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Original Article

Neogambogic acid relieves myocardial injury induced by sepsis *via* p38 MAPK/NF-κB pathway

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ABSTRACT Sepsis-associated myocardial injury, an invertible myocardial depression, is a common complication of sepsis. Neogambogic acid is an active compound in garcinia and exerts anthelmintic, anti-inflammatory, and detoxification properties. The role of neogambogic acid in sepsis-associated myocardial injury was assessed. Firstly, mice were pretreated with neogambogic acid and then subjected to lipopolysaccharide treatment to induce sepsis. Results showed that lipopolysaccharide treatment induced up-regulation of biomarkers involved in cardiac injury, including lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and troponin I (cTnI). However, pretreatment with neogambogic acid reduced levels of LDH, CK-MB, and cTnI, and ameliorated histopathological changes in the heart tissues of septic mice. Secondly, neogambogic acid also improved cardiac function in septic mice through reduction in left ventricular end-diastolic pressure, and enhancement of ejection fraction, fractional shortening, and left ventricular systolic mean pressure. Moreover, neogambogic acid suppressed cardiac apoptosis and inflammation in septic mice and reduced cardiac fibrosis. Lastly, protein expression of p-p38, p-JNK, and p-NF-κB in septic mice was decreased by neogambogic acid. In conclusion, neogambogic acid exerted anti-apoptotic, anti-fibrotic, and anti-inflammatory effects in septic mice through the inactivation of MAPK/NF-κB pathway.

INTRODUCTION

Sepsis, a systemic inflammatory disease, is usually caused by maladjusted response of host to infection [1]. Sepsis often leads to life-threatening organ dysfunction, such as multiple organ dysfunctions and systemic inflammatory response syndrome, thus referring to leading causes of death [2]. Heart is a common target organ of sepsis, and sepsis induced cardiomyopathy, as common complication of sepsis, aggravates deterioration of sepsis and attributes to mortality of septic patients [3]. Vasopressors, dobutamine, levosimendan, ivabradine, and mechanical support are currently used in the management of sepsis induced cardiomyopathy [3]. However, more effective therapeutic strategies are still urgent needed for sepsis induced cardiomyopathy.

Previous studies have shown that myocardial fibrosis, mitochondrial dysfunction, apoptosis injury, autophagy impairment, autonomic nervous system disorders, calcium regulation disorders, oxidative stress, and inflammatory response disorders are involved in the pathogenesis of sepsis induced cardiomyopathy [4,5]. Strategies to repress the pathological processes might contribute to amelioration of pathological processes [6].

Gambogic acid is an active compound in traditional Chinese herb, *Garcinia hanburyi*, and exhibits a wide range of properties, including anti-inflammatory, antioxidant, anti-bacterial; and anti-proliferative activities [7]. For example, gambogic acid reduced lipopolysaccharide-stimulated secretion of proinflammatory factors in macrophages [8]. Neogambogic acid, as the isoform of gambogic acid, exerts a broad anti-cancer activity with low toxic-

ity [9]. Neogambogic acid inhibited biofilms formation to protect against methicillin-resistant *Staphylococcus aureus* infection [10]. Moreover, neogambogic acid reduced silica-induced inflammation and fibrosis to prevent silicosis [11]. However, the role of neogambogic acid in pathological processes involved in sepsis induced cardiomyopathy remains unclear.

In this study, effects of neogambogic acid on cardiac apoptosis, inflammation, fibrosis in lipopolysaccharide-stimulated mice were investigated. The meaning results might provide potential agent for the treatment of sepsis induced cardiomyopathy.

METHODS

Animal model

Thirty of seven-week-old male C57BL/6 mice (22–25 g weight) were acquired from Hunan SJA Laboratory Animal Co., Ltd. (Hubei, China). The experiment was approved by Second Affiliated Hospital of Nanchang University. Mice were randomly separated into 5 groups with six mice for each group: sham, lipopolysaccharide (LPS), LPS with 1.5 mg/kg neogambogic acid (NGA), LPS with 3 mg/kg NGA, and LPS with 6 mg/kg NGA. Mice were intraperitoneal injected with different concentrations of NGA (Shanghai Ronghe Medical Technology Co., Shanghai, China) dissolved in DMSO twice a day for consecutive 10 days. Mice in the sham and LPS groups were received an equal volume of DMSO. On day 11, mice in the LPS groups were then intraperitoneal injected with 10 mg/kg LPS (Sigma-Aldrich, St. Louis, MO, USA), and mice in the sham group were received an equal volume of saline. Echocardiography was performed 12 h later, and then the mice were sacrificed.

Another group of mice (n = 6) were injected with 6 mg/kg NGA and 2.5 mg/kg anisomycin (Sigma-Aldrich) twice a day for consecutive 10 days. Mice were also then injected with LPS.

Echocardiography

Mice were anesthetized with 1.5% isoflurane, and fastened to a heating pad. Vevo770 (30 Hz; VisualSonics, Toronto, ON, Canada) was used to detect transthoracic echocardiography. LV dimensions were recorded under M-mode tracings with short-axis view during course of systole and diastole. Left ventricular systolic mean pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were calculated, ejection fraction (EF) and fractional shortening (FS) were also recorded.

Determination of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB)

Mice post echocardiography were sacrificed, the blood samples were harvested from right ventricle with vacuum tubes. Serum samples were then collected from the blood, and levels of LDH and CK-MB were calculated using automatic biochemical analyzer (Rayto, Guangdong, China).

ELISA

Commercial ELISA kits (MultiSciences Biotech Co., Ltd., Wuhan, China) were used to determine serum levels of cardiac troponin I (cTnI), interleukin (IL)-1β, IL-18, IL-6, and tumor necrosis factor-α (TNF-α).

Histopathological analysis

Heart tissues were were fixed in 10% formalin and embedded in paraffin. The tissues were sliced into 5 μm sections. The sections were deparaffinized in toluene and hydrated in gradient concentrations of alcohol. Sections were then stained with hematoxylin and eosin (Sigma-Aldrich) and Masson's trichrome (Sigma-Aldrich). The sections were observed under microscope (Olympus, Tokyo, Japan).

TUNEL staining

Cardiac sections were implemented at 90°C, and then treated with Protease K (Sigma-Aldrich). Section were suspended in TDT buffer (Sigma-Aldrich), and incubated overnight with anti-serum alkaline phosphatase and anti-digoxin complex (Sigma-Aldrich). Following counter staining with DAPI, sections were observed under microscope. The apoptotic ratio was quantified using Image-Pro Plus.

qRT-PCR

Heart tissues were lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and the isolated RNAs were reverse-transcribed into cDNAs. The cDNAs were subjected to qRT-PCR analysis with SYBR Green Master (Roche, Mannheim, Germany). GAPDH (Forward: 5'-GGATTTGGTCGTATTGGG-3' and Reverse: 5'-GGAAGATGGTGATGGGATT-3') served as endogenous control. Expression of IL-1β (Forward: 5'-CACCTTCTTTTCCTTCATCTTTG-3' and Reverse: 5'-GTCGTTGCTTGTCTCTCCTTGTA-3'), IL-18 (Forward: 5'-AAAGTGCCAGTGAACCC-3' and Reverse: 5'-TTTGATGTAAGTTAGTGAGAGTGA-3'), TNF-α (Forward: 5'-ACTGAACTTCGGGGTGATTG-3' and Reverse: 5'-GCTTGGTGGTTTGCTACGAC-3'), and IL-6 (Forward: 5'-TGATGGATGCTTCCAAACTG-3' and Reverse: 5'-GAGCATTGGAAGTTGGGGTA-3') were determined by $2^{-\triangle\triangle CT}$ method.

Western blot

Cardiac tissues were lysed in RIPA buffer (Beyotime, Beijing, China). Protein samples were segregated using SDS-PAGE, and then transferred onto nitrocellulose membranes. Membranes were blocked in 5% dry milk, and then incubated with primary antibodies: anti-cleaved caspase-3 and anti-β-actin (1:2,000), anti-BAX and anti-BCL-2 (1:2,500), anti-collagen I and anti-collagen III (1:3,000), anti-p-JNK and anti-JNK (1:3,500), anti-p-p38 and anti-p38 (1:4,000), anti-TLR4 (1:4,500), anti-p-NF-κB and anti-NF-κB (1:5,000). The membranes were then incubated with secondary antibodies (1:6,000), and subjected to chemiluminescence reagent kit (Beyotime). All the proteins were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

All the data were expressed as mean ± SEM and analyzed by Student's t-test or one-way analysis of variance. p < 0.05 was considered as statistically significant.

RESULTS

Neogambogic acid alleviated cardiac injury and dysfunction in septic mice

To induce sepsis, mice were treated with LPS. Histopathological analysis showed that LPS induced disordered myofilament arrangement and edema of myocardial tissues (Fig. 1A). Moreover, levels of LDH (Fig. 1B), CK-MB (Fig. 1C) and cTnI (Fig. 1D) were increased by LPS. LPS also induced cardiac dysfunction in mice, as evidenced by increase of LVEDP, decrease of EF, FS and LVSP (Table 1). However, injection with neogambogic acid attenuated histopathological changes in myocardial tissues of LPS-induced mice (Fig. 1A). Neogambogic acid also reduced LDH (Fig. 1B), CK-MB (Fig. 1C) and cTnI (Fig. 1D) of LPS-induced mice in a dosage dependent way. Neogambogic acid ameliorated cardiac dysfunction in LPS-induced mice through decrease of LVEDP, increase of EF, FS and LVSP (Table 1).
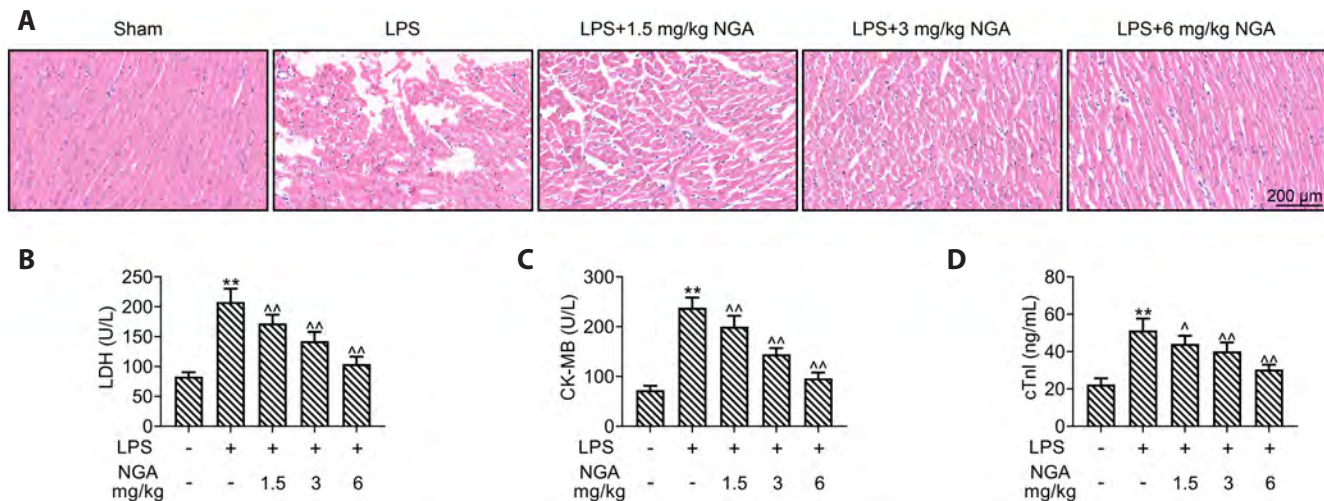
Fig. 1. Neogambogic acid (NGA) alleviated cardiac injury and dysfunction in septic mice. (A) Lipopolysaccharide (LPS) induced disordered myofilament arrangement and edema of myocardial tissues, injection with neogambogic acid attenuated histopathological changes in myocardial tissues of LPS-induced mice using H&E staining. Magnification: x200. (B) NGA reduced serum level of lactate dehydrogenase (LDH) in septic mice. (C) NGA reduced serum level of creatine kinase-MB (CK-MB) in septic mice. (D) NGA reduced serum level of creatine troponin I (cTnI) in septic mice. ** *vs.* sham, p < 0.01. ^,^^ *vs.* LPS, p < 0.05, p < 0.01.

Table 1. Myocardial function of mice in each group (12 h after LPS)

| Group	Sham	LPS	LPS + 1.5 mg/kg	LPS + 3 mg/kg	LPS + 6 mg/kg
EF (%)	72.04 ± 1.88	49.62 ± 4.28*	55.34 ± 2.15#	61.16 ± 5.43#	65.85 ± 1.48#
FS (%)	44.25 ± 3.13	20.65 ± 2.26*	27.16 ± 2.04#	32.78 ± 4.90#	40.09 ± 5.21#
LVSP (mmHg)	108.94 ± 1.65	50.25 ± 3.63*	68.33 ± 3.54#	79.43 ± 2.12#	88.21 ± 4.21#
LVEDP (mmHg)	4.09 ± 2.32	10.47 ± 1.23*	8.75 ± 0.23	6.41 ± 1.19#	5.02 ± 2.09#

Values are expressed as mean ± SE (n = 6 per group). LPS, lipopolysaccharide; EF, ejection fraction; FS, fractional shortening; LVSP, left ventricular systolic mean pressure; LVEDP, left ventricular end-diastolic pressure. *p < 0.05 *vs.* Sham; #p < 0.05 *vs.* LPS.

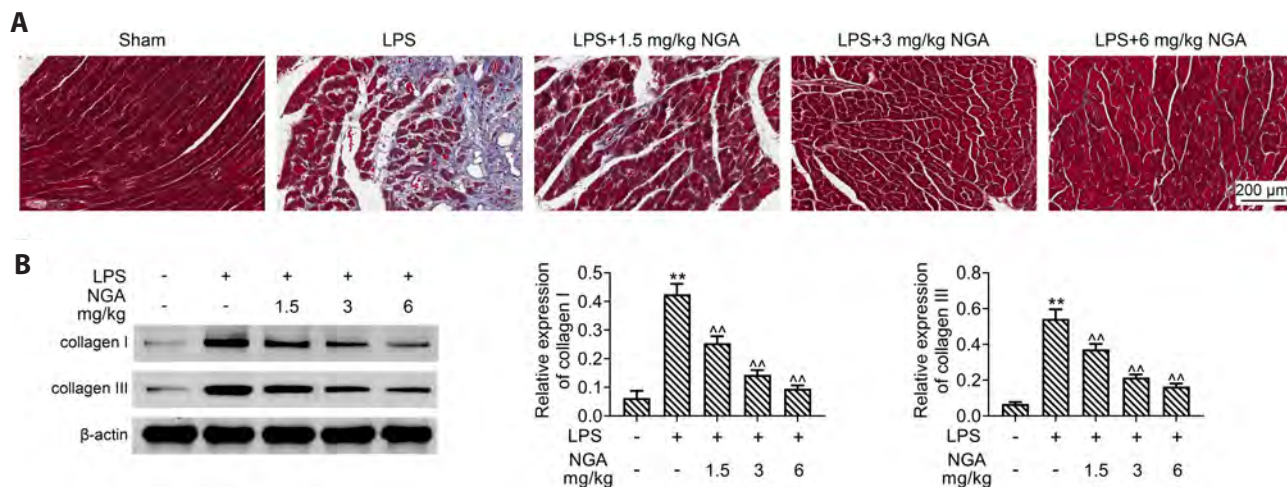## Neogambogic acid alleviated cardiac apoptosis in septic mice

Myocardial apoptosis was promoted by LPS (Fig. 2A) with down-regulation of Bcl-2, up-regulation of Bax and cleaved caspase-3 (Fig. 2B). Neogambogic acid reduced the number of TUNEL positive cells in myocardial tissues of LPS-induced mice (Fig. 2A). Moreover, protein expression of Bcl-2 in LPS-induced mice was increased, Bax and cleaved caspase-3 were decreased by neogambogic acid (Fig. 2B).

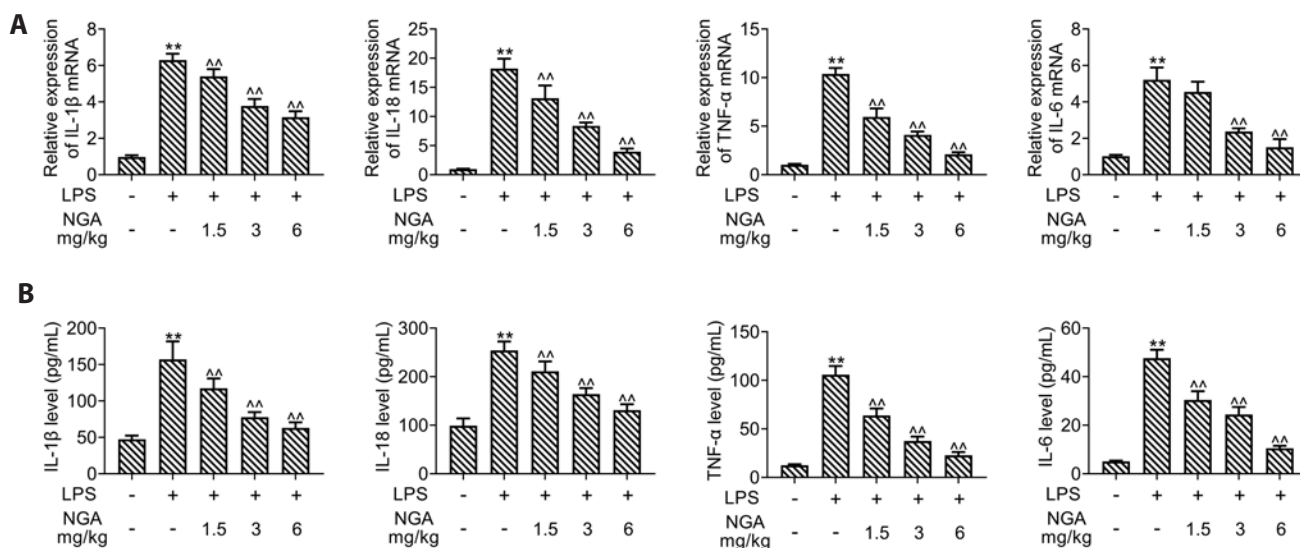## Neogambogic acid alleviated cardiac fibrosis in septic mice

Mice treated with LPS showed increase of fibrotic reactions in the heart tissues as demonstrated by Masson's trichrome (Fig. 3A). However, neogambogic acid reduced LPS-induced cardiac fibrosis in mice (Fig. 3A). Neogambogic acid also attenuated LPS-induced up-regulation of collagen I and collagen III in heart tissues of mice (Fig. 3B).
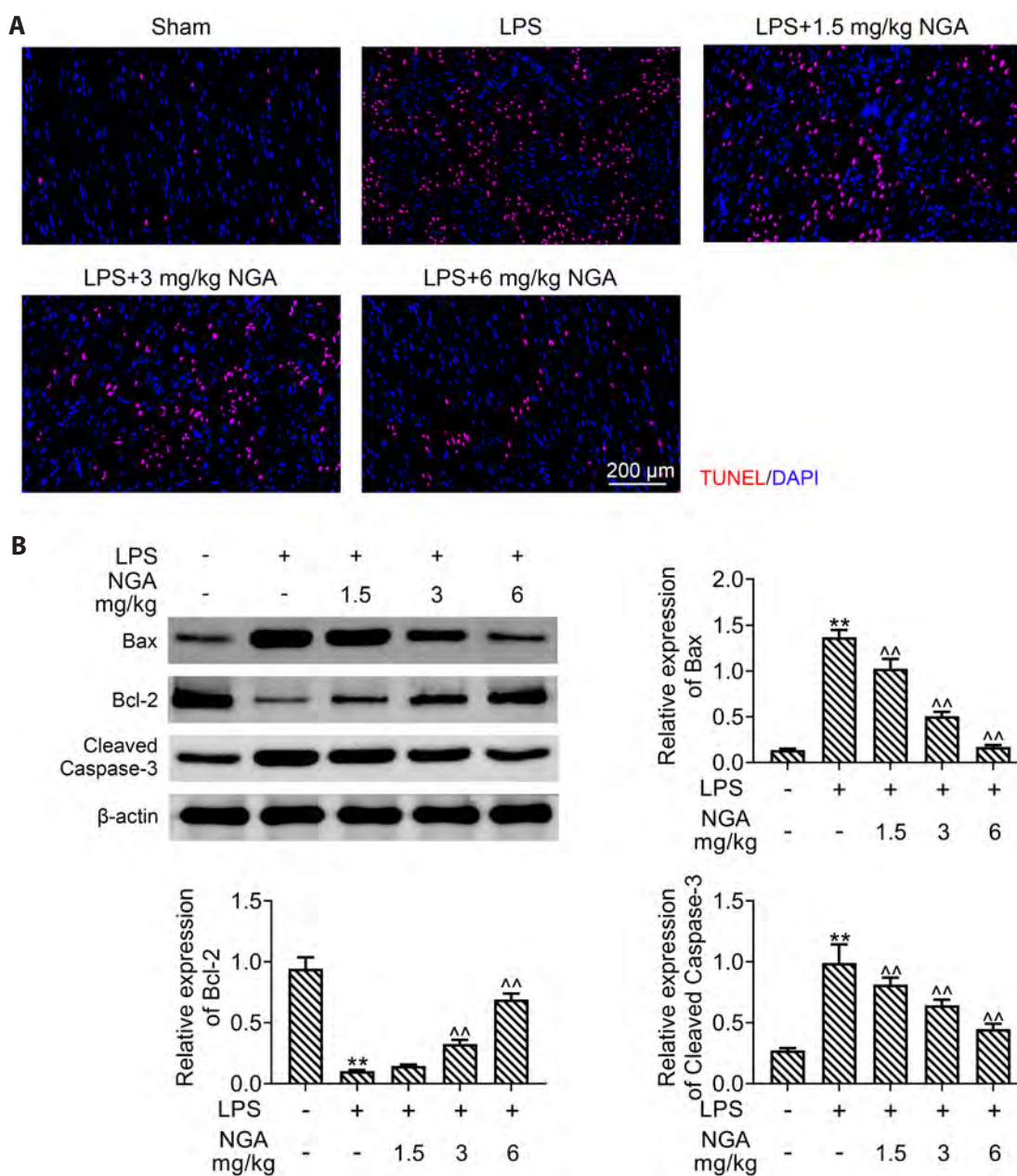
Fig. 2. Neogambogic acid (NGA) alleviated cardiac apoptosis in septic mice. (A) NGA reduced number of TUNEL positive cells in myocardial tissues of lipopolysaccharide (LPS)-induced mice (×200). (B) NGA enhanced protein expression of Bcl-2, reduced Bax and cleaved caspase-3 in LPS-induced mice. ** *vs.* sham, p < 0.01. ^^ *vs.* LPS, p < 0.01.

Fig. 3. Neogambogic acid (NGA) alleviated cardiac fibrosis in septic mice. (A) NGA reduced lipopolysaccharide (LPS)-induced cardiac fibrosis in mice. (B) NGA reduced protein expression of collagen I and collagen III in heart tissues of LPS-induced mice. ** *vs.* sham, p < 0.01. ^^ *vs.* LPS, p < 0.01.

Fig. 4. Neogambogic acid (NGA) alleviated cardiac inflammation in septic mice. (A) NGA reduced mRNA expression of interleukin (IL)-1β, IL-18, IL-6, and TNF-α in heart tissues of lipopolysaccharide (LPS)-induced mice. (B) NGA reduced serum levels of IL-1β, IL-18, IL-6, and tumor necrosis factor-α (TNF-α) in LPS-induced mice. ** *vs.* sham, p < 0.01. ^^ *vs.* LPS, p < 0.01.

Neogambogic acid alleviated cardiac inflammation in septic mice

Neogambogic acid decreased mRNA expression of IL-1β, IL-18, IL-6, and TNF-α in heart tissues of mice (Fig. 4A). Serum levels of IL-1β, IL-18, IL-6, and TNF-α in septic mice were down-regulated by neogambogic acid (Fig. 4B).
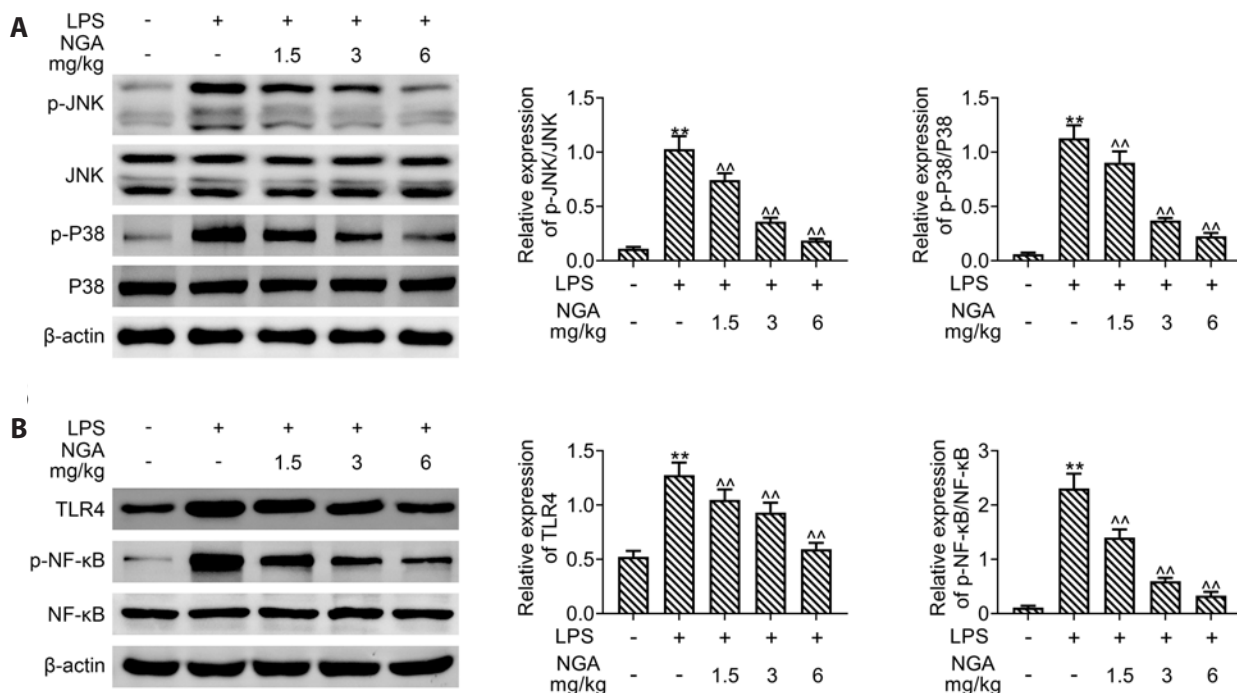
Neogambogic acid suppressed p38 MAPK/NF-κB signaling in septic mice

Neogambogic acid attenuated LPS-induced increase of p-JNK and p-p38 in heart tissues of mice (Fig. 5A). Moreover, protein

expression of TLR4 and p-NF-κB in septic mice were reduced by neogambogic acid (Fig. 5B). Treatment with JNK activator, anisomycin, weakened neogambogic acid-induced decrease of p-JNK and p-p38 in heart tissues of LPS-induced mice (Fig. 6A). Moreover, neogambogic acid-induced decrease of p-NF-κB in LPS-induced mice was also enhanced by anisomycin (Fig. 6B). Anisomycin attenuated neogambogic acid-induced decrease of IL-1β and IL-6 in LPS-induced mice (Fig. 6C).

DISCUSSION

Garcinia hanburyi is widely known as traditional Chinese

Fig. 5. Neogambogic acid (NGA) suppressed p38 MAPK/NF-κB signaling in septic mice. (A) NGA reduced protein expression of p-JNK and p-p38 in heart tissues of lipopolysaccharide (LPS)-induced mice. (B) NGA reduced protein expression of TLR4 and p-NF-κB in heart tissues of LPS-induced mice. ** *vs.* sham, p < 0.01. ^^ *vs.* LPS, p < 0.01.

medicine, and gambogic acid that is isolated from *Garcinia hanburyi* exhibits anti-inflammatory, anti-bacterial; anti-proliferative, and antioxidant effects in numerous chronic diseases [12]. This study found that neogambogic acid, an isoform of gambogic acid, alleviated sepsis induced cardiomyopathy through anti-apoptotic, anti-inflammatory and anti-fibrotic properties.

LPS, as endotoxin in Gram-negative bacteria, stimulates cardiac apoptosis and secretion of pro-inflammatory factors [13]. Therefore, LPS was widely used in the establishment of *in vivo* model of sepsis induced cardiomyopathy [14]. Results in this study also showed that LPS induced cardiac injury with up-regulation of biomarkers, including LDH, CK-MB and cTnI. Moreover, cell apoptosis and inflammation in heart tissues were also promoted by LPS.

Previous study has shown that cardiomyocyte apoptosis and inflammation were implicated in the pathogenesis of sepsis induced cardiomyopathy [15]. Pro-inflammatory factors, such as IL-1β, IL-6, and TNF-α, were associated with depression of LVEF in canine model with septic shock [16]. The pro-inflammatory factors reduced myocardial contractility, induced septic myocardial dysfunction [17]. Suppression of cardiomyocyte apoptosis and inflammation contributed to amelioration of cardiac dysfunction in septic mice [18]. LPS-induced up-regulation of IL-1β, IL-1α, IFN-β, IL-12b, IL-23a and TNF-α in macrophages were reduced by gambogenic acid [8]. This study also demonstrated that neogambogic acid suppressed cardiac apoptosis in heart tissues of LPS-induced septic mice, and reduced expression of IL-1β,

IL-18, IL-6, and TNF-α in septic mice. These results suggested anti-apoptotic and anti-inflammatory effects of neogambogic acid on sepsis induced cardiomyopathy. Oxidative stress induced by excessive accumulation of reactive oxygen species also stimulates development of sepsis induced cardiomyopathy [15]. Gambogic acid inhibited angiotensin II-induced oxidative stress [19]. Neogambogic acid might also exert anti-oxidant effect against sepsis induced cardiomyopathy.

LPS has been shown to stimulate cardiac fibrosis in animals, and attenuation of LPS-induced fibrosis facilitate for the amelioration of sepsis induced cardiomyopathy [20]. Silica-induced alveolar fibrosis has been reported to be inhibited by neogambogic acid [11]. Results in this study indicated that neogambogic acid reduced fibrotic reactions in LPS-induced septic mice through down-regulation of collagen I and collagen III. Therefore, neogambogic acid exerted anti-fibrotic effect against sepsis induced cardiomyopathy.

Increasing evidence has shown that p38 MAPK/NF-κB is involved in inflammation, and sustained activation of p38 MAPK/NF-κB was positively associated with cardiac injury [14]. LPS stimulated activation of p38 MAPK/NF-κB signaling, and inactivation of p38 MAPK/NF-κB signaling alleviated LPS-induced cardiac insufficiency [21]. Silica-induced up-regulation of p-p38 in macrophages was decreased by neogambogic acid [11], and neogambogic acid reduced p-JNK and p-NF-κB in receptor activator of nuclear factor κB ligand-induced mice [22]. This study revealed that neogambogic acid down-regulated protein expres-
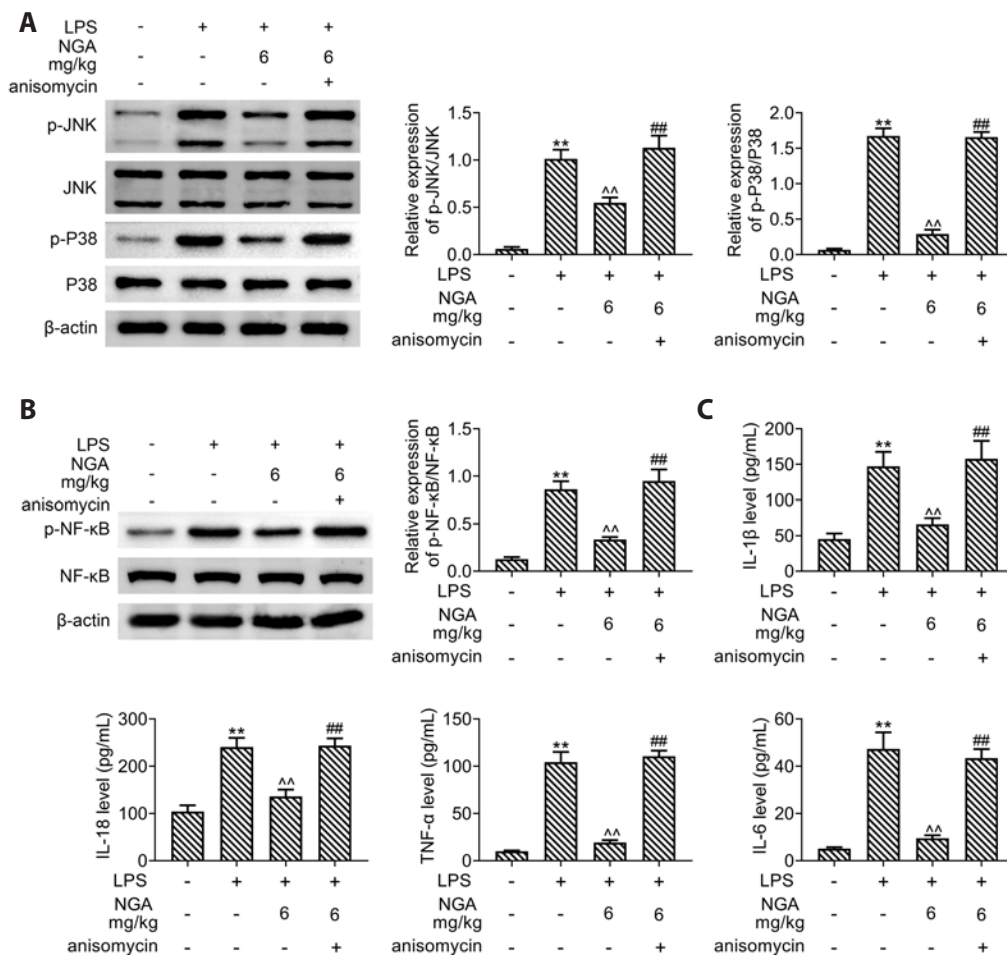
Fig. 6. Activation of JNK reversed neogambogic acid (NGA)-mediated cytokine release in septic mice. (A) Treatment with JNK activator, anisomycin, weakened NGA-induced decrease of p-JNK and p-p38 in heart tissues of lipopolysaccharide (LPS)-induced mice. (B) Treatment with JNK activator, anisomycin, weakened NGA-induced decrease of p-NF-κB in LPS-induced mice. (C) Anisomycin attenuated NGA-induced decrease of interleukin (IL)-1β and IL-6 in LPS-induced mice. ** *vs.* sham, $p < 0.01$. ^^ *vs.* LPS, $p < 0.01$. ## *vs.* LPS+NGA, $p < 0.01$.

sion of p-p38, p-JNK and p-NF-κB in heart tissues of septic mice, suggesting that neogambogic acid might alleviate sepsis induced cardiomyopathy through inactivation of p38 MAPK/NF-κB signaling.

Collectively, neogambogic acid ameliorated cardiac injury and dysfunction in LPS-induced septic mice, suppressed cardiac apoptosis, inflammation and fibrosis through down-regulation of p-JNK and p-NF-κB. Therefore, neogambogic acid might be a potential agent for the treatment of sepsis induced cardiomyopathy.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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