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To Explore the Protective Mechanism of PTEN-Induced Kinase 1 (PINK1)/Parkin Mitophagy-Mediated Extract of *Periplaneta Americana* on Lipopolysaccharide-Induced Cardiomyocyte Injury

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Background: Sepsis is defined as a systemic inflammatory response syndrome caused by an infection (suspected or confirmed). Its essence is inflammatory mediators and cytokines mediated by host immune response. The present study aimed to investigate the role of *Periplaneta americana* extracts (XML) on PTEN-induced kinase 1 (PINK1)/Parkin mediated mitophagy in cardiomyocyte injury by sepsis.

Material/Methods: H9C2 cells were cultured and transfected with Mdivi-1 and Atg7 siRNA. The cell viability and drug toxicity were detected using Cell Counting Kit-8 assay. ELISA (enzyme-linked immunosorbent assay) was used to assess cardiac injury factors and inflammatory factors. Fluorescence levels of LC3 were detected using immunofluorescence assay. Then, the protein and mRNA expression levels were analyzed using western blot and qRT-PCR. Intracellular adenosine triphosphate (ATP) levels were measured using an ATP kit. Finally, flow cytometry was used to detect apoptosis.

Results: The result showed that XML significantly increase cell viability in H9C2 cells. Compared with XML+LPS (lipopolysaccharide) group, the level of cTNI, CK-MB, interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α was significantly upregulation in LPS+XML+Mdivi-1 or LPS+XML+Atg7 siRNA group. In addition, the release of LC3 was significant decreased. The protein and mRNA expression of PINK1, Parkin, Nix, Beclin-1 was significantly increased, but decreased expression of Mitofusin1, Mitofusin2, Opa1, Drp1, and P62 in LPS+XML+Mdivi-1 or LPS+XML+Atg7 siRNA groups. More importantly, we found that cell apoptosis was induced by Mdivi-1 and Atg7 siRNA.

Conclusions: The study provided evidence that XML regulated the process of LPS-induced cardiomyocyte injury through mitophagy by the PINK1/Parkin pathway.

MeSH Keywords: **Lipopolysaccharides • Mitochondrial Degradation • Myocytes, Cardiac**

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Background

Sepsis is defined as a systemic inflammatory response syndrome caused by an infection (suspected or confirmed). Its essence is inflammatory mediators and cytokines mediated by host immune response [1]. A recent report found that sepsis is one of the commonly diseases with high morbidity and mortality rates in intensive care units (ICU) [2]. In the United States, approximately 215 000 deaths from sepsis occur each year [3]. The condition of sepsis, especially rapidly developed sepsis, leads to severe sepsis, septic shock, and multiple organ dysfunction syndrome (MODS), including heart dysfunction, which induces both systolic and diastolic dysfunction [4,5]. Studies have confirmed that myocardial dysfunction is not associated with tissue hypoperfusion, and adequate O₂ supply in sepsis has been demonstrated in experiments on human and animals [6]. A number of studies have identified the mechanism of myocardial dysfunction in sepsis to mainly include nitric oxide, oxidative stress, energy metabolic derangements, and apoptosis. Myocardial mitochondrial damage, and decreased mitochondrial inner and outer membrane potential prevents ATPase from synthesizing ATP (adenosine triphosphate) to reduce productivity, thus activating mitochondria-dependent apoptotic pathway; additionally, mitochondrial damage also increases cytochrome c release, further activating mitochondria-dependent apoptotic pathways [7]. It has been suggested that the mechanism of sepsis is inseparable from damage of mitochondria.

Autophagy plays an important role in the degradation to cytoplasmic constituents of protein and organelles, which are subsequently delivered to lysosomes. Autophagy protects cells from damaging factors and maintains intracellular stability [8]. Especially, mitophagy selectively removes damaged mitochondrial, to regulate the number of mitochondria to adapt to the living environment of cells. Mitophagy is tightly regulated by molecules, of which Parkin and PTEN-induced putative kinase 1 (PINK1) are in a pathway critical for the maintenance of mitochondrial integrity and function. PINK1 is a key initiator of mitophagy, which stabilization or phosphorylating Parkin and ubiquitin. When the membrane potential of mitochondria initiates mitophagy, PINK1 accumulates on the outer mitochondrial membrane, then its kinase activity recruits Parkin to the mitochondria. Activated Parkin then builds ubiquitin chains on damaged mitochondria to tag them for degradation through mitophagy.

The *Periplaneta americana* is a kind of traditional Chinese medicine. Studies suggest that higher dosage extracts of adult *Periplaneta americana* can play important roles in reducing swelling and decreasing pain [9]. It can tonifying the spleen, stimulating circulation and diuresis. Some typical prescriptions include Kangfu Xinye, Xinmailong injection, and Xiaozheng Yigan tablets. Especially noted is the role of Xinmailong to

promote Ca²⁺ influx in myocardial cells, permanently increase myocardial contractility, effectively enhance cardiac function and delay heart failure. Therefore, the present study may aid in elucidating the underlying molecular mechanism of mitophagy by PINK1/Parkin pathway under the protection of *Periplaneta americana* extract (XML), which may contribute to the development of novel treatments for the therapy for the sepsis.

Material and Methods

Models of sepsis and groups

The H9C2 cells were cultured in DMEM medium (Thermo Fisher Scientific, MA, USA), with 10% fetal bovine serum (FBS; Gemini Bio, Calabasas, USA) and 1 nM glutamine, and 1% penicillin/streptomycin at 37°C in 5% CO₂ incubator.

The H9C2 cells were randomly divided into 6 groups as follows: control group, XML group (Tengyao, Yun Nan, China), LPS group (Sigma, USA), LPS+XML group, LPS+ XML+ mitophagy inhibitor (Mdivi-1, Abcam, MA, USA) group, and LPS+ XML+ Atg7 siRNA (50 µg/mL, CST, MA, USA) group. H9C2 cell in the XML group were treated with 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL, and experimental times were 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours. The concentration of LPS in the LPS group and the XML group was 1 µg/mL.

Transfection of Atg7 siRNA

After culturing for 24 hours, Atg7 siRNA was transfected into the H9C2 cells and cells were seeded into 6-well plates and incubated in standard conditions. According to the Lipofectamine™ RNAi MAX transfect manufacturer's protocol, the H9C2 cells were transfected with NC siRNA and Atg7 siRNA. Cell cultures were maintained for 48 hours to stabilize the lentiviral transduction.

Cell counting kit-8 (CCK-8) assay

The cells were cultured at a cell density of 1×10⁴/mL for each well in the 96-well plate, the CCK-8 kit was used to detect cell viability. The H9C2 cells were treated with different concentration of XML (0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL). After which the cells were treated by the different concentration of XML for 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours. The drug toxicity experiment was divided into control group, XML group, LPS group, LPS+XML group, LPS+ XML+ Mdivi-1 group, and LPS+ XML+ Atg7 siRNA group. Then, 10 µL of CCK-8 solution was added to each well and absorbance values were measured after 1 hour of incubation. The optical density (OD) of each well was read by wavelength of 450 nm.

Table 1. Primers used for quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
P62	GCACCCCAATGTGATCTGC	CGCTACACAAGTCGTAGTCTGG
PINK1	GCCTCATCGAGGAAAAACAGG	GTCTCGTGTCCAACGGGTC
Parkin	CATGATCAGTTCATGATCACT	ATCTAGCATTAGCTAACTC
Mitofusin1	AGTGTATCTCGCAGTCAG	TTCCCATACCATCCTCTA
Mitofusin2	CTCTCGATGCAACTCTATCGTC	TCCTGTACGTGTCTTCAAGGAA
Opa1	TGTGAGGTCTGCCAGTCTTTA	TGTCCTTAATTGGGGTCTGTTG
Drp1	CTGCCTCAAATCGTCGTAGTG	GAGGTCTCCGGGTGACAATTC
Nix	ATGTCGTCCCACCTAGTCGAG	TGAGGATGGTACGTGTTCCAG
Beclin-1	CCATGCAGGTGAGCTTCGT	GAATCTGCGAGAGACACCATC
Atg7	CAGTTTCCCCCTTTAGTAGTGC	CCAGCCGATACTCGTTCCAGC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGCATACTTCTCATGG

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of the culture cells were collected after treatment and used to measure cytokine secretion using a commercial ELISA kit according to the manufacturer's Instructions (Biolegend, CA, USA).

Immunofluorescence assay

The H9C2 cells were cultured on 24-well slides (Costar, NY, USA) and treated as manufacturer's protocol. When the myocardial cell growth reached 60–70% confluence, we discarded the medium and wash cells 3 times with pre-cooled phosphate buffered saline (PBS) for 5 minutes each time. Cells were fixed with 4% paraformaldehyde-PBS for 15 minutes at 4°C, then washed and permeabilized with 0.2% TritonX-100 at room temperature for 5 minutes, then washed again. Then cells were incubated with 5% bovine serum albumin (BSA) using a horizontal shaker for 45 minutes at room temperature. The cells were incubated with rabbit LC-3 antibody (1: 300, CST, MA, USA) in 5% BSA-PBS at 4°C overnight, washed, and incubated with fluorescent dye-conjugated secondary antibody (1: 200, Beyotime, Shanghai, China) in PBS at room temperature for 2 hours. The nuclear staining was analyzed by incubating cells with DAPI (Beyotime, Shanghai, China) for 10 minutes. Finally, under confocal microscopy, the number of green spots formed by endogenous LC3II represented the formation of autophagosomes. We used Image Pro Plus Version 6.0 analysis system.

For determination of intracellular ATP, the cells were fully lysed, centrifuged at 1000 rpm for 5 minutes at 4°C, and the supernatant was analyzed for detection of ATP according to manufacturer's protocol.

Real-time PCR

The H9C2 cells were cultured on 6-well slides (Costar, USA) with logarithmic growth stage are used for treatment, total RNA was extracted using total RNA miniprep kit (Axyprep, CA, USA), and steps were carried out according to manufacturer's instructions. Subsequently, Genomic DNA removal and reverse transcription reactions were performed by PrimeScript@RT reagent Kit and gDNA Eraser (TaKaRa, Kyoto, Japan). The reverse transcription cDNA was measured by T043 NanoDrop (Thermo, MA, USA), and then appropriately dispensed and stored at –20°C. GAPDH was used as an internal control for detection mRNA expression. The experiment was completed in a reaction mixture using PrimeScript@RT reagent Kit (TaKaRa, Kyoto, Japan), which was reverse transcribed in 20 µL. The thermal cycling conditions were: 60 seconds at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 45 seconds. We use $2^{-\Delta\Delta C_T}$ to calculate the relative expression of mRNA. The gene PINK1, Parkin, Mitofusin1, Mitofusin2, Opa1, Drp1, Nix, Beclin-1, P62, and Atg7 primers sequences are presented in Table 1.

Western blot analysis

Western blot analysis was used for detection of the protein expression in PINK1, Parkin, Mitofusin1, Mitofusin2, Opa1, Drp1, Nix, Beclin-1, P62, and GAPDH (Abcam, MA, US). After different experimental group treatments, the radioimmunoprecipitation assay buffer was used to collect the total protein concentration as determined by the BCA protein assay kit, and saved at –20°C. The proteins were separated by SDS-PAGE, then transferred to polyvinylidene fluoride membranes, with 5% nonfat milk incubation for 2 hours at room temperature, then washed with TBST. Subsequently, the primary antibodies (1: 1000 in 5% BSA-PBS) were applied to the membranes overnight at 4°C.

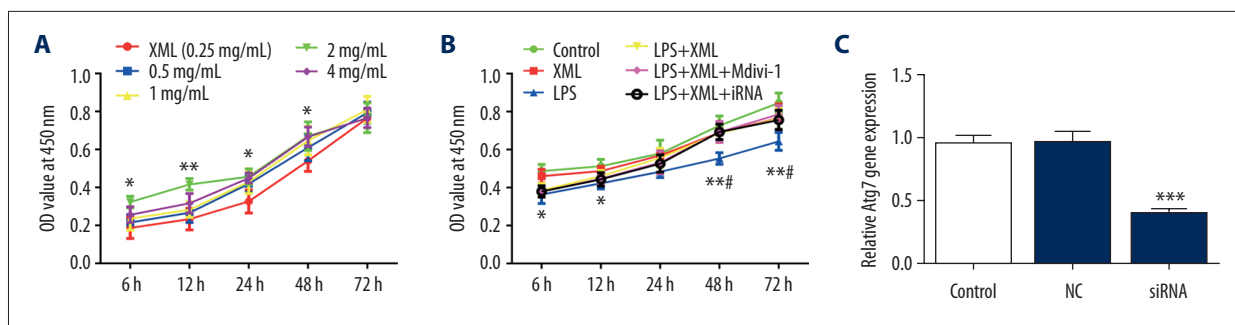


Figure 1. (A) Atg7 gene expression in H2C9 cells was significantly decreased in the Atg7 siRNA group. (B, C) The cell viability and drug toxicity were detected using Cell Counting Kit-8 assay. Compared with the control group, * $P < 0.05$ was statistically significant. Compared with XML+LPS group, # $P < 0.05$ was statistically significant. XML – *Periplaneta americana* extracts; LPS – lipopolysaccharide.

The primary antibody was discarded, and the NC membrane was washed 6 times with TBST for 5 minutes each time; the secondary antibody (1: 5000 in 5% BSA-PBS) was incubated on the membranes at room temperature for 120 minutes. Finally, the signals were detected using detection reagents according to the manufacturer's instructions.

Flow cytometry analysis

Apoptosis of H9C2 cells were detected by Annexin V-FITC/PI apoptosis double staining kit (Becton, Dickinson and Company, NJ, USA). The supernatant was collected, trypsinized, and then lightly pipetted with 1 mL of PBS; centrifuged at 1500 rev/minutes for 10 minutes. The cells were washed twice, and a blank group (no dye group), a FITC single staining group, a PI single staining group, and a double staining treatment group (FITC and PI double staining) were created. After adding the dye to the dye groups, cells were incubated for 30 minutes at room temperature in the dark. Then 1×10^6 loading buffer 500 μ L was used to resuspend the cells, which were filtered through 200 mesh nylon mesh; we treated each group 3 times. Flow cytometry analysis was performed within 1 hour, and 10^4 cells were detected in each group.

Statistical analysis

Statistical significance was assessed by one-way ANOVA analysis. Statistical analysis for data was performed using SPSS 22.0 statistical software (SPSS, Inc, Chicago, IL, United States), P values of < 0.05 were considered statistically significant.

Results

CCK-8 assay detected cell viability

The experiment data from CCK-8 assay indicated that the viability of H2C9 cells was increased in a dose-time-dependent

manner after treatment with 0.25, 0.5, 1.0, 2.0, and 4.0 mg/mL XML for 6, 12, 24, 48, 72 hours (Figure 1A). Additionally, in the drug toxicity experiment, the results showed that cell viability was increased in a time-dependent (Figure 1B). These data revealed that XML could upregulate the growth of H2C9 cells.

In order to investigate the role of Atg7 in H2C9 cells, Atg7 was silenced using Atg7 siRNA. At 48 hours after cell transfection, the effective downregulation of the mRNA levels of Atg7 was confirmed by RT-qPCR analysis, respectively, as compared with the non-transfected and control-transfected cells (Figure 1C).

XML upregulated myocardial injury factors and inflammatory factors in H2C9 cells

To test the effect of XML on inflammatory factors and myocardial injury factors, we exposed H2C9 cells to LPS, followed by treatment with XML. As shown in Figure 2, compared with the control group, the levels of cTNI, CK-MB, IL-1 β , IL-6, and TNF- α in the LPS group were significantly upregulated. However, compared with the LPS+XML group, the pretreatment of XML suppressed the upregulation of the expression levels of cTNI, CK-MB, IL-1 β , IL-6, and TNF- α . Then, the transfection of mitophagy inhibitor and Atg7 siRNA, the expression of cTNI, CK-MB, IL-1 β , IL-6, TNF- α antagonized the effects of XML. This suggested that XML could inhibit the cardiac injury factors cTNI, CK-MB, and inflammatory factors IL-1 β , IL-6, and TNF- α , and is related to mitochondrial autophagy.

The fluorescence expression of LC3

Autophagy is an essential homeostasis mechanism that regulates the elimination of damaged macromolecules and promotes protection and cell survival. Therefore, in order to determine the activation and function of autophagy under mitochondrial dysfunction conditions, we used detection of LC3 by fluorescence in H2C9 cells. Compared with the control group, the LPS treatment group upregulated the release of LC3.

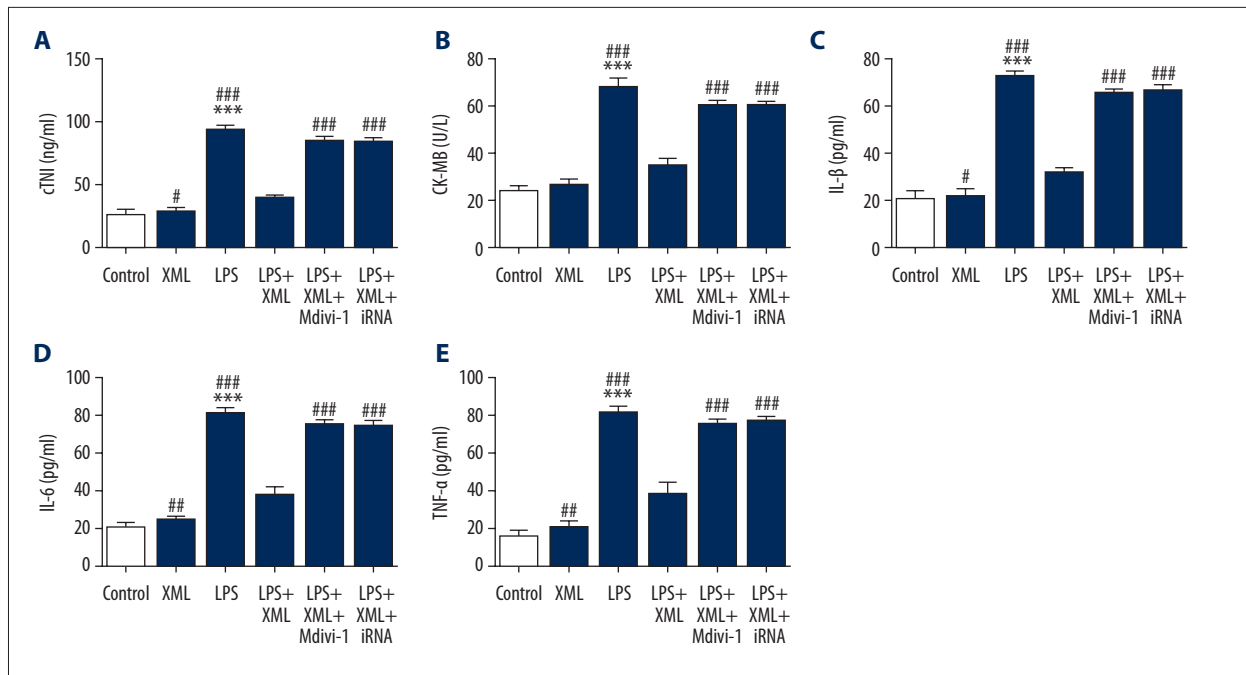


Figure 2. (A, B) CK-MB and cTNI were upregulated in the LPS group, XML suppression expression. (C–E) LPS activates the expression of pro-inflammatory factors IL-6, TNF- α , and IL- β are offset by XML. Compared with the control group, * $P < 0.05$ was statistically significant. Compared with XML+LPS group, # $P < 0.05$ was statistically significant. XML – *Periplaneta americana* extracts; LPS – lipopolysaccharide, IL – interleukin, TNF – tumor necrosis factor.

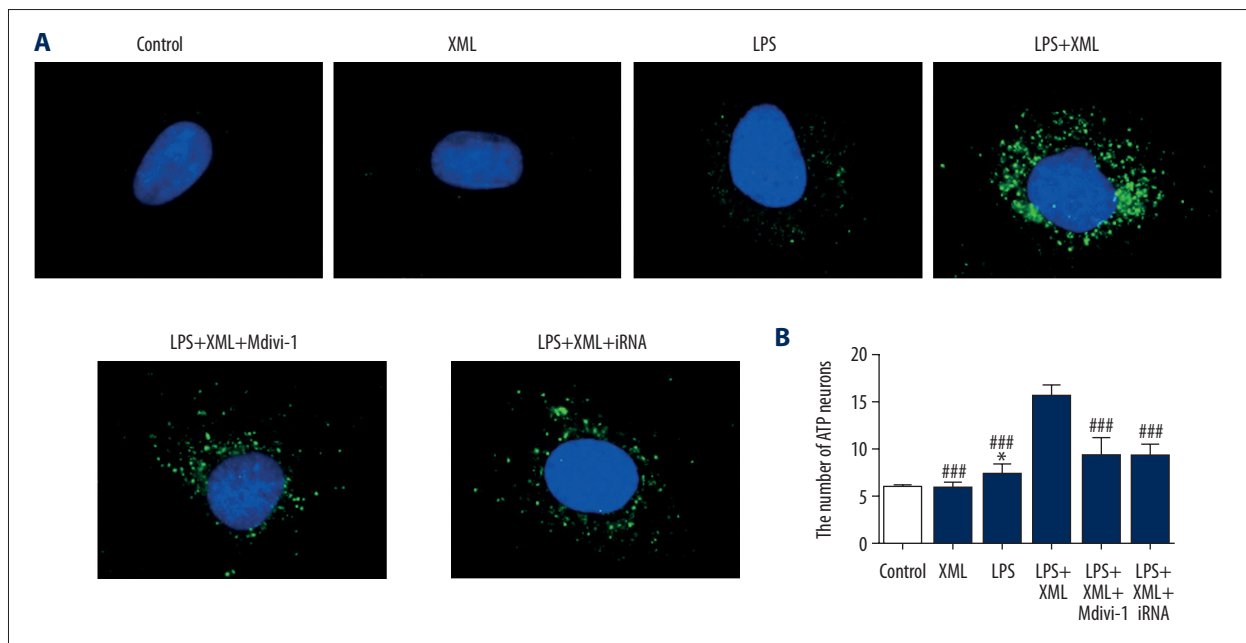


Figure 3. (A) The H2C9 cells were immediately double stained with LC3 and DAPI and visualized by confocal microscopy. (B) Intracellular ATP levels were measured using an ATP kit. Compared with the control group, * $P < 0.05$ was statistically significant. Compared with the XML+LPS group, # $P < 0.05$ was statistically significant. XML – *Periplaneta americana* extracts; LPS – lipopolysaccharide.

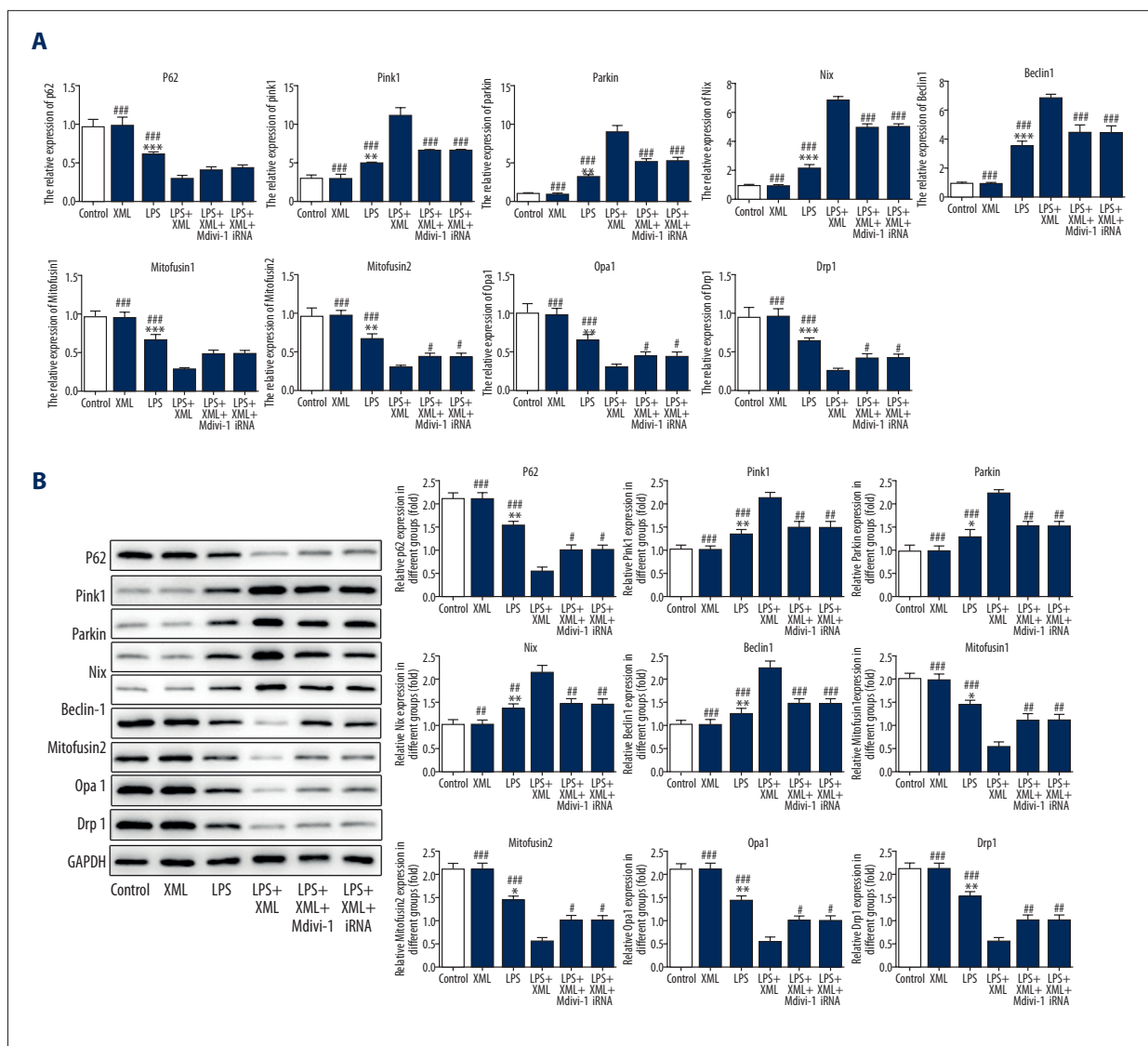


Figure 4. (A, B) The protein and m RNA levels of PINK1, Parkin, Mitofusin1, Mitofusin2, Opa1, Drp1, Nix, Beclin-1, and P62 were determined by western blot and RT-qPCR. GAPDH protein and m RNA expression monitored as controls showed in the bottom. Compared with the control group, * $P < 0.05$ was statistically significant. Compared with the XML+LPS group, # $P < 0.05$ was statistically significant. XML – *Periplaneta americana* extracts; LPS – lipopolysaccharide.

However, compared with the XML+LPS group, the treatment of XML significantly decreased the effect of LPS-induced cardiomyocyte injury; the application of Mdivi-1 and Atg7 siRNA partially increased the effect of LPS (Figure 3A). The detection of intracellular ATP was consistent with the aforementioned results (Figure 3B). These data show that LPS upregulated the release of LC3, and XML inhibited these effects.

The regulation of mitophagy by PINK1/Parkin pathway in H2C9 cells

To explore the mechanism of mitophagy action in cardiomyocyte injury we examined the expression of mitophagy-key proteins,

including PINK1, Parkin, Mitofusin1, Mitofusin2, Opa1, Drp1, Nix, Beclin-1, and P62 (Figure 4). The results showed that, compared with the control group, the protein expression of Mitofusin1, Mitofusin2, Opa1, Drp1, and P62 in the LPS group were significantly downregulated, but the expression of PINK1, Parkin, Nix, and Beclin1 were upregulated. Subsequently, compared with the XML+LPS group, the protein and mRNA level of PINK1, Parkin, Nix, and Beclin-1 was significantly increased; however, the level of Mitofusin1, Mitofusin2, Opa1, Drp1, and P62 was significantly decreased in the LPS+XML+Mdivi-1 and the LPS+XML+Atg7 siRNA group.

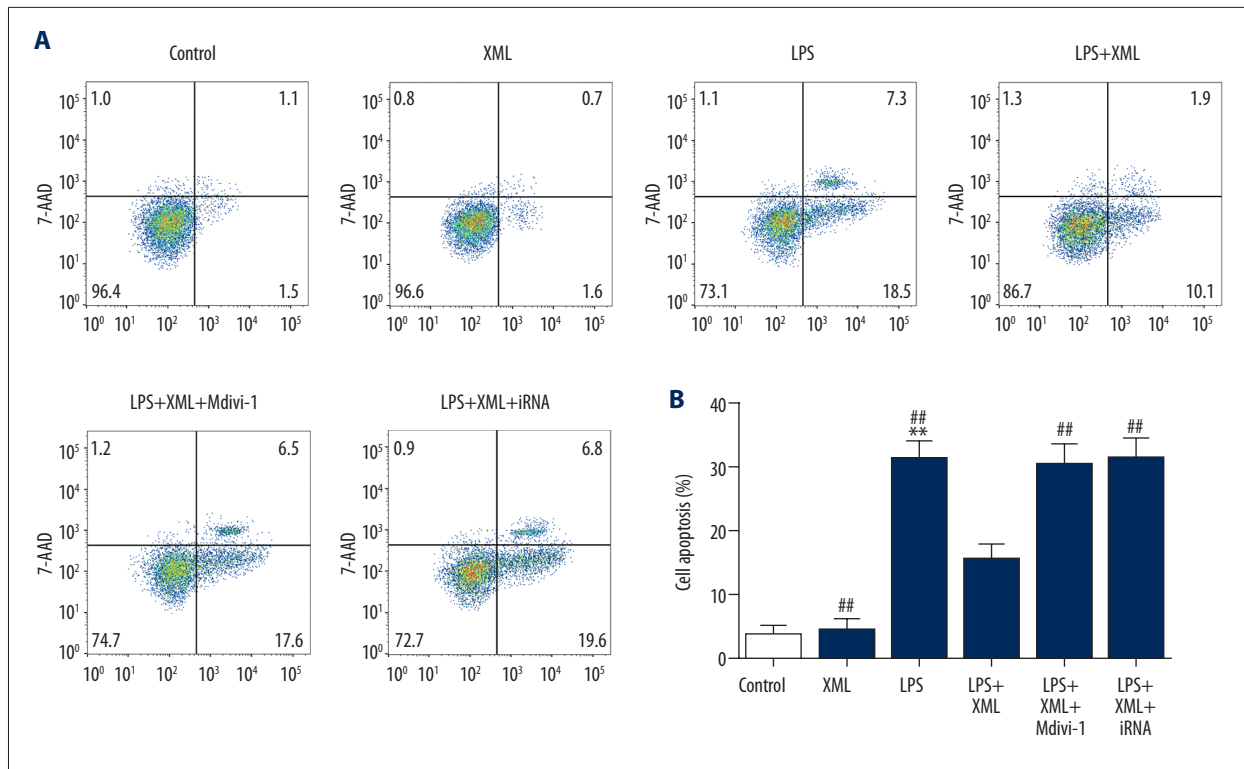


Figure 5. (A, B) Flow cytometry was conducted to detect the cell apoptosis, and cell apoptosis rate was calculated. LPS upregulation increases the apoptosis of H2C9 cells. Compared with the control group, * $P < 0.05$ was statistically significant. Compared with the XML+LPS group, # $P < 0.05$ was statistically significant. XML – *Periplaneta americana* extracts; LPS – lipopolysaccharide.

The effect of mitophagy on cell apoptosis

To detect cell apoptosis, we used Annexin V-FITC/PI apoptosis detection kit (Figure 5). Our findings suggested that LPS treatment significantly increased cell apoptosis rate. However, the addition of XML reversed the effect of LPS on the apoptosis rate. Furthermore, the transfection of mitophagy inhibitor and Atg7 siRNA markedly inhibited the downregulated apoptosis in the XML group. The data indicated that mitophagy downregulation was capable of promoting H2C9 cell apoptosis.

Discussion

In the present study, it was demonstrated that XML attenuated LPS-induced cardiomyocyte injury, inflammatory factors expression, and cardiomyocyte injury factors via regulation of mitophagy by the PINK1/Parkin pathway.

In patients with severe sepsis, the extent of mitochondrial dysfunction is associated with clinical outcomes. The mechanism of mitochondrial damage in the pathogenesis of sepsis involves flaws in energy production and inflammation induction. The inflammatory response is driven by the production of soluble proinflammatory cytokines and inflammatory mediators

produced by immune cells as well as provides host defense against infection and injury [10]. It was recently reported that sepsis-induced inflammation was limited by activation of mitochondrial biosynthesis by the upregulation of the anti-inflammatory cytokine, IL-10 [11]. Chen et al. suggested that the pro-inflammatory cytokines took part in the occurrence and regulation of inflammatory response of sepsis-induced cardiomyocyte injury, for instance, TNF- α and IL-6 [12]. Furthermore, sepsis-induced acute kidney injuries and acute lung injury are connected with the inflammatory response [13,14]. CK-MB and cTNI are commonly recognized indicators of myocardial injuries and testing is necessary. In our study, LPS pretreatment of H2C9 cells gave rise to CK-MB, cTNI, IL-6, TNF- α , and IL- β in ELISA; however, XML pretreatment obviously inhibited the upregulation of CK-MB, cTNI, IL-6, TNF- α , and IL- β , which was consistent with a previous report.

Autophagy falls into 3 categories: macro-autophagy, micro-autophagy, chaperone-mediated autophagy. The important role of LC3 in autophagy is that LC3 is transformed from LC3-I into LC3-II when autophagy is induced, so the release rate of LC3-I/LC3-II can be treated as a marker for the quantification of autophagy activity [15]. Moreover, P62 has been shown to be a component of intracellular polyubiquitinated protein inclusion and serves as a marker of autophagy [16]. However,

the mitophagy was categorized as a macro-autophagy. PINK1/Parkin pathway was considered the most important way to regulate mitophagy, and PINK1 selectively accumulates dysfunctional mitochondria, and the recruitment of depolarized mitochondria by Parkin and subsequent Parkin-induced mitophagy is strictly dependent on the mitochondrial targeting signal of PINK1 and the accumulation of depolarization induction. Additionally, evidence suggests that Beclin-1 increase LC3-II associated with mitochondria and factors of the PINK1/Parkin pathway, but suppresses an increase in mitophagy receptors following LPS-stimulated sepsis-induced cardiomyocyte injury [17]. Therefore, in our present study, LPS injection significantly increased the mRNA and protein expression of Parkin, PINK1, Nix, and Beclin-1, but decreased P62. XML pretreatment obviously inhibited the upregulation of Parkin, PINK1, Nix, and Beclin-1 and downregulation of P62 in the present study, suggesting that the activation of the PINK1/Parkin signaling pathway induced by LPS stimulation was protected by XML; fluorescence expression of LC3 was consistent with the aforementioned results. Subsequently, the promotion mitophagy effect of XML was inhibited by the mitophagy inhibitor (Mdivi-1) and Atg7 siRNA in LPS-induced cardiomyocyte injury. These findings suggest that XML exerted its protective effect in H2C9 cells with LPS-induced cardiomyocyte injury through Parkin/PINK1-mediated mitophagy, and further suggest that Atg7 was involved in the regulation of this mechanism.

Mitochondrial fusion in mammalian cells is controlled by Mitofusin1, Mitofusin2, Opa1, and Drp1 [18]. Kageyama et al. [19] demonstrated that mitophagy was linked to cardiovascular disease and cooperated with Drp1 and Parkin to maintain the integrity of mouse cardiac mitochondrial structure and function. Our findings suggested that LPS injection significantly downregulated Mitofusin1, Mitofusin2, Opa1, and Drp1. However, XML pretreatment significantly increased these proteins. Then, Mdivi-1 and Atg7 siRNA application markedly inhibited the effects of XML. These findings suggest that XML promoted

cell mitophagy by increasing the expression of Mitofusin1, Mitofusin2, Opa1, and Drp1. Additionally, another important discovery of our study was that XML promoted cell mitophagy by inhibiting apoptosis in LPS-induced myocardial injury.

In summary, XML pretreatment alleviated LPS-induced myocardial injury to a certain extent, and reversed the LPS-induced upregulation in mitophagy and inflammatory factor expression. Furthermore, the application of Mdivi-1 and Atg7 siRNA significantly suppressed the effects of XML. Taken together, these data demonstrate that the protective effect of XML may be via PINK1/Parkin-mediated mitophagy.

Conclusions

Sepsis occurs at a high incidence rate in cardiomyocyte injury and affects prognosis. Furthermore, the occurrence of sepsis cardiomyocyte injury is related to the damage of mitochondria in myocardial tissue and the level of mitophagy in cardiomyocyte. Therefore, our results indicate that the PINK1/Parkin pathway-induced mitophagy plays a vital role in cardiomyocyte injury from sepsis, and is probably one of the mechanisms of sepsis. At the same time, the extract of *Periplaneta americana* has a protective effect on sepsis heart dysfunction, and provides a new target and important theoretical basis for clinical Chinese medicine treatment.

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Conflict of interests

None.

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