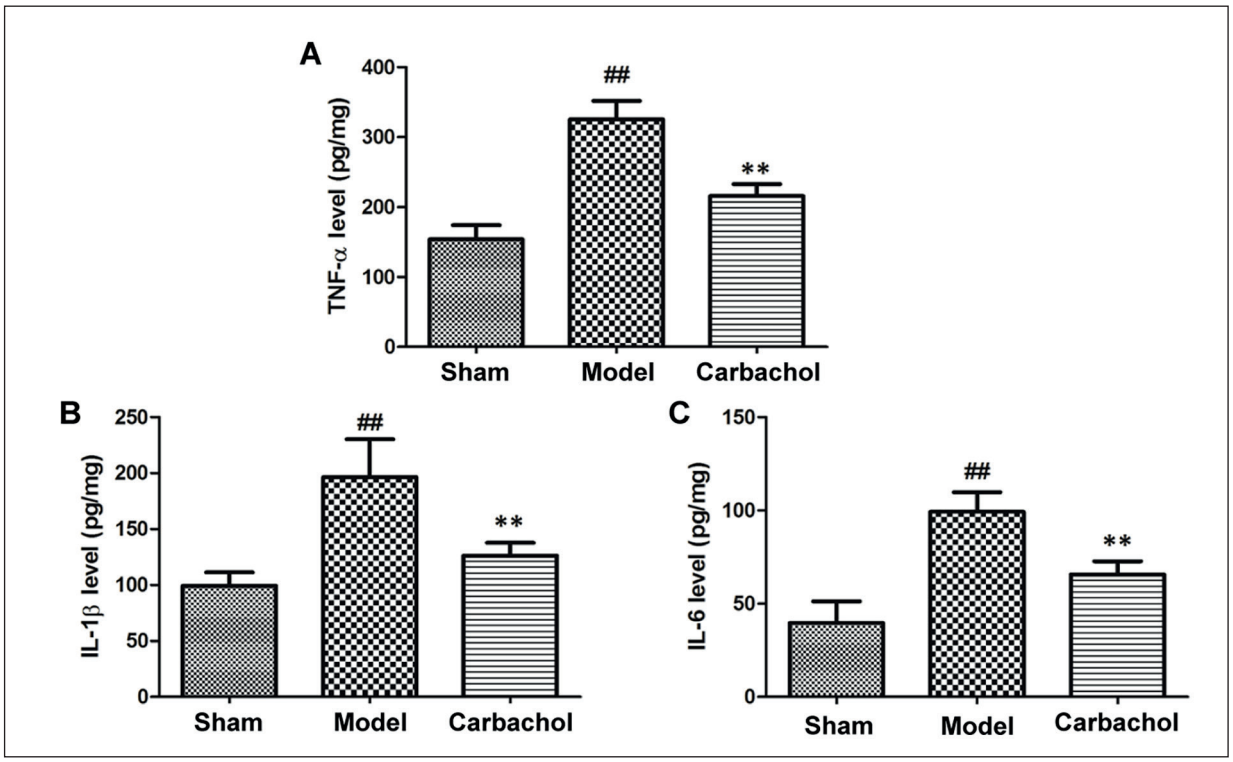

Figure 5. Content of inflammatory factors in myocardial tissues. **A,** Content of TNF-α. **B,** Content of IL-1b. **C,** Content of IL-6. The content of TNF-α, IL-1b, and IL-6 in myocardial tissues in model group is all markedly higher than that in sham group and carbachol group. $^{**}p<0.01$ *vs.* model group, $^{\#\#}p<0.01$ *vs.* sham group.

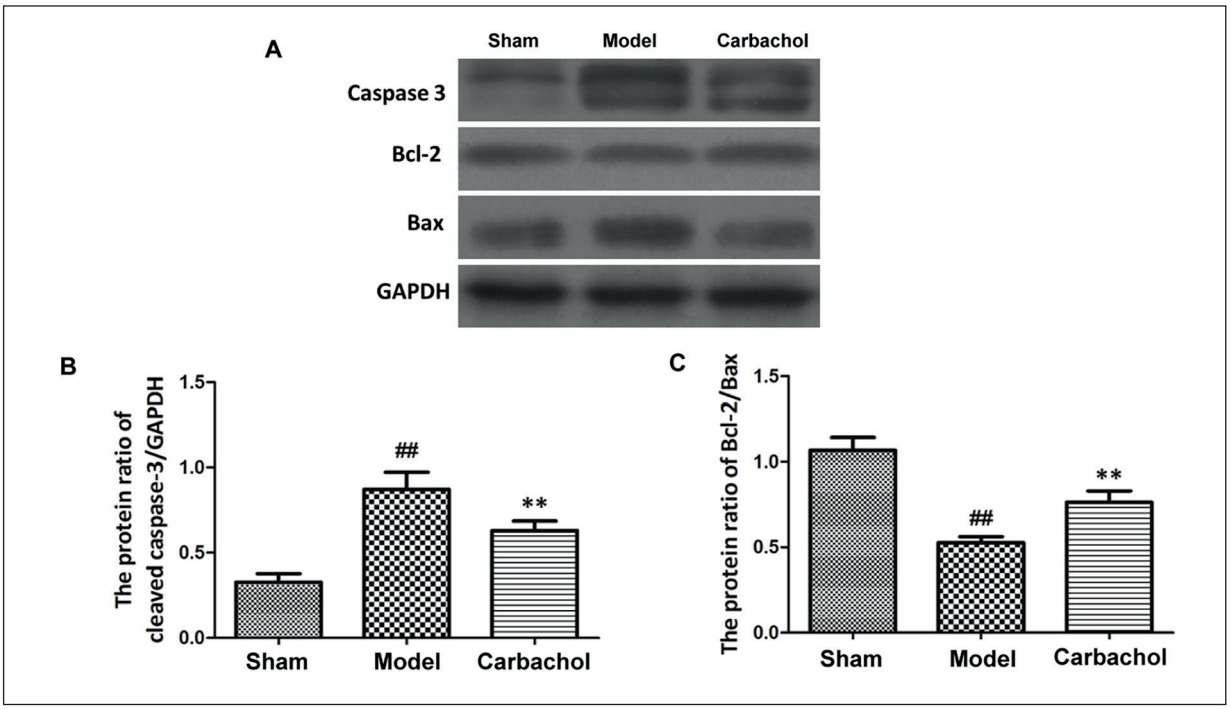

Figure 6. Expression levels of apoptosis-related proteins. In model group, the expression of Caspase-3 in myocardial tissues is significantly higher, while that of Bcl-2/Bax is notably lower than those in sham group and carbachol group. $^{**}p<0.01$ *vs.* model group, $^{\#\#}p<0.01$ *vs.* sham group.

el group compared with those in sham group (p<0.01), while the contrary is the case in carbachol group (p<0.01, p<0.01).

Expression Levels of PI3K/AKT Signaling Pathway-Related Proteins

At 48 h after operation, the rats in each group were sacrificed and the myocardial tissues were isolated to detect the expression levels of the PI3K/AKT signaling pathway-related proteins in myocardial tissues in each group through Western blotting. According to Figure 7, the expression levels of p-PI3K, p-AKT, NLRP3, and Caspase-1 in myocardial tissues rose dramatically in model group compared with those in sham group (p<0.01), while the contrary is the case in carbachol group (p<0.01).

Discussion

Sepsis leads to a high prevalence of organic injury to the heart, often dominated by arrhythmia and heart failure[12]. The mechanism of sepsis-induced heart injury has not been fully understood, and research evidence[13] suggests that the inflammatory response, calcium overload, oxidative stress response, and energy metabolism abnormality may be the main causes. Alotaiby et al[14] studied and found that the level of serum TNF-a in patients with myocardial infarction significantly rises, and it is positively correlated with the changes in serum myocardial enzymes. TNF-a is an inflammatory factor playing a key initial role, and the increase of its content can directly or indirectly stimulate the release of IL-6

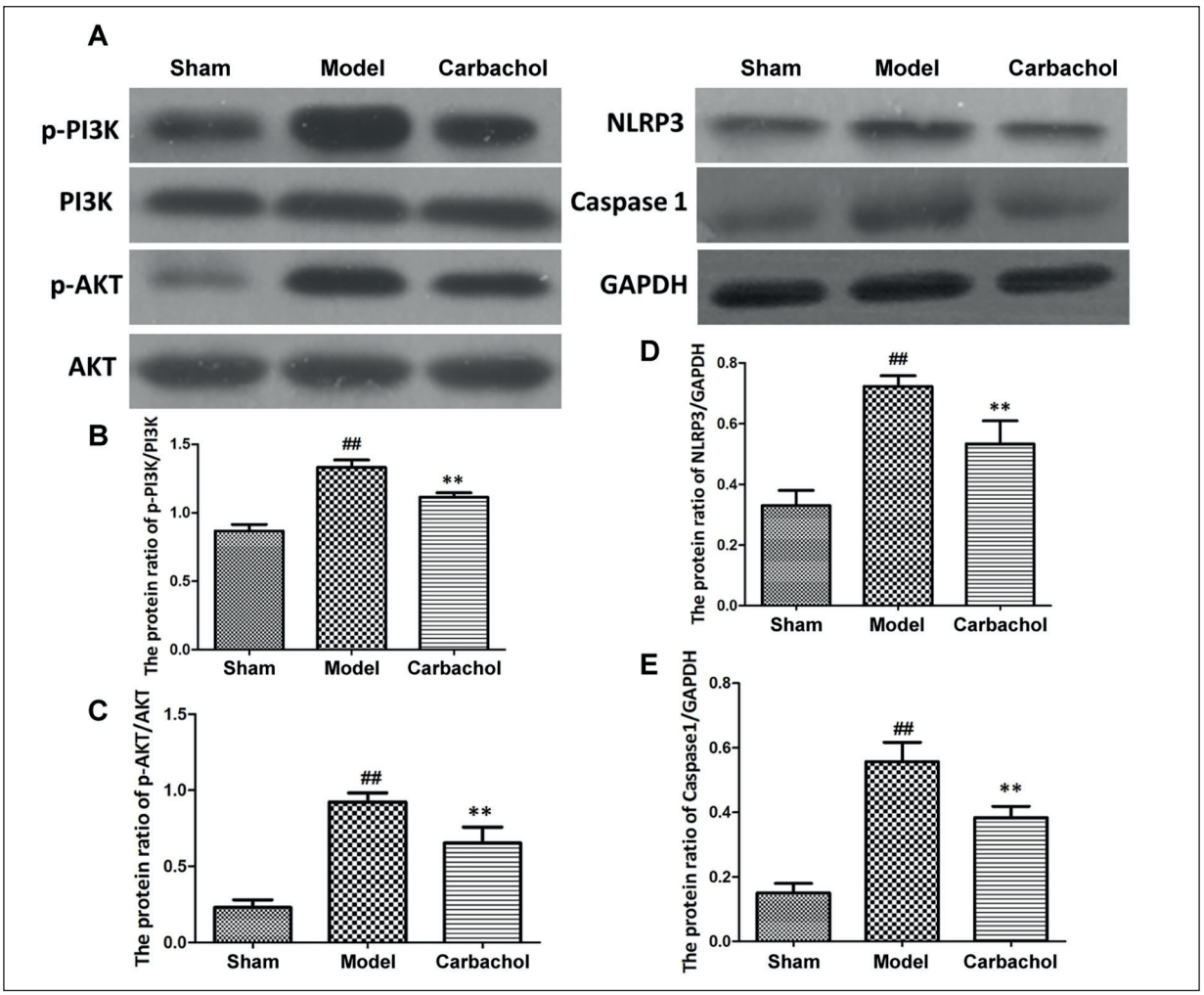

Figure 7. Expression levels of PI3K/AKT signaling pathway-related proteins. **A,** Protein bands. **B,** Expression level of p-PI3K. **C,** Expression level of p-AKT. **D,** Expression level of NLRP3. **E,** Expression level of Caspase-1. The expression levels of p-PI3K, p-AKT, NLRP3 and Caspase-1 in myocardial tissues in model group are all dramatically higher than those in sham group and carbachol group. **p<0.01 *vs.* model group, $^{\#\#}p$<0.01 *vs.* sham group.

and other inflammatory factors *in vivo*. The infiltration of a large number of inflammatory factors can directly damage tissues or organs and lead to organic injury[15]. In this study, it was found that in septic rats, the level of serum CK-MB significantly rose, LVIDs was apparently increased, both LVEF (%) and LVFS (%) distinctly declined, typical myocardial injury occurred, and the content of inflammatory factors TNF-a, IL-1b and IL-6 in myocardial cells was significantly raised. The above results indicate that sepsis-induced cardiac dysfunction may be closely related to the massive infiltration of inflammatory factors. Bach et al[16] showed that there is infiltration of a large number of inflammatory factors in peripheral blood of stroke patient, further leading to significant tissue ischemia, edema, and functional damage, and a large quantity of inflammatory factors are closely related to systemic infection, septic shock, etc.

Carbachol is an artificially synthesized cholinomimetic drug mainly used in the treatment of abdominal distension, gastric retention, and glaucoma in clinic. Carbachol has potent anti-inflammatory, anti-oxidative, and anti-apoptotic effects[17]. It is believed that the "cholinergic anti-inflammatory pathway" can effectively exert a systemic anti-inflammatory effect by stimulating the release of cholinergic neurotransmitters in the central nervous system. Hong et al[18] argued that electrically stimulating the vagus nerve or directly giving cholinergic neurotransmitters can effectively inhibit the release of inflammatory factors in plasma and small intestine, reduce the content of inflammatory factors, and remarkably alleviate the damage of tissue cells caused by ischemia. In this study, the rat model of sepsis was established, and carbachol in an effective dose was administered for protection immediately after treatment. The results manifested that carbachol could effectively lower the content of CK-MB in peripheral blood of septic rats, evidently improve cardiac function, and evidently reduce the apoptosis level of myocardial cells and content of inflammatory factors in myocardial tissues. Villapol et al[19] revealed that carbachol preconditioning for cerebral ischemia-reperfusion injury rats can protect against brain tissue damage caused by ischemia-reperfusion, whose mechanism may be that carbachol binds to cholinergic N receptor subunit on the cell membrane to significantly reduce the release of inflammatory factors.

In the case of severe infection or hypoxia in the body, the release of lysosomes can be activated to degrade the damaged macromolecules and organelles, thereby maintaining the homeostasis in the body and providing a suitable environment for the cell repair and renewal[20]. AKT is an important target protein in the PI3K/AKT signaling pathway, which possesses many physiological effects of regulating cell cycle, proliferation, and apoptosis. The PI3K/AKT signaling pathway is also an important signaling pathway affecting lysosomal effect *in vivo*, which can significantly influence the release of NLRP3 inflammasomes[21]. NLRP3 is a kind of non-specific inflammatory mediator mainly distributed in the cytoplasm and cell membrane, and activating NLRP3 can promote the release of Caspase-1. Then, a large amount of Caspase-1 can further cleave IL-1b and IL-6 in cells, and facilitate the maturation and release of inflammatory factors, leading to massive infiltration of inflammatory factors *in vivo*[22]. In this study, it was observed that the PI3K/AKT signaling pathway was activated in myocardial tissues of septic rats, further resulting in the release of NLRP3 and Caspase-1, promoting the release of inflammatory factors and damaging myocardial cells. Carbachol, by inhibiting the PI3K/AKT signaling pathway, could lower the release of NLRP3 and Caspase-1, reduce the apoptosis of myocardial cells, and protect the cardiac function.

Conclusions

In summary, carbachol can reduce the release of inflammatory factors in myocardial cells, the expression of apoptotic proteins and the apoptosis of myocardial cells, and improve the cardiac function and survival rate of septic rats by inhibiting the PI3K/AKT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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