

## Original Article

# Poly (ADP-ribose) synthetase inhibitor has a heart protective effect in a rat model of experimental sepsis

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**Abstract:** The aim of this study is to investigate whether PARP inhibitor could reduce cell apoptosis and injury in the heart during sepsis. Materials and methods: 60 healthy male Sprague-Dawley (SD) rats were randomly divided into 4 groups—sham group, modal group, 3-AB pretreatment group and 3-AB treatment group, 15 rats per group. The cecal ligation and puncture (CLP) model of sepsis was used. The following were determined—levels of malondialdehyde (MDA), ATP and nicotinamide adenine dinucleotide (NAD<sup>+</sup>), expression of PARP, Bcl-2, Bax, cytochrome C and caspase 3 activity in the myocardium tissue, levels of serum creatine kinase muscle brain (CK-MB) fraction and troponin I. Results: Histological and molecular analyses showed that myocardial cells apoptosis were associated with mitochondria injury, with an increase in the amount of PARP and a decrease in ATP and NAD<sup>+</sup> levels in model group. In addition, the levels of Bax, cytochrome C and caspase 3 activity, serum levels of CK-MB and troponin I increased, but levels of Bcl-2 significantly decreased. Inhibition of PARP upregulated the levels of ATP, NAD<sup>+</sup> and Bcl-2, and significantly reduced the activation of PARP and caspase 3, decreased the levels of MDA, cytochrome C, CK-MB and troponin I. As a result, apoptosis in the heart was attenuated. Conclusion: These results indicate that PARP activation may be involved in apoptosis in the heart induced by sepsis and 3-AB could improve it.

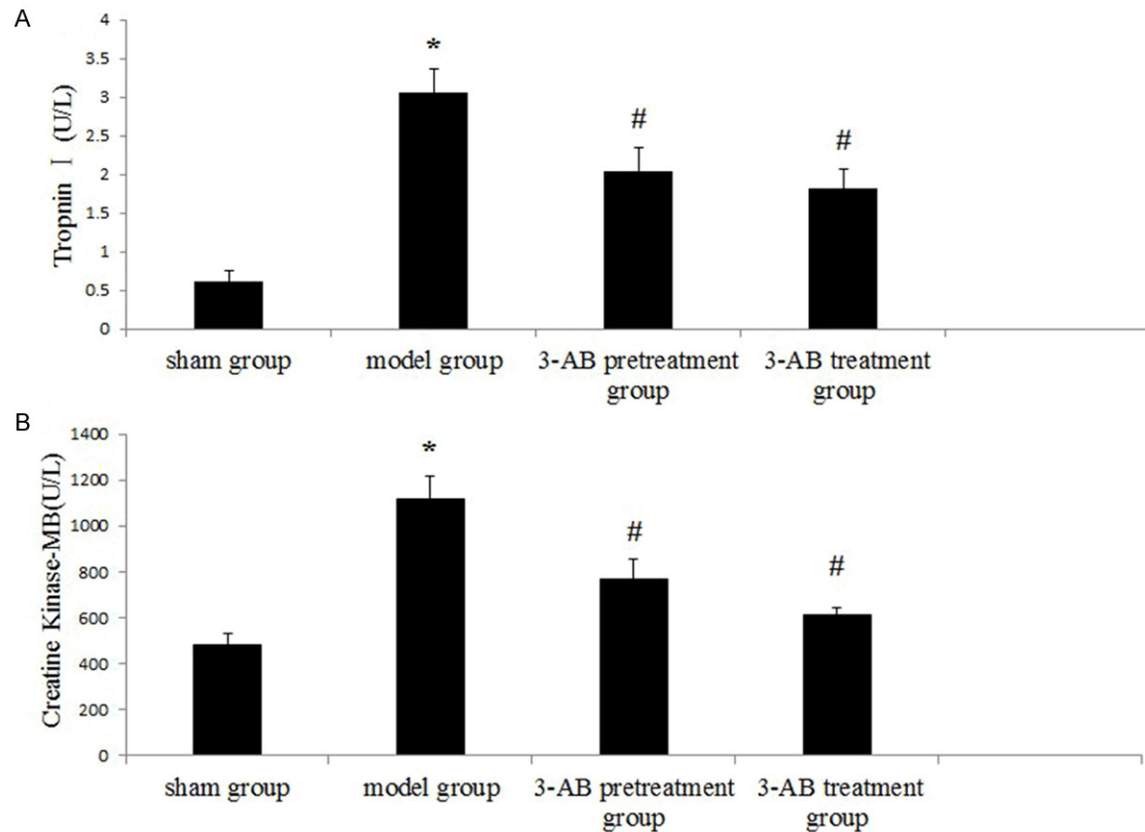
**Keywords:** Sepsis, 3-AB, heart, rats

## Introduction

Sepsis, a serious medical condition that is characterized by an overwhelming systemic response to bacterial infection, can lead to multiple organ failure, shock, and death [1] and is a leading cause of mortality in critically ill intensive care unit patients [2, 3]. In the U.S, sepsis is the leading cause of death, accounting for 215,000 deaths and \$17 billion in health care expenditures annually [4]. Despite advances in critical care medicine and use of anti-sepsis therapy, the mortality remains high and the long term outcome is poor for patients that survive sepsis [5, 6]. The severe reality suggests a need for additional therapies to the conventional approach to sepsis, thus, there is a urgent need for effective and safe drugs for the treatment of sepsis [7, 8]. PARP, a DNA nick-sensor enzyme and also an abundant nuclear enzyme in eukaryotic cells, is activated by DNA breakage and plays a critical role in physiological cellular functions, including DNA repair, gene tran-

scription, apoptosis and cell death. In massive DNA damage, PARP is over activated, exhausting nicotinamide adenine dinucleotide and leading to cell death. One apoptotic mechanism is induced by the over activation of PARP, which leads to intracellular depletion of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD<sup>+</sup>) [9], and the mechanism of PARP-dependent apoptosis is the occurrence of intracellular energy depletion in excessive oxidative stress-related pathophysiological processes such as stroke and ischemia/reperfusion injury [10]. Previous studies observed that myocardial PARP-dependent apoptosis is involved in the pathogenesis of sepsis-related myocardial depression [11, 12]. Several studies have showed that an increase in PARP activity causes damage of several organs such as brain and kidney that is significantly attenuated by PARP inhibition [13-16].

3-aminobenzamide (3-AB), the first inhibitor of PARP, is used to investigate the role of PARP in



**Figure 1.** Effects of 3-AB on serum levels of blood troponin I and CK-MB in rat CLP sepsis. In separate 24 time killed studies serum levels of blood troponin I and CK-MB were measured in rats with CLP sepsis. \*represents significantly different when compared to sham group ( $P < 0.05$ ), #represents significantly different when compared to model group ( $P < 0.05$ ).

a wide range of pathophysiological status in many animal models. Recent studies have investigated significant protective effects in myocardial ischemia/reperfusion injury by inhibiting PARP activity in vivo and in vitro animal models. And it has been demonstrated that PARP inhibition decreased infarct size, along with attenuation in heart mechanical dysfunction in PARP deficient mice models [17-19].

However, the role of PARP inhibition in heart injury of sepsis is not completely understood. According to the classical study, the essential mechanism of PARP-dependent apoptosis is the occurrence of intracellular energy depletion in excessive oxidative stress-related pathophysiological processes such as ischemia/reperfusion, stroke, injury, and diabetes [20, 21].

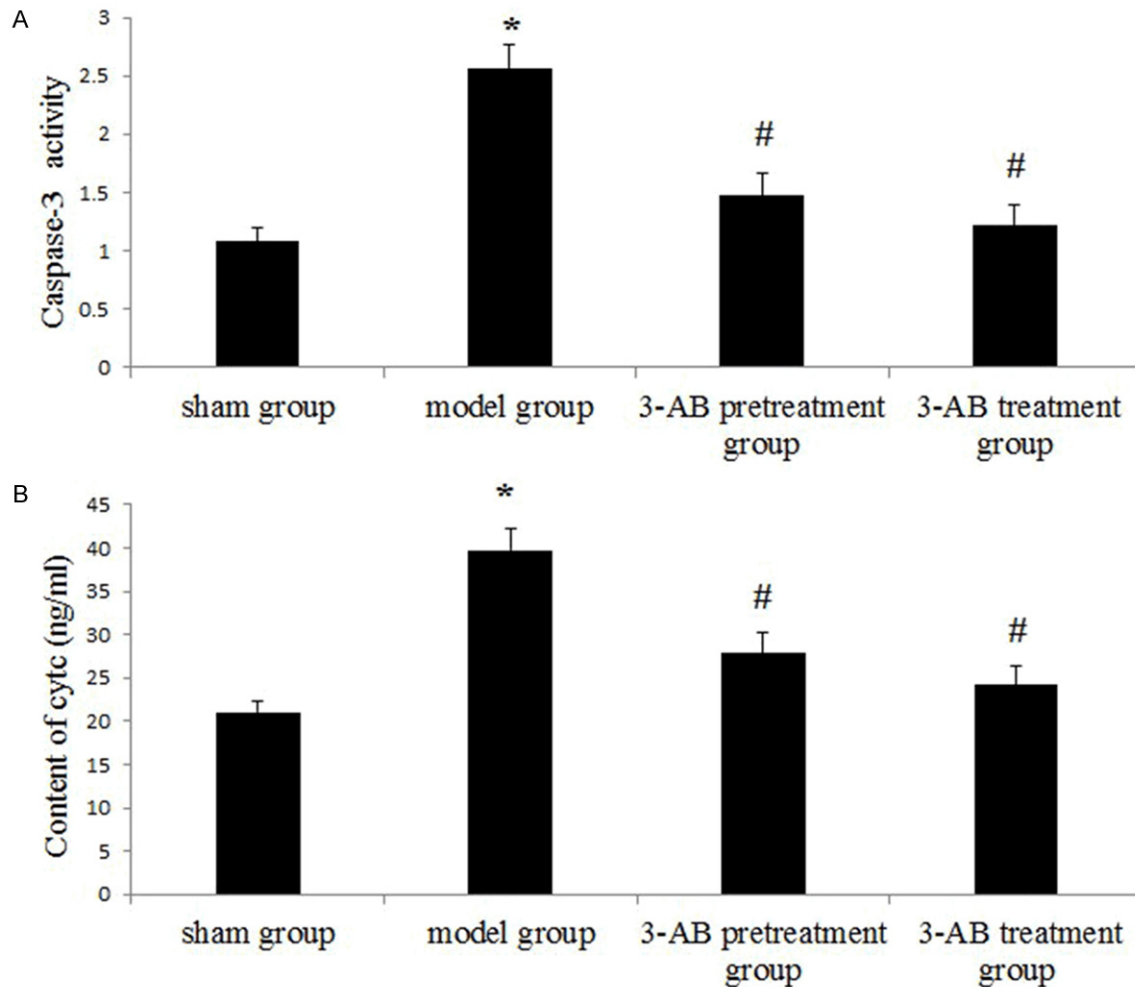
The aims of this study are to investigate whether PARP inhibition contributes to protect heart by reducing cardiomyocyte apoptosis, and to investigate the underlying molecular and cellular mechanisms.

So the parameters related with oxidative stress (MDA), energy metabolism (PARP, NAD, ATP) and mitochondrial apoptotic pathway (Bax, Bcl-2, CytC and Caspase-3) were measured in this study.

## Materials and methods

### Experimental design and rat model of sepsis

60 healthy male Sprague-Dawley (SD) rats (8 weeks old, 200 to 250 g) were purchased from Experiment Animal Center of Shandong Lu-ye pharmaceutical Limited by Share Ltd in Yantai (SCXK (Shandong) 20090009). The animals were allowed to acclimate for 1 week and maintained at a room temperature of  $22 \pm 2^\circ\text{C}$  and a relative humidity of  $60 \pm 10\%$ , with a 12 h light/dark cycle and free access to food and water. After 1-week-acclimation, the animals were randomly divided into 4 groups, 15 rats per group, sham group, modal group, 3-AB pretreatment group and 3-AB treatment group.



**Figure 2.** Effect of 3-AB on activity of caspase-3 and cytc levels in rat CLP sepsis. Activity of caspase-3 and cytc levels was evaluated by the ELISA-based method and expressed as a fold change compared to the control. \*represents significantly different when compared to sham group ( $P < 0.05$ ), #represents significantly different when compared to model group ( $P < 0.05$ ).

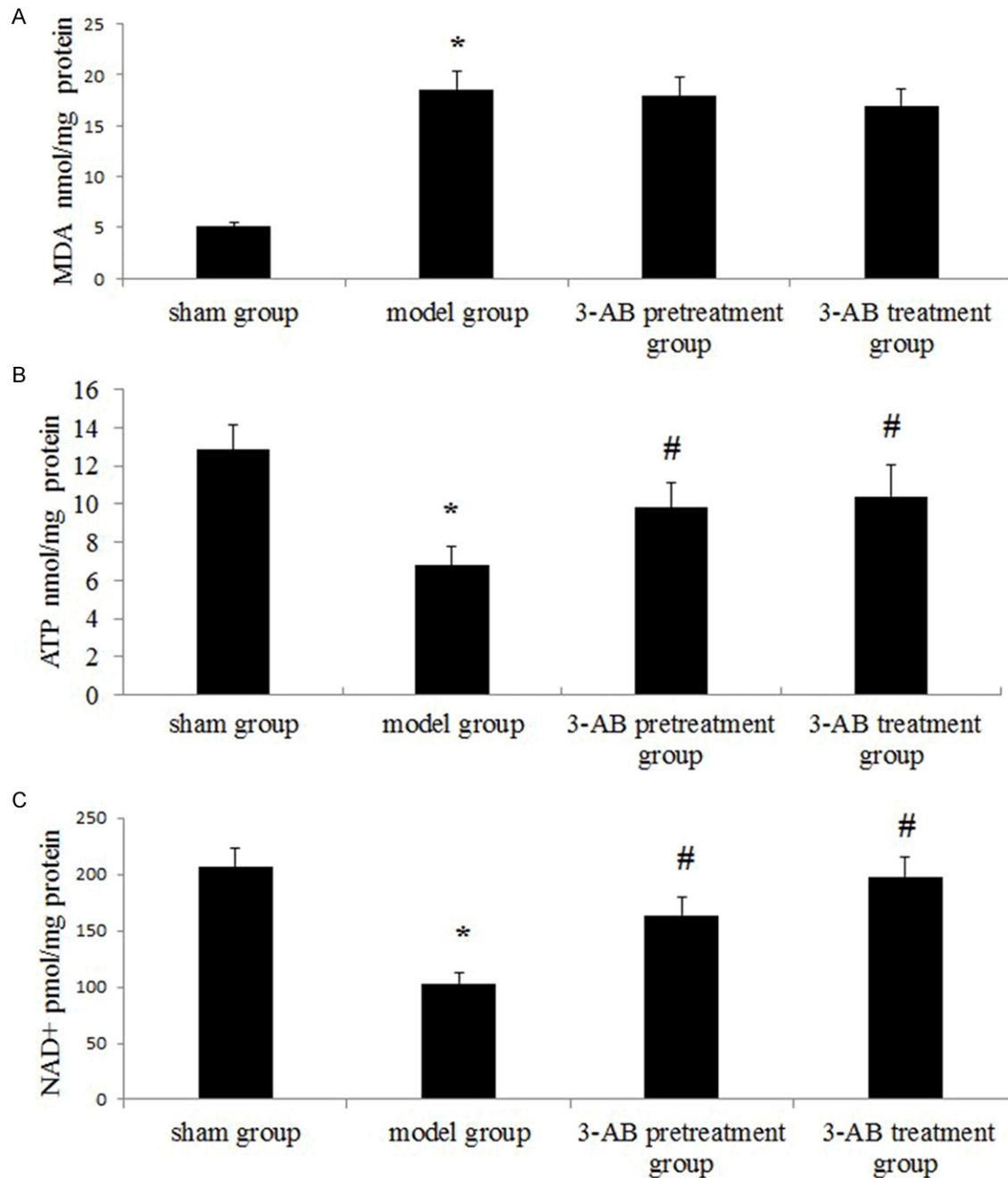
All the animals were anesthetized with 5 ml/kg pentobarbital (7%) by intraperitoneal injection during the surgery and about a sterile 3 cm midventra abdomina incision was made to expose the intestines. The ileocecal valve were ligated with 3-0 silk and three perforations were then made in the cecum by passing a 19-gauge needle "through and through". To ensure consistent cecal damages among all animals, the perforated cecum was squeezed until 50-80  $\mu$ l offeces extruded onto both surfaces, and then the bowel was reinserted into the abdomen and the incision was closed. A single post-operative saline (NS) bolus was provided (45 ml/kg subcutaneous) for fluid support. All surgical procedures and animal care were in accordance with National Institutes of Health (NIH) guidelines and are approved by

the Animal Care and Use Committee of Binzhou Medical University.

Animals were monitored as the time interval of three hours for clinical signs (degree of piloerection, presence of periorbital bleeding or nasal discharge, change of appetite, body temperature, body weight and blood count ). Rats were prepared for time-sacrifice at 24 h by induction of deep 5 ml/kg pentobarbital (7%) anesthesia.

#### Treatment groups

The rat in each group following CLP was received one of the following treatments, sham group (with laparotomy and bowel manipulation but no CLP, i.v. infusion of sterile saline, 10 mg/kg); CLP modal group (i.v. infusion of sterile



**Figure 3.** Effect of 3-AB on MDA, ATP and NAD<sup>+</sup> concentrations. \*represents significantly different when compared to sham group ( $P < 0.05$ ), #represents significantly different when compared to model group ( $P < 0.05$ ).

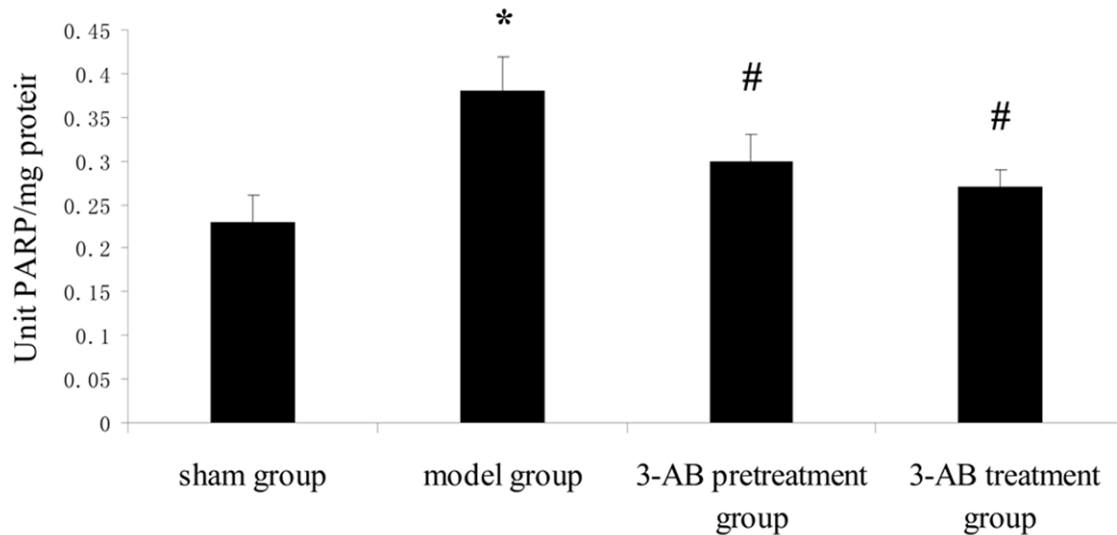
saline, 10 mg/kg); 3-AB pretreatment group (i.v. infusion of sterile 3-AB before 2 h of CLP, 10 mg/kg); treatment group (i.v. infusion of sterile 3-AB after 2 h of CLP, 10 mg/kg).

#### Measurement of serum creatine kinase muscle brain fraction and troponin I

The rats were sacrificed at 24 hours. First, blood was obtained from aorta abdominalis

after anesthesia. Blood samples, collected at baseline, were centrifuged at 3000 rpm for 10 minutes. Serum samples were obtained and analyzed for levels of blood Troponin I and Creatine Kinase Muscle Brain Fraction (CK-MB). Serum levels of CK-MB and Troponin I were measured in the top supernatant using the commercial kits via a Fuji DRI-CHEM 4000 i system and an i-STAT analyzer, respectively.

## The heart protective effect of Poly (ADP-ribose) synthetase inhibitor in rats



**Figure 4.** Effects of 3-AB on the PARP activity of myocardium. \*represents significantly different when compared to sham group ( $P < 0.05$ ), #represents significantly different when compared to model group ( $P < 0.05$ ).

### Measurement of cytochrome C levels and caspase-3 activity

The protein was extracted from myocardium tissues and quantified by the kits. The cytochrome C levels and activity of caspase-3 in myocardium tissues were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer protocol, and the value of absorbance (450 nm) was read with microplate reader (Thermo Multiskan MK-3, US).

### Measurement of malondialdehyde, ATP, and NAD<sup>+</sup>

The protein was extracted from myocardium tissues and quantified by the kits. The levels of malondialdehyde (MDA), ATP and NAD<sup>+</sup> in the myocardium tissues were determined using the Bioxytech MDA-586 assay kit, ATP Colorimetric/Fluorometric Assay Kit according to the manufacturer protocol. The levels of MDA, ATP and NAD<sup>+</sup> were normalized based on the protein concentrations in the tissue.

### PARP activity assay

PARP activity was measured with the universal colorimetric PARP assay kit (Trevigen, USA) based on the incorporation of biotinylated ADP-ribose onto histone proteins. The protein was extracted from myocardium tissues and quantified by the kits, 50 µg of protein were loaded

into a 96-well plate coated with histones and biotinylated poly ADP-ribose, allowed to incubate for 1.5 h, treated with strep-HRP, and read at 450 nm in a spectrophotometer.

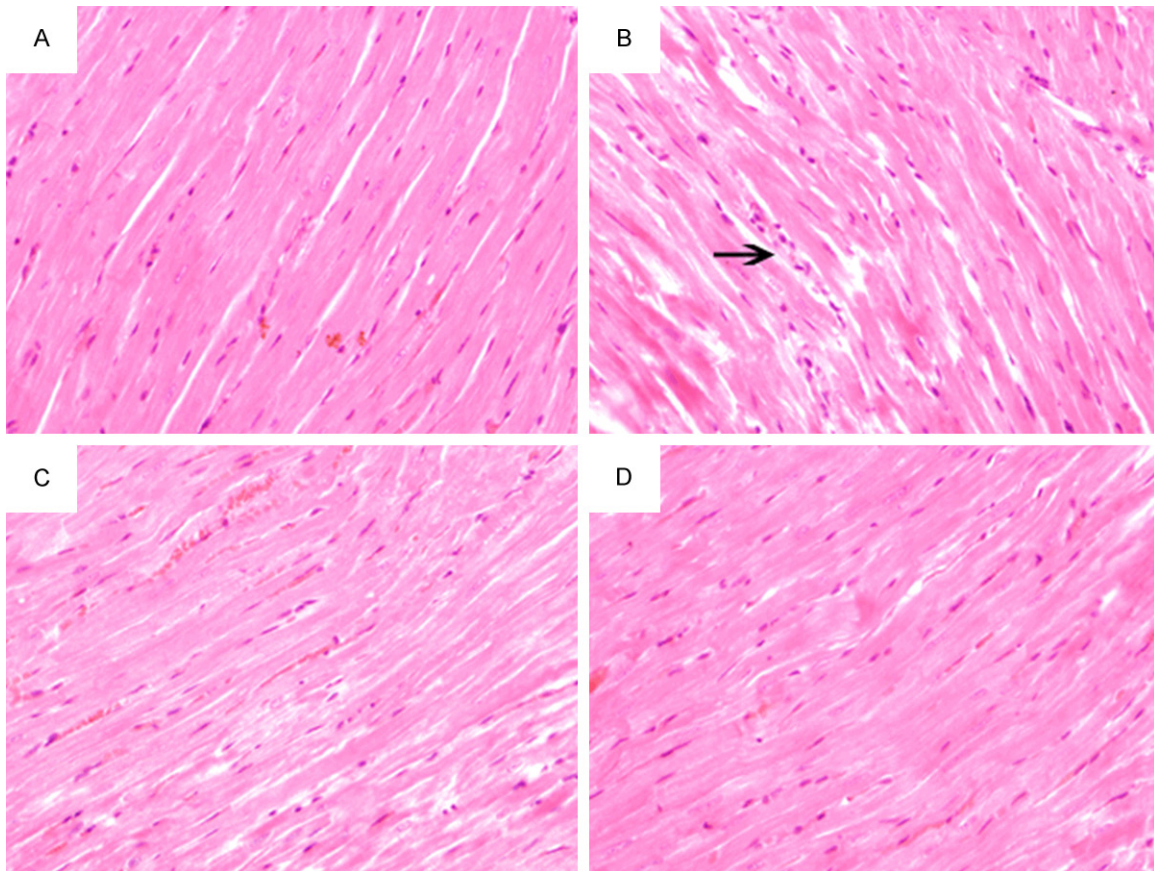
### Histology and transmission electron microscopy (TEM)

Isolated rat myocardium tissue was fixed in 10% methanal, embedded with paraffin, and cut into 4-µm thick sections. Morphology of myocardial tissue was observed under light microscope (Olympus X71-F22PH, Japan). For electron microscopic analysis, myocardial tissue were first fixed in 2.5% pentane 1, 5-dial (glutaraldehyde) for 3 hours, followed by 1% osmium tetroxide in 100 mmol/L phosphate buffer for additional 30 minutes. After dehydration, tissues were embedded in epoxy resin. Ultrathin sections were stained with uranyl ethanoate and lead citrate, and were visualized under TEM (JEM2100, JEOL, Japan).

### Immunohistochemistry

The myocardial tissue was fixed in 10% formalin for 48 h and embedded in paraffin. Tissues were sectioned at a thickness of 4 µm, deparaffinized, and stained with anti-rat PARP-1 antibody (diluted 1:300), anti-rat Bax antibody (diluted 1:500), anti-rat Bcl-2 antibody (diluted 1:500) followed by horseradish peroxidase coupled anti-rabbit IgG antibody. Immunostaining was performed by 5-min incubation with diami-





**Figure 5.** Histopathological results of myocardium (HE, original magnification  $\times 400$ ). A. Sham group. B. model group. C. 3-AB pretreatment group. D. 3-AB treatment group.

nobenzidine (DAB). PBS was used as the primary antibody in the negative control. DAB staining intensity was assessed with microscopic image analysis system (GX51, Japan).

#### Statistical analysis

All the data were analyzed by the statistical software package SPSS 16.0. Differences among all groups were analyzed by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) multiple range test to detect significant differences between groups. All the values were expressed as the means  $\pm$  standard deviation (SD). The results were considered significant at  $P < 0.05$ .

### Results

#### Measurement of troponin I and CK-MB levels

There was an obvious increase in troponin I and CK-MB levels in model group when compared

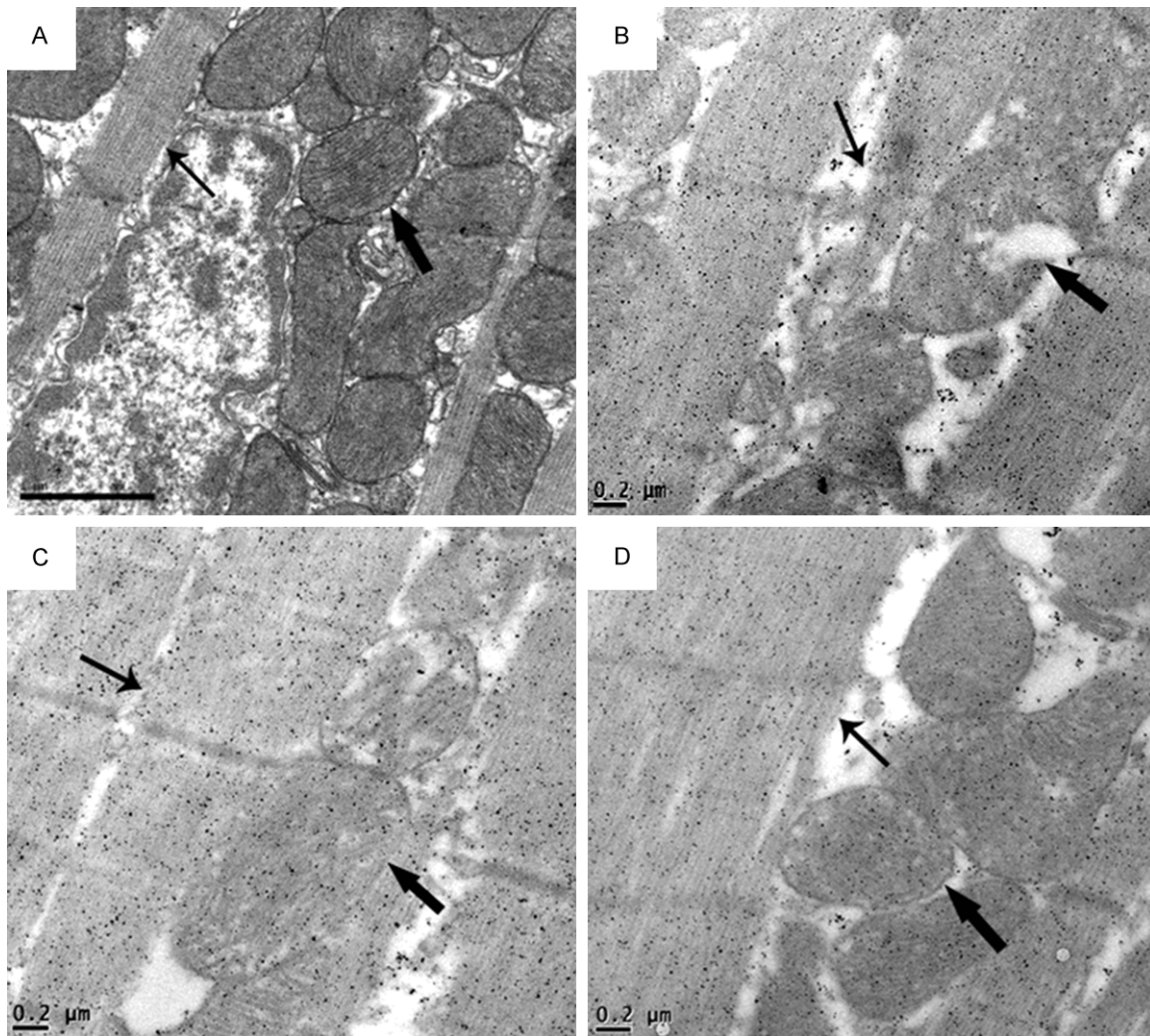
with sham group. 3-AB decreased the levels of troponin I and CK-MB in both the treatment group and pre-treatment group when compared with model group. (**Figure 1A, 1B**).

#### The activity of caspase-3 and levels of cytochrome C assay

The caspase-3 activity and cytochrome C levels were significantly higher in the model group than in the sham group; however, the activity of caspase-3 and level of cytochrome C significantly decreased after 3-AB treatment and pre-treatment (**Figure 2A, 2B**).

#### Measurement of MDA, ATP and NAD<sup>+</sup>

MDA concentrations were significantly higher in the model group than that in the sham group, but no difference was observed in the 3-AB pre-treatment and treatment groups compared to model group ( $P > 0.05$ ) (**Figure 3A**). Compared to the sham group, the model group showed



**Figure 6.** Electron microscopic results of myocardium (A. Original magnification  $\times 6000$ , B-D. Original magnification  $\times 8000$ ). A. Sham group. B. model group. C. 3-AB pre-treatment group. D. 3-AB treatment group. The thick arrows denote mitochondria, and the thin arrows denote myofiber.

significantly lower ATP and NAD<sup>+</sup> concentrations, whereas their concentrations were significantly increased in the 3-AB pre-treatment and treatment groups (**Figure 3B, 3C**).

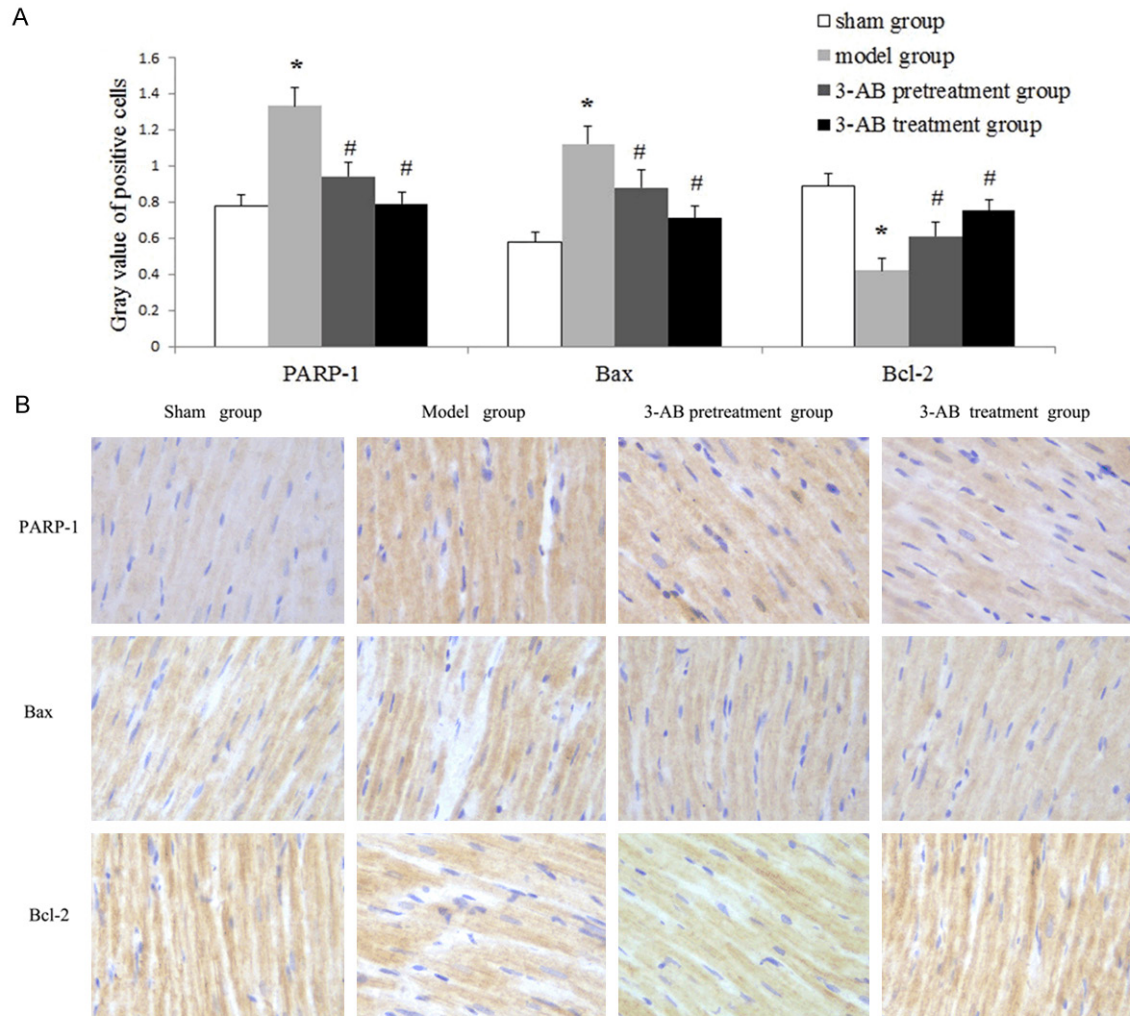
#### *The activity of PARP*

The myocardial tissue PARP activity was significantly increased in the model group than that in the sham group (**Figure 4**). It has been demonstrated that activation of PARP resulted in increased PARP1 expression [22, 23]. In line with this finding, we found that PARP1 expression increased in myocardial tissue. But the activity of PARP1 significantly decreased after 3-AB treatment and pre-treatment (**Figure 7A, 7B**).

#### *Histopathological and TEM results of myocardium*

Micrographs showed variable degrees of cardiocyte injury in model group. The obvious changes appeared in the model group in which the cardiocyte membrane partially disappeared and numerous inflammatory cells infiltrated. The injury degree in the 3-AB pre-treatment and treatment groups were significantly reduced compared with the model group (**Figure 5**). TEM illustration showed myofilament dissolved, no sarcomeres, mitochondrial crista disordered and disrupted in the model group. In contrast, there were no obvious cellular morphological changes in the 3-AB groups, except for a few





**Figure 7.** A. Bar graphs represent optical density of positive cells. \*represents significantly different when compared to sham group ( $P < 0.05$ ), #represents significantly different when compared to model group ( $P < 0.05$ ). B. Immunohistochemical analysis of PARP, Bax and Bcl-2 (Original magnification  $\times 400$ ). A. Sham group. B. model group. C. 3-AB pretreatment group. D. 3-AB treatment group.

narrow degenerated mitochondria and the distended sarcoplasmic (**Figure 6**).

#### PARP-1, Bax and Bcl-2 expression by immunohistochemistry

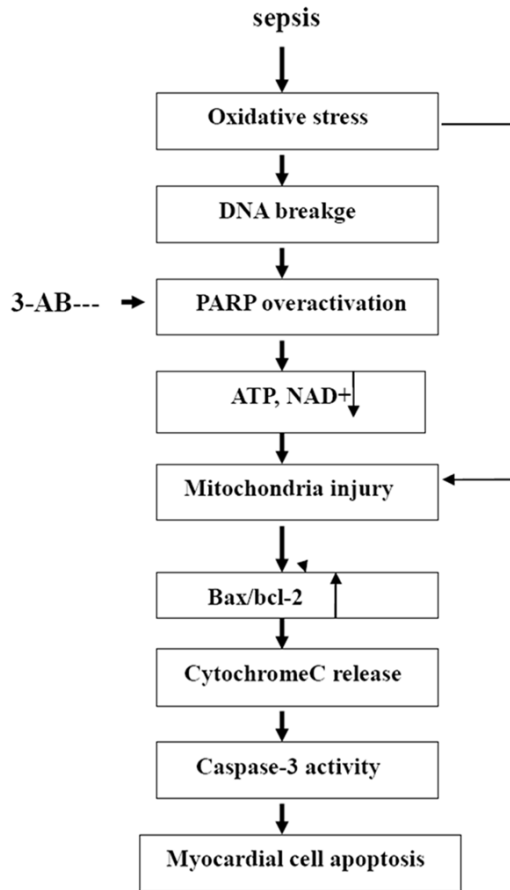
PARP-1, Bax and Bcl-2 expression shows as brown staining in cells. The PARP and Bax expressions in the model group increased significantly higher than that in the sham group. There were significantly lower PARP-1 and Bax expressions in the 3-AB group compared with model group. On the other hand, there was significantly less Bcl-2 expression in the model group compared with sham group, but more Bcl-2 expression in the 3-AB group compared

with model group. It has been demonstrated that the activation of PARP resulted in increased PARP-1 expression [24, 25]. In line with this finding, we found that PARP-1 expression was increased in model group. However, the pre-treatment and treatment of 3-AB decreased the expression of PARP-1 (**Figure 7A, 7B**).

#### Discussion

The novel finding of this study was that oxidative stress and activation of PARP-1 was associated with the induction of mitochondria-pathway apoptosis in the heart after sepsis. In addition, the PARP-1 inhibitory effects decreased apoptosis of myocardium cell and improved survival rate of rats.





**Figure 8.** The schematic diagram of myocardial cell apoptosis induced by sepsis and the probable protective effects of 3-AB.

Oxidative stress is induced in sepsis [26, 27], and as an index marker of oxidative stress, MDA levels was increased in model group and 3-AB group, this indicated that the persistent oxidative stress and the levels of MDA were not affected by the 3-AB. Deoxyribonucleic acid (DNA) could be damaged through oxidative stress [28, 29] and PARP could be activated by the breakage of DNA and plays an important role in physiological cellular functions and apoptosis [9]. Previous studies have proved that oxidative stress induces DNA breaks and subsequent increases in PARP activation that activate apoptotic pathways and induce cell death in vitro and in vivo [20].

Recent studies have succeeded in reducing cellular damage in ischemia/reperfusion by inhibiting PARP. One apoptotic mechanism is induced by the over activation of PARP, which leads to intracellular depletion of NAD<sup>+</sup> and ATP [9].

In addition to energy depletion, PARP can also regulate the activation caspase-independent apoptosis through mitochondria [30, 31]. A major cause of death in patients with severe sepsis is multiple organ failure, but the underlying pathogenesis is not fully understood. Mitochondrial damage and dysfunction has been recognized as an important molecular pathology in sepsis [32-36]. Our results also observed the injury of mitochondria under TEM. On the other hand, when mitochondria injured, the increased production of cellular reactive oxygen species (ROS) of mitochondrial origin during sepsis can cause significant oxidative stress and further aggravate the damage of the cells [37-40].

Activation of the mitochondrial apoptotic pathway is related to mitochondrial injury, which is regulated by pro- and anti-apoptotic Bcl-2 family proteins. The study indicated that the activation of PARP decreased the Bcl-2/Bax ratio via phosphorylated Akt [41, 42]. Proapoptotic (Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-xl) members of the Bcl-2 family of proteins regulate cytochrome C release through an increase or reduction of mitochondrial permeability, and subsequently activate caspase-dependent and independent apoptotic pathways [43]. This is consistent with the present study in that the levels of cytochrome C and caspase-3 increased in model group but decreased in 3-AB group.

Serum CK-MB and troponin I are indicators for myocardial injury. The mitochondria injury and ATP decreasing can induce the damage of cell membrane, thus lead to the release of troponin I and CK-MB.

All these factors triggered by the activation of PARP-1, such as motichondrial injury, caspase-dependent apoptotic, led to the damage of myocardial cells just as the histopathological showed. Several studies showed that inhibition of PARP-1 had a protective effect on various organs during I/R [13, 44]. The PARP inhibitor 3-AB thus seems to play protective effects in reducing myocardial cells mitochondrial cristae rupture, myofibrils break and edema or structure disorder. And the inhibitory effects were better in 3-AB treatment group than pre-treatment group. The reason may be pretreatment of 3-AB probably blocked partial activated PARP, which plays an important role in the

repairing of DNA breakage during the early phase of sepsis.

## Conclusion

The present study has provided evidence that the activation of PARP may be involved in mitochondrial injury of heart during sepsis and the subsequent mitochondria-mediated pathway apoptosis. The results of this study have deepened the understanding about the pathophysiology, molecular mechanisms of apoptosis and organ failure in sepsis, and provided some novel insights for developing improved treatments (Figure 8).

## Acknowledgements

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## Disclosure of conflict of interest

None.

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