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**Jujuboside A attenuates Sepsis-induced Cardiomyopathy by inhibiting
inflammation and regulating autophagy**

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Abstract

Background: Jujuboside A (JuA), as a main effective component of Jujubogenin, which was extracted from the seed of *Ziziphus jujuba* Mill, has long been known as a sedative-hypnotic drug. The aim of the current study was to investigate the potential effect of JuA on sepsis-induced cardiomyopathy (SIC) induced by lipopolysaccharide (LPS) in mouse models.

Method: Wide type C57BL/6J mice were randomly divided into four groups: ddH₂O+control, ddH₂O+JuA, LPS+NS and LPS +JuA, and the cardiac function of septic mice were detected by echocardiography. Moreover, the survival rate at each time point was calculated for 7 days. ELISA assays were used to analyze inflammatory factors in serum. Furthermore, Western blotting, flow cytometry and TUNEL staining were performed to assess cell apoptosis and transmission electron microscopy detecting the number of autophagosomes. Finally, the expression of autophagy-related and oxidative stress-related proteins was analyzed by western blotting and immunohistochemistry staining.

Results: Results showed that JuA pretreatment significantly improved the survival rate and cardiac function, and suppressed systemic inflammatory response in septic mice. Further study revealed that JuA could decrease cell apoptosis and enhanced autophagy. Moreover, JuA pretreatment also significantly decreased oxidative stress and nitroductive stress, as evidenced by downregulating iNOS and gp91 expression in vivo. In addition, the autophagy inhibitor 3-MA significantly abolished the effect of JuA on autophagic activity in SIC.

Conclusion: In conclusion, the findings indicated that JuA enhanced autophagy blocking inflammasome-mediated cardiomyocyte apoptosis and suppress myocardial iNOS and gp91 expression to improve cardiac function of SIC in septic mice.

Key Words: Jujuboside A; autophagy; sepsis-induced cardiomyopathy; apoptosis; oxidative stress.

Abbreviations

SIC: sepsis-induced cardiomyopathy; JuA: Jujuboside A; LPS: lipopolysaccharide; Bax/Bcl-2: Bcl-2 associated X, apoptosis regulator; Bcl-2: Bcl2 apoptosis regulator; NS: normal saline; LVEF: left ventricular ejection fraction; LVFS: left ventricular fraction shortening; LVESV: left ventricular end- systolic volume; LVEDV: left ventricular end-diastolic volume; TNF- α : tumor Necrosis Factor- α ; IL-1 β : interleukin-1 β ; IL-18: interleukin-18; ELISA: enzyme linked immunosorbent assay; QPCR: quantitative real-time polymerase chain reaction; HE: hematoxylin-eosin; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI: 4',6-Diamidino-2-Phenylindole, Dihydrochloride; SD: Standard Deviation; qPCR: quantitative real-time polymerase chain reaction; FITC: Fluorescein Isothiocyanate; PI: Propidium iodide; CCK8: Cell Counting Kit-8; 3-MA: 3-Methyladenine; iNOS: inducible nitric-oxide synthase; WT: wild type

1.1 Introduction

Sepsis, which is a common condition often caused by a dysregulated host response to infection, can lead to life-threatening organ dysfunction as reported previously [1]. Severe sepsis and septic shock are major healthcare problems of morbidity and mortality worldwide annually [2]. Sepsis-induced cardiomyopathy (SIC) is a global but reversible cardiac dysfunction caused by sepsis and has long been a research hotspot in the pathogenesis of sepsis [3], such as dysregulation of inflammatory mediators, oxidative stress, mitochondrial dysfunction, autonomic nervous system dysregulation. [4].

Autophagy is the major intracellular degradation system, which maintains protein quality and organelle function by degrading the damaged or dysfunctional cellular components such as endoplasmic reticulum, mitochondria and peroxisomes, as well as eliminating intracellular pathogens in the lysosome[5]. Autophagy is a process of self-degradation, which that is important for balancing energy sources during critical periods of development and in response to nutritional stress[6]. Autophagy has been demonstrated to be involved in numerous physiological processes, such as the starvation response, cell growth control and innate immunity [7]. However, the purpose of autophagy is not simply to eliminate materials, but instead as a dynamic recycling system that generates new building blocks and energy for cellular repair and homeostasis. Previous studies have found that autophagy is associated with apoptosis and mobilized in the early stage of sepsis through increased accumulation of autophagic vacuoles and increased expression of autophagy-associated proteins [8, 9].

Jujuboside A (JuA) is a main effective component of Jujubogenin, isolated from the seed of *Ziziphus jujuba* Mill var *spinosa* (Bunge) Hu ex H F Chou (*Ziziphus*), and traditionally used as a folk medicine, due to its anxiolytic and sedative effects [10-12]. Recently, previous studies have reported that JuA have the notable neuroprotective activities in dementia diseases such as Alzheimer's disease via anti-inflammation, anti-apoptosis, anti-oxidant and neuro-protection [13-15]. However, there was almost rarely study showed the potential effect of JuA on cardiac disease. Han et al. indicated that JuA can significantly reduce the damage of isoproterenol to H9c2 cells via activating

the phosphoinositide 3-kinase/AKT/mammalian target of the rapamycin pathway [16]. In addition, Wan et.al reported that pretreatment with JuA can reverse norepinephrine-induced decrease in cell viability and increase in H9c2 apoptosis by regulating the MAPK and AKT signaling pathways [17]. However, to the best of our knowledge, the above two researches were only focused attention to H9c2 cells but not animal disease model; moreover, whether JuA has the potential therapeutic effect on SIC has not been explained, or/and the underlying mechanism remains to be resolved.

This study aims to examine the protective effects of JuA on SIC and to clarify the underlying mechanism of JuA. In the present study, we demonstrated that JuA significantly attenuated the cardiac function and reduced the inflammatory response in SIC. JuA treatment effectively weaken the apoptosis through enhancing the autophagy of myocardium, involving in suppressing oxidative and nitrosative stress in sepsis-mice model.

1.2 Methods

Animal, Sepsis model and treatment

Wild-type (WT) C57BL/6 male mice (8-10 weeks old, 20-25g) were purchased from Slac Laboratory (Shanghai, China) and were fed a standard rodent diet with free access to food and tap water. All experimental procedures were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (8th Edition, 2011), and all protocols were approved by the Institute's Animal Ethics Committee of Shanghai Jiao Tong University. All the mice were kept at constant temperature (21 ± 2 °C) in a light and dark cycle with relative humidity of $50 \pm 5\%$, and free access to food and water. The experimental mice were randomly divided into 4 groups (10 mice per group): **The control group, the JuA treatment group (JuA), the LPS + normal saline group (LPS+NS) and the LPS + JuA group (LPS+JuA)**. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Sigma, Saint Louis, MO, USA) was dissolved in PBS and JuA was purchased from MedChemExpress (HY-N0659, Monmouth Junction, NJ, USA).

To investigate the potential effects, mice were given JuA (20mg/kg, 0.2ml, p.o.) or the equal volume of saline (0.2ml, p.o.) for consecutive 7 days. On the last day, all the mice were given a single intraperitoneal injection of LPS (20mg/kg) or the same volume of saline to mimic sepsis-induced cardiomyopathy. After 12 hours, cardiac function was detected by M-mode echocardiography and all the mice were sacrificed to harvest hearts.

The survival rate was observed using other 20 mice in each group. These LPS/saline-induced mice were returned to their cages after JuA/NS-treatment and were closely monitored for up to 7 days, as well as given ad libitum access to food and water.

Echocardiographic assessment

Cardiac function and structure were assessed 12 hours after injecting LPS using echocardiography (Vevo 2100, VisualSonics, Toronto, Canada) with a 25MHz imaging transducer. Mice were anesthetized by inhalation of 2% isoflurane. Cardiac function parameters such as left ventricular ejection fraction (LVEF), left ventricular fraction shortening (LVFS), left ventricular end-diastolic diameter (LVEDD), left ventricular

end-systolic diameter (LVESD), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV) were calculated using computer algorithms.

Cell Lines and Cell Culture

The cardiomyoblast cell line H9c2 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, CA, USA) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and maintained at 37 °C in 5% CO₂. And the cells were passaged every 2-3 days to maintain growth. For in vitro treatment, the cells were pretreated with JuA (0 μ M and 20 μ M) or an equal volume of PBS for 1 h, and then were treated with LPS (20 μ g/mL) for 24 h.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)

TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions to assess apoptosis in heart tissues. Diphenyl phenylindole (DAPI) staining was used to stain the cell nucleus. The apoptosis index was defined as the number of TUNEL-positive myocytes/the total number of myocytes stained with DAPI. Images were obtained using the Leica laser fluorescence microscope at a magnification of $\times 200$ and analyzed with ImageJ software (ImageJ version 1.43r; NIH).

Transmission electron microscopy (TEM)

The heart tissues were harvested to determine the quantification of autophagosomes. The heart tissues were fixed with 2.5% glutaraldehyde and stored at 4 °C. According to the manufacturer's instructions, each specimen was performed by a series of manipulation, including fixation, dehydration, embedding, curing, biopsy, and dyeing. The autophagosomes were observed by a transmission electron microscope (JEOL, Tokyo). Random sections were imaged and analyzed by two technicians blinded to the experiment. The mean number (and standard deviation) of autophagosomes per field was calculated.

Cytokine measurement

At the predetermined time points, these mice were anesthetized by isoflurane, the plasma samples were obtained from blood after centrifugation for 15 minutes at 3000g at 4 °C, and stored at -80 °C until use in various biochemical assays. The concentrations of TNF- α , IL-6, IL-1 β , IL-18 were measured by ELISA kits according to the manufacturer's instructions (Sangon Biotech, Shanghai, China). OD450 was calculated by subtracting the background, and standard curves were plotted. Values are expressed as pg/ml of total protein.

Flow Cytometry

Flow cytometry was used to determine the apoptosis rate in LPS-induced H9c2 cells. Apoptotic cells were differentiated from viable or necrotic cells by the combined application of annexin V (AV)–fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the manufacture of the Annexin V-FITC cell apoptosis kit (C1062, Beyotime Biotechnology). Cells were washed twice and adjusted to a concentration of 10^6 cells/ml. AV–FITC (5 μ l) and PI (10 μ l) were added to 195 μ l of binding buffer in each sample and incubated for 20 min at room temperature in the dark. Then without washing, samples were analyzed by using flow cytometry. Each experiment was performed at least in three independent experiments.

Western blot analysis

H9c2 Cells and the heart tissue were lysed by RIPA lysis buffer with protease inhibitors and phosphatase inhibitors according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). Protease inhibitor cocktail was from Sigma Chemicals (St. Louis, MO, USA). The protein concentration was determined with the BCA assay kit (Thermo Fisher Scientific, Inc.). Total protein from myocardium tissues and cardiomyocytes was separated by SDA-PAGE gels and were electro-blotted onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and then incubated overnight at 4°C with primary antibodies against Bax (1:1000, ab32503,

Abcam), Bcl-2 (1:500, sc-7382, Santa Cruz Biotechnology), β -actin (1:500, Santa Cruz Biotechnology); GAPDH (1:1000, ab9485, Abcam); LC3B (1:1000, AL221, Beyotime Biotechnology); p62 (1:1000, A19700, ABclonal Technology); Beclin1(1:1000, AF5123, Beyotime Biotechnology); caspase-3 (1:1000, 9662, Cell Signaling Technology); iNOS (1:1000, ab178945, Abcam); gp91^{phox} (1:1000, sc-130543, Santa Cruz Biotechnology) antibodies. After incubation with the primary antibodies, blots were incubated with horseradish peroxidase-linked secondary antibodies (1:5000) for 60 min at room temperature. Blots were again washed three times with TBST and immunoreactive bands were detected using an ECL chemiluminescence. Band intensities were quantified using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Silver Spring, MD, USA).

Quantitative reverse transcription PCR (q-PCR) analysis

Total RNA of myocardial tissues was isolated using TRIzol Reagent (Invitrogen). The Concentration of mRNA was determined using absorbance at 260 and 280 nm. The sample RNA was reversely transcribed to cDNA with reverse transcription reagent kit (Takara BIO) according to the manufacturer's instructions. q-PCR was performed using a SYBR® Premix Ex Taq™ Perfect Real Time Kit (Takara BIO) in the 7300 System SDS Software (Roche Applied Science). The primer sequences utilized for real-time PCR are presented as fellows: TNF- α Forward: ATGTCTCAGCCTCTTCTCATTC, Reverse: GCTTGTCACCTCGAATTTTGAGA; IL-10 Forward: TTCTTTCAAACAAAGGACCAGC, reverse: GCAACCCAAGTAACCCTTAAAG; IL-1 β Forward: TCGCAGCAGCACATCAACAAGAG; Reverse : TGCTCATGTCCTCATCCTGGAAGG. Results from 7300 System SDS Software are presented as Ct values, normalized against GAPDH, and shown as $2^{-\Delta\Delta Ct}$.

Cell viability assay

Cell Counting Kit-8 (CCK8) was applied to assess the number of viable cells. Cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/mL, and were allowed to acclimatize overnight. and then cultured in complete medium in the presence or

absence of LPS and JuA (20 μ M). The cells were pre-treated with various concentrations of the JuA (0 μ M to 20 μ M) for 1 h, and then treated with LPS 20 μ g/mL for 24 h. The OD₄₅₀ was tested at 3h, 6h, 12h, 24 h using a microtiter plate reader (SkanIt Software 5.0, RE, ver. 5.0.0.42). The results were calculated using the following formula: Cell viability = (Treatment Group OD – Blank Group OD)/ (Control Group OD – Blank Group OD)[18].

Statistical analysis

All data are presented as mean $\pm$ standard deviation at least three experiments. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL). Unpaired t-tests were used to determine the statistical differences between 2 groups in each analysis. A one-way analysis of variance (ANOVA) was used for multiple comparisons. Survival was analyzed with Kaplan–Meier survival curves and compared with the log-rank test. A p value of less than 0.05 was considered significant. P<0.05 was considered to be statistically significant.

1.3 Results

JuA treatment attenuated LPS-induced cardiac dysfunction and ameliorated survival rate in sepsis mice

To investigate whether JuA treatment protected against sepsis, mice were received JuA (20mg/kg, p.o.) for 7days before LPS injection in LPS+JuA group. Subsequently, cardiac function of these sepsis mice was detected by echocardiography as shown in Figure1. As expected, mice injected LPS for 12 hours displayed significant cardiac dysfunction with decreased LVEF, LVFS and increased LVESV, LVEDV, LVESD, LVEDD compared with that in the control group. Conversely, pre-treatment with JuA significantly improved cardiac function in LPS-treated mice by increased LVEF, LVFS and decreased LVESV, LVEDV, LVESD, LVEDD compared with the cardiac function of those with saline-treated sepsis mice (Figure 1A-E).

Furthermore, the survival rate was analyzed in LPS+saline group and LPS+JuA group. The mortality rate in the LPS group was 50-70%. Significantly, pre-treatment (20mg/kg by p.o. for 7 days) with JuA improved survival rate of sepsis mice (Figure 1F), compared with the group of LPS + NS. Therefore, these results showed that early treatment of JuA in sepsis could promote the cardiac function and prognosis of these septic mice.

JuA treatment suppressed cardiac inflammation and apoptosis in LPS-induced sepsis mice

To investigate the protective effects of JuA in vitro, further experiments were performed to examine the effects of JuA on the inflammation in septic mice. ELISA showed that the level of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-18 were increased in the LPS-treated mice compare with the control group, which on the contrary were largely reduced in LPS + JuA group by JuA treatment (Figure 2A). Moreover, JuA treatment significantly inhibited the mRNA expression of TNF- α , IL-1 β and IL-18 in the heart of LPS-treated mice (Figure 2B), whilst elevated levels of IL-10, which was also reflected by decreased pathology scores compared with saline-treated mice (Figure 2B). Furthermore, to evaluate the effects of JuA on cardiac apoptosis, a TUNEL assay

was performed (Figure 2C). Significantly, there were more TUNEL-positive cardiomyocytes detected in the heart of LPS-treated mice than that of the control group, in contrast, lower percentages of apoptotic cells were found in LPS + JuA group compared with the LPS+NS group (Figure 2C). Western blotting showed that the levels of cleaved caspase-3 were significantly increased in the group of LPS-treated mice compared with the control group, but decreased in the LPS + JuA group (Figure 2D). Further detection also revealed that JuA pretreatment downregulated the expression of Bax but promoted Bcl-2 induced by LPS(Figure 2D). These results demonstrated that pretreatment with JuA had a cardioprotective effects against inflammation and apoptosis in sepsis mice.

JuA treatment reduced LPS-induced inflammatory response and apoptosis in H9c2 cells

Considering the protective effects of JuA in vivo, we further verified the role of JuA in vitro. As shown in Figure 3, H9c2 cells were used to explore the protective effect of JuA on LPS-induced cardiomyocyte damage. Apoptosis in H9c2 cells was detected by flow cytometry, which showed that JuA pretreatment could decrease H9c2 cell apoptosis with LPS condition (Figure 3A). Western blot also confirmed that JuA not only decreased Bax expression but also increased Bcl2 expression in LPS-treated H9c2 (Figure3B). In addition, when LPS decreased cell viability in vitro, JuA pretreatment could block this effect (Figure3C). Immunofluorescence showed that JuA could also prevent LPS-induced cardiomyocyte apoptosis in vitro according to TUNEL staining (Figure3D). These results demonstrated that JuA could suppress the apoptosis induced by LPS-treatment.

JuA enhances cardiomyocyte autophagy in LPS-induced sepsis mice

Increasing evidence have found that autophagy plays a critical role in cell repairment in sepsis and impairment of autophagy may contribute to myocardial dysfunction and trigger apoptotic death in cardiomyocyte of sepsis[19]. We next investigated whether JuA could induce autophagy, which in turn contributed to the therapeutic effects of JuA

on LPS-induced myocardial injury. As shown in Figure 4A, TEM analysis observed more autophagosomes and messy myocardium in the LPS group compared with that in the control group. And JuA treatment further increased the number of autophagosomes and attenuated the structure of myocardium in the heart of septic mice. Meanwhile, western analysis revealed that JuA pretreatment promoted autophagy-related protein Beclin1 and LC3II expression, while decreased the expression of p62 protein in the heart of LPS-induced sepsis mice compared with the control group (Figure 4B-E), revealing the activation of autophagy in response to myocardial injury caused by LPS and JuA. These results demonstrated that JuA could enhance autophagy pathway to attenuate myocardial injury.

JuA treatment downregulated the production of iNOS and gp91 in the heart of LPS-induced septic mice

Oxidative damage was known as consequences of sepsis and may contribute to myocardial dysfunction. To explore the mechanisms underlying the potential role of JuA against LPS-induced sepsis, we subsequently assessed JuA in regulating sepsis-induced oxidative stress and nitrosative stress. Compared with the LPS group, mice pretreatment with JuA displayed a reduction of iNOS (a mediator of endoplasmic reticulum-stress apoptosis pathway) in the heart of sepsis mice (Figure 5A-B). Moreover, western blot analysis confirmed that JuA significantly downregulated the expression of gp91phox (the major component of NADPH oxidase responsible for the generation of superoxide anions) in LPS-challenged mice ($P < 0.01$, Figure 5A-C). Furthermore, immunohistochemistry staining in the heart of septic mice indicated that JuA could decrease the expression of iNOS and gp91phox (Figure 5D). In all, these results demonstrated the attenuation of myocardial injury by JuA may be due to the inhibited role against oxidative stress, which facilitated cardiomyocyte survival and improved cardiac function.

Inhibition of autophagy reversed the suppressive effect of JuA on LPS-induced inflammation and apoptosis in sepsis mice

To establish the autophagy in the protective role of JuA against sepsis, 3-MA (20 mg/kg)

was administered intraperitoneally 6 h before LPS injected. However, inhibition of autophagy with 3-MA pretreatment reversed the protective effect of JuA on LPS-induced cardiomyocyte inflammation, as evidenced by the pathways involving apoptosis and oxidative stress and nitrosative stress. As shown in Figure 6A-D, western blotting showed that the effect of JuA on autophagic activity in cardiomyocytes of sepsis mice was abolished by autophagy inhibitor 3-MA, as evidenced by the decrease in Beclin1 and LC3B II expression and the increased expression in p62. Furthermore, the inhibition of autophagy by 3-MA could significantly neutralize the effect of JuA in attenuating the oxidative stress and nitrosative stress by increasing the expression of iNOS and gp91 (Figure 6E-G). In addition, 3-MA pretreatment significantly diminished the inhibitory effects of JuA pretreated on LPS-induced cardiomyocytes apoptosis, with an increase in the Bax / Bcl-2 ratio (Figures 6H-I), and the activation of cleaved caspase-3 (Figures 6H-J) compared with the LPS+JuA group. These results demonstrated that autophagy activation is essential for JuA to combat oxidative stress and nitrosative stress under LPS-induced sepsis. autophagy inhibitor 3-MA significantly abolished the effect of JuA on apoptosis in JuA pretreated LPS-induced sepsis. Taken together, these results confirmed the autophagy activation involving oxidative stress and nitrosative stress pathway in the protective role of JuA on apoptosis.

1.4 Discussion

In this study, we found that JuA, a main effective component of jujubogenin extracted from the seed of *Ziziphus jujuba* Mill var *spinosa* (Bunge) Hu ex H F Chou (*Ziziphus*) [20, 21], significantly ameliorates LPS-induced myocardial injury by enhancing autophagy. These results in present study were consistent with Wan et.al, who reported that JuA played a protective role on norepinephrine-induced decreased cell viability and increased apoptosis of H9c2 [22] and Han et al, JuA could notably reduce the damage cause by isoproterenol via promoting the phosphorylation of PI3K, Akt, and mTOR and inhibiting LC3 conversion to attenuate the injury of H9c2[16], which indicated that JuA may be a potential choice for the treatment of heart diseases.

In the present study, mice were subjected to LPS to construct sepsis animal model. The results of this study showed that the cardioprotective effect of JuA against SIC was revealed by ameliorating the cardiac function and reducing levels of the inflammatory cytokines TNF- α , IL-1 β and IL-18, as well as alleviating cardiomyocyte apoptosis in vitro and in vivo. Furthermore, our results demonstrated that JuA increases autophagic activity in the heart of sepsis mice. However, this action of JuA was abolished by 3-MA in cardiomyocytes treated with LPS, suggesting that regulating autophagy by JuA may be essential in maintaining cardiac function in response to sepsis injury. Furthermore, the cardioprotective effects of JuA against SIC were associated with oxidative stress and nitroductive stress signaling was strongly involved in these protective effects.

JuA is a natural product isolated from the seeds of *Zizyphus jujuba*, possessing numerous biological effects [16], which is widely available for treating symptoms of insomnia and anxiety [23]. Previous studies have reported that JuA exerts anti-injury effects, cardioprotective and neuroprotective activity via anti-inflammatory and antioxidative effects in animal models of dementia in vivo [14, 16, 24]. As reported, SIC is a complication of severe sepsis and septic shock characterized by an invertible myocardial depression, as evidenced by the major hemodynamic characteristics with decreased ejection fraction and LV systolic dysfunction [25]. The possible beneficial effects of JuA on SIC have not yet been fully demonstrated. In this study, the results detected from CCK8 assay indicated that JuA (0-20 μ M) did not exhibit a cytotoxic

effect on H9c2 cells. Moreover, Zhu et al. reported that JuA effectively reversed the decreased cell viability caused by norepinephrine and reduced norepinephrine-induced H9c2 cell apoptosis [22]. Consistently in the present study, we found that pre-treatment with JuA in mice with SIC behaved with increased LVEF, LVFS values and decreased LVESV and LVEDV values by echocardiography. To the best of our knowledge, the present study is the first to report that JuA was significantly attenuated the cardiac function in mice with SIC.

Notably, the inflammatory response is the initial process in the hallmark development of SIC [26]. Mayer et al. reports that excessive levels of releasing pro-inflammatory mediators result in the inflammatory response in sepsis, while the compensatory anti-inflammatory reaction fails to suppress the immune response, resulting an imbalance between pro-inflammatory response and anti-inflammatory response occurred during infection [27]. TNF- α , IL-1 and IL-6 are the main inflammatory mediators which could lead to myocardial depression in sepsis [28]. Accumulating studies have reported that treatment with anti-inflammatory antibodies could improve cardiac function in patients with septic shock [29, 30]. In this study, the inflammatory factors of TNF- α , IL-1 β and IL-18 were increased in the serum of septic mice and in the supernatant of LPS-stimulated H9c2. Moreover, JuA pretreatment ameliorated the cardiac dysfunction of SIC by reducing the expression of inflammatory factor both in vivo and in vitro, suggesting that the cardioprotective effects of JuA are in connection with its anti-inflammatory activity in septic mice, which was consistent with the previous reported studies [31, 32]. Furthermore, we firstly demonstrated that JuA exhibited inhibitory effects on LPS-induced inflammation and possessed the potentially protective role in the treatment of LPS-induced sepsis.

In the present study, C57BL/6 mice and H9c2 cells were exposed to LPS to establish myocardial toxicity models, and apoptosis has been extensively implicated as a determining process in myocardial depression of SIC [33]. Previous studies have found that JuA may protect against norepinephrine-induced apoptosis of cardiomyocytes via modulation of the mitogen-activated protein kinase and AKT signaling pathways [22]. Positively, we found that JuA effectively reversed the decreased cell viability caused by

LPS and inhibited apoptosis induced by LPS both in vivo and in vitro, as evidenced by decreased TUNEL-positive cardiomyocytes by TUNEL staining and decreased H9C2 cell apoptosis detected by Flow Cytometry. In addition, the expression of caspase enzymes and Bax/Bcl-2 ratio was measured to demonstrate the molecular basis of the antiapoptotic effects of JuA. JuA treatment attenuated the expression of cleaved caspase-3 and decreased Bax/Bcl-2 ratio both in vivo and in vitro. These data indicate that JuA ameliorated sepsis-induced myocardial dysfunction by reducing myocardial apoptosis both in vivo and in vitro.

Another significant finding was that JuA could enhance autophagy in the heart of LPS-induced sepsis mice. Previous studies have shown that autophagy, a primary mechanism for maintaining cellular homeostasis, activated initially in sepsis, followed by a subsequent stage of myocardial impairment [34, 35] and apoptosis has been extensively regarded as the decisive process in SIC [36]. In the present study, autophagy was activated by LPS in vivo and vitro, as revealed by the increasing number of autophagosomes in vivo, the aggravation of cell apoptosis in vitro, and the increased expression of LC3II and Beclin1, decreased expression of p62 consistent with previous studies [37, 38]. Recent investigations suggest that appropriate autophagy modulation has a potential role to improve cardiac function by regulating mitochondria and attenuating inflammation in SIC with unclear mechanism in recent years [39]. Previous studies have shown that multiple medicine and bioactive molecules exert cardioprotective effects by regulating autophagy in sepsis [40-42]. In this study, we demonstrated that autophagic activity was enhanced by JuA pretreatment both in vivo and vitro, as shown by increasing the expression levels of LC3II and Beclin1, decreasing SQSTM1/p62 expression. Moreover, TEM data illustrated the number of autophagosomes in the heart of septic mice were significantly decreased followed by JuA treatment. 3-Methyladenine (3-MA), an autophagy inhibitor, exerts a cardioprotective effect in a lethal model of murine endotoxemia and polymicrobial sepsis by inducing autophagy [43]. In our study, we found that 3-MA could reverse the upregulation of autophagy induced by JuA, accompanied by an increase in the expression of Bax/Bcl-2 ratio and cleaved caspase3, indicating that JuA treatment may

play a protective role in sepsis by increasing autophagy.

The increasing evidence has indicated SIC was a consequence of uncontrolled inflammation [44], mitochondrial dysfunction [45, 46], excessive apoptosis [47], oxidant/antioxidant imbalance [48], and autonomic nervous system malfunction [49]. To investigate the mechanisms underlying the potential effect of JuA on LPS-induced SIC, this study subsequently explored JuA in regulating sepsis-induced oxidative stress and nitrosative stress. As expected, this study demonstrated a potentially protective mechanism of JuA in the pathophysiology of SIC by suppressing the expression of iNOS and gp91, acting by western blot and immunohistochemistry in the heart of sepsis mice and LPS-induced cardiomyocytes. In addition, the data in this study identified a potentially novel mechanism that links autophagy with abnormal oxidative stress and myocardial dysfunction. Importantly, autophagy inhibition (3-MA) neutralized the effect of JuA in ameliorating oxidative stress revealed by the expression of iNOS and gp91 and increasing the apoptosis in the heart of sepsis mice. Taken together, it can thus be suggested that the suppression of oxidative stress in myocardium by JuA could associate with the autophagy of cardiomyocyte, which might contribute to the alleviation of myocardial dysfunction during sepsis.

Limitation

However, there are still several limitations exist in this study. Firstly, sepsis-induced cardiomyopathy was established by injected LPS intraperitoneally, but mice with cecal ligation and puncture (CLP)-induced sepsis was not constructed in this study. Secondly, the H9C2 rat cardiomyoblast cell line were used in in vitro experiment. However, whether H9C2 cells can accurately mimic the hypertrophic responses of primary cardiomyocytes has not yet been fully determined. Furthermore, H9c2 cells and primary neonatal mouse cardiomyocytes have the functions of differentiation and proliferation, while the adult mouse cardiomyocytes are terminally differentiated cells and have no differentiation and proliferation function at the same time [50]. H9c2 cells can't fully mimic the primary neonatal mouse cardiomyocytes and the adult cardiomyocytes. Furthermore, ROS level in the myocardium should be detected to further clarify the oxidative stress induced by sepsis. In addition, this study did not investigate the effect

of JuA at multiple time points or in *in vitro* cell models. Since the current experimental settings may have also produced bias in the results, further extensive research is needed before a reliable conclusion can be drawn. These problems should be considered and resolved in future experiments.

1.5 Conclusion

In conclusion, the findings of this study confirmed that JuA may play a cardioprotective role in sepsis, which may be achieved through its ability to inhibit inflammation and apoptosis, and enhance autophagy and antioxidant activity. These findings may provide a clinical basis for the use of JuA as a new therapeutic option for the treatment of sepsis-induced myocardial dysfunction.

Data availability

The data used to support the findings of this study are included within the article.

Conflicts of interest

No conflicts.

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Disclosure statement

No potential conflicts of interest were disclosed.

Authors' contributions

WZ and JQQ designed the study. WZ, JQQ and LYJ performed the experiments. Data were collated by CZH and the results of data were discussed by SLH and HB. WZ and JQQ wrote the draft of the manuscript. All authors read and approved the final version

of the manuscript.

Ethics approval and consent to participate

All procedures were approved by the Institute's Animal Ethics Committee of Shanghai Jiao Tong University (Shanghai, China) and were performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

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Figure Legends

Figure 1. Effects of JuA on cardiac function in LPS-induced septic mice. **(A)** Representative echocardiographic images were shown. **(B)** LVEF. **(C)** LVFS. **(D)** LVESV. **(E)** LVEDV. **(F)** Kaplan-Meier survival curve of WT and JuA (20mg/kg)-treated WT mice after LPS-induced sepsis within 7days (n = 20 per group). *P < 0.05 vs. Control by log-rank test. WT The columns and error bars represent the means and standard error of the mean (n=10). *P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus Control group. #P<0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 versus LPS+NS group. LPS, lipopolysaccharide; NS: normal saline; LVEF, left ventricular ejection fraction; LVFS, left ventricular fraction shortening; LVESV, left ventricular end-systolic volume; LVEDV, left ventricular end-diastolic volume; JuA, Jujuboside A.

Figure 2. JuA decreased serum inflammatory factor levels and suppressed the myocardial apoptosis in LPS-induced septic mice. A. Serum inflammatory factors TNF- α , IL-1 β and IL-18 were detected by ELISA. B. The mRNA expression of inflammatory factors TNF- α , IL-1 β and IL-18 were detected in myocardium by QPCR. C. Representative images of the HE staining and TUNEL assay were shown in the myocardium of different groups. D. Western blotting showed expression of caspase-3, Bcl-2, and Bax. GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. *P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus Control group. #P<0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 versus LPS+NS group. LPS, lipopolysaccharide; NS: normal saline; TNF- α : tumor Necrosis Factor- α ; IL-1 β : interleukin-1 β ; IL-18: interleukin-18; ELISA: enzyme linked immunosorbent assay; QPCR: quantitative real-time polymerase chain reaction; HE: hematoxylin-eosin; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI: 4',6-Diamidino-2-Phenylindole, Dihydrochloride; SD: Standard Deviation.

Figure 3. JuA treatment reduced LPS-induced inflammation and apoptosis in H9c2

cells. A. Apoptosis was analyzed by flow cytometry after double labeling with Annexin V- FITC and PI. The percentage of apoptotic cells in each group was analyzed in the right of Figure 3A. Data are presented as the mean $\pm$ SD. ** $P < 0.01$ versus Control group. # $P < 0.05$ versus LPS + NS group. B. Western blotting showed expression of Bcl-2, and Bax in LPS-induced H9c2 cells. GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus the corresponding group. C. Cell viability was detected in different groups by CCK8 assay. LPS, lipopolysaccharide; FITC: Fluorescein Isothiocyanate; PI: Propidium iodide; CCK8: Cell Counting Kit-8; SD: Standard Deviation; JuA, Jujuboside A.

Figure 4. JuA enhances myocardial autophagy in LPS-induced sepsis mice. A. Representative transmission electron micrographs of left ventricular specimens (magnification x26,500, the white arrows indicate myocardium; the red arrows indicate mitochondria; the yellow arrows indicate autophagosomes). B. Western blotting showed expression of p62 (C), Beclin1 (D), and LC3B (E). GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus the corresponding group. LPS, lipopolysaccharide; SD: Standard Deviation; JuA, Jujuboside A.

Figure 5. JuA treatment decreased the expression of iNOS and gp91 in nitrosative stress and oxidative stress in the myocardium of LPS-induced septic mice. A. Western blotting showed expression of iNOS (B), and gp91 (C). GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus the corresponding group. (D) Representative immunohistochemical images of iNOS and gp91 in the myocardium of LPS-induced septic mice. The scale bar was 200 μ m. LPS, lipopolysaccharide; iNOS: inducible nitric-oxide synthase; SD: Standard Deviation; JuA, Jujuboside A.

Figure 6. Inhibition of autophagy reversed the suppressive effect of JuA on LPS-

induced inflammation and apoptosis in the heart of sepsis mice. A. Western blotting showed expression of p62 (B), Beclin1 (C), and LC3B (D) under the treatment of 3-MA in the heart of LPS-induced septic mice. E. Western blotting showed expression of iNOS (F) and gp91 (G) under the treatment of 3-MA in the heart of LPS-induced septic mice. H. Western blotting showed expression of Bax and Bcl-2 (I), caspase 3 (J) under the treatment of 3-MA in the heart of LPS-induced septic mice. GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. *P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus the corresponding group. *P<0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus Control group. #P<0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 versus LPS+NS group. \$ P<0.05, \$\$ P < 0.01 versus LPS+JuA group. LPS, lipopolysaccharide; 3-MA: 3-Methyladenine; iNOS: inducible nitric-oxide synthase; SD: Standard Deviation; JuA, Jujuboside A.

Figure 1

Figure 2

Figure 3

A

B

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Figure 4

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Figure 5

Figure 6

Figures

Figure 1

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Inhibition of autophagy reversed the suppressive effect of JuA on LPS-induced inflammation and apoptosis in the heart of sepsis mice. A. Western blotting showed expression of p62 (B), Beclin1 (C), and LC3B (D) under the treatment of 3-MA in the heart of LPS-induced septic mice. E. Western blotting showed expression of iNOS (F) and gp91 (G) under the treatment of 3-MA in the heart of LPS-induced septic mice. H. Western blotting showed expression of Bax and Bcl-2 (I), caspase 3 (J) under the treatment of 3-MA in the heart of LPS-induced septic mice. GAPDH was used as an endogenous control. Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus the corresponding group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus Control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ versus LPS+NS group. \$ $P < 0.05$, \$\$ $P < 0.01$ versus LPS+JuA group. LPS, lipopolysaccharide; 3-MA: 3-Methyladenine; iNOS: inducible nitric-oxide synthase; SD: Standard Deviation; JuA, Jujuboside A.