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Original Article

Protective effects of paeoniflorin on myocardial injury induced by sepsis via calpain/caspases-3

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Abstract: Objective: This study is to investigate the application of paeoniflorin (PF) on myocardial injury as well as its underlying mechanism on calpain signal transduction pathways. Methods: Rat myocardial injury models were established using Cecal ligation and puncture (CLP) method and intervened with 30, 60, and 120 mg/kg PF, respectively. After 24 h post operation, creatine kinase isoenzymes (CK-MB) and cardiac troponin I (cTNI) levels in rat sera were assessed. Pathological alters in cardiac muscle tissues were observed by Hematoxylin-eosin (HE) staining and apoptosis was measured by TUNEL. Then, expressions of calpain and calpastatin mRNA were measured by Real time polymerase chain reaction (RT-PCR). Additionally, the expression of calpain, calpastatin, caspase-3, caspase-8, B-cell lymphoma-2 (Bcl-2), and Bid proteins were evaluated by Western blot. Results: CLP model had higher CK-MB and cTNI levels than the sham group. After adding PF, the levels of CK-MB and cTNI decreased. HE staining showed that PF attenuated myocardial injury and 30 mg/kg PF had better function comparing with other concentrations. TUNEL demonstrated that PF alleviated myocardial apoptosis in septic rats. Comparing with CLP group, mRNA expression of calpain and calpastatin were significantly increased after treatment of PF, and the effect of PF was reduced in a concentration-dependent manner. Calpain-1, caspase-3, caspase-8 and Bid proteins were increased whereas calpastatin and Bcl-2 proteins were down-regulated in CLP models compared with the sham group. PF drastically weakened the expression profiles of these proteins. Conclusion: PF is praised to be a novel therapeutic indicator for myocardial injury protection and provides new insight in improving limitations and efficacy of sepsis therapy.

Keywords: Paeoniflorin, myocardial injury, sepsis, calpain, apoptosis

Introduction

Sepsis has been a systemic inflammatory response syndrome caused by infections [1, 2]. Sepsis can trigger damage to various organs and change body temperature, circulation, and breath. Approximately 40% sepsis patients undergo life-threatening injury on myocardium, appearing as cardiac dysfunction [3, 4]. Myocardial injury is one of complications of sepsis with high incidence, characterized with arrhythmia, hypotension and cardiac insufficiency, which commonly aggravates other organ dysfunctions [5]. Currently, the mechanism of myocardial injury induced by sepsis remains limited. More and more animal and clinical researches have proved that the apoptosis existed in the progression and development of myocardial injury induced by other diseases [6, 7]. Thus, the apoptotic mechanism of cardiomyocytes and drug intervention in sepsis are of great significance.

Calpains are calcium-dependent cysteine proteases usually constitutively expressed in cardiomyocytes, which have been shown to be closely related to apoptosis either in concert with caspases or independently [8]. The endogenous inhibitor calpastatin can suppress activity of calpain through its C extremity, meanwhile regulate Ca^{2+} channel. Cardiomyocyte apoptosis in myocardial injury induced by sepsis is mainly by the two signal transductions. The first pathway is exogenous apoptotic cascade, which is mediated by caspases [9]. The tumor necrosis factor receptor associated death domain

(TRADD) activates caspase-8. Subsequently, caspase-3 is activated to cell apoptosis, contributing to irreversible death [10]. The other pathway is endogenous apoptotic cascade, which is activated by stress stimulation. After stimulating factors disturbs mitochondria, pro-apoptosis factors Smac and cytochrome C are released and Bax proteins up-regulate [11]. The exogenous and endogenous apoptotic cascades ultimately activate caspase-3 to induce cell apoptosis, which damage cardiomyocyte functions.

Paeoniflorin (PF), a monoterpene glycoside, is a bioactive component extracted from dried roots of *Paeonia* [12]. As a main effective monomer of *Paeonia*, PF shows multiple pharmacological effects including liver protection, anti-oxidative and anti-inflammatory activities, composure, analgesia, enhancing immune functions with low toxicity [13, 14]. Chen *et al.* revealed that PF could attenuate the liver fibrosis at least in part by reducing tumor necrosis factor (TNF)-alpha in the serum and improving the anti-oxidative defense [13]. PF had protective effects on SH-SY5Y cell apoptosis from hydrogen peroxide [15]. Moreover, PF is considered to have a protective effect against doxorubicin (DOX)-induced cardiomyocyte apoptosis by suppressing NADPH oxidase (NOX)2, NOX4 expression and NOX activity [16]. However, the effect of PF on calpain of cardiomyocyte apoptosis in myocardial injury induced by sepsis is still poorly understood. In this study, we established rat sepsis-induced myocardial injury models using Cecal ligation and puncture (CLP) method so as to investigate the application of paeoniflorin (PF) on myocardial injury as well as its underlying mechanism on calpain signal transduction pathways.

Materials and methods

Construction of CLP models

The animal models of sepsis were constructed using 60 healthy male Sprague-Dawley (SD) rats (weighing within 200-220 g) purchased from Shanghai Sipper-BK Lab Animal Co. Ltd. (Shanghai, China). Rats were randomly assigned to six groups (each group n=10) with a random number table: a control group, a sham-operated group (sham group), a sepsis model group (CLP group), a PF low-concentration group (30 mg/kg; Chengdu Mansite Technology Ltd., Chengdu, China), a PF middle-concentration group (60 mg/kg) and a PF high-concentration group (120 mg/kg). They were raised in separate cages for 7 d when water was allowed ad libitum before the operations. All rats were given by gavage for 7 d. PF groups were given 10 ml/kg different concentrations of PF. The control, sham and CLP groups were given 10 ml/kg normal saline.

After gavages of the seventh day, fasting was performed among all rats for 12 h with free drinking. CLP method was applied to establish myocardial injury models induced by sepsis. Briefly, after rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (3.5 ml/kg), a 2 cm abdominal midline incision was made to expose the cecum. The cecum was ligated approximately at 1/3 of its root. The terminal cecum was crossed with an 18-gauge needle and a little stool was squeezed through the puncture site. Then the cecum was situated back in the abdomen and the incision was sutured. The control group was not treated by CLP and sham group underwent a similar operation with CLP group without cecal ligation or puncture. All animals received fluid resuscitation with 50 ml/kg saline solution post operation.

Assessment of myocardial injury in sera

After 24 h post operation, all rats were put to execution. From the inferior vena cava, 5 mL sera were taken and 2.5 mL blood were placed in an anticoagulation tube containing heparin for 4°C storage. An automatic biochemistry analyzer was used to measure creatine kinase isoenzymes (CK-MB) in sera. The cardiac troponin I (cTNI) in sera was assessed by immune chemiluminescence method.

Hematoxylin-eosin (HE) staining of cardiac muscle tissues

After rats were killed at 24 h after surgery, the heart was taken out rapidly. It was cut to 10 ml/g cardiac muscle tissues and made to homogenate. After centrifuged for 20 min, the supernate was collected and stored at -70°C. The above operations were performed at 4°C. The cardiac muscle tissues were fixed by 10% methanal for 30-50 min. Then 50%, 70%, 80%, 95% ethyl alcohol and anhydrous alcohol (Huadong Medicine Co., Ltd., Hangzhou, China) were successively used to dehydrate for 1 h. Tissues were embedded by paraffin. Tissue

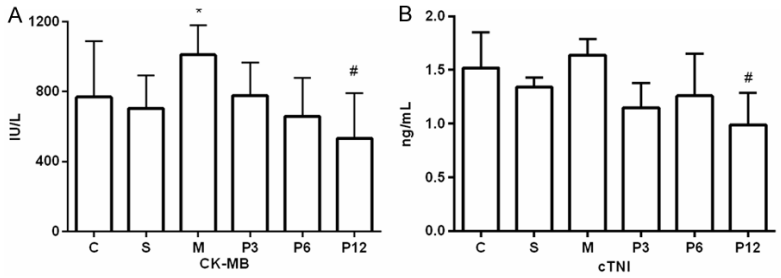

Figure 1. Effect of PF on serum indicators CK-MB and cTNI. (A) CK-MB assessment in six groups; (B) cTNI assessment in six groups. CK-MB, Creatine kinase isoenzymes; cTNI, Cardiac troponin I; C, control group; S, sham group; M, Cecal ligation and puncture model group; P3, 30 mg/kg PF group; P6, 60 mg/kg PF group; P12, 120 mg/kg PF group; PF, paeoniflorin; *, $P < 0.05$, compared with C group; #, $P < 0.05$, compared with M group.

sections of the myocardium were sliced up and stained with HE staining. Morphological changes were observed and recorded under a light microscopy.

TUNEL apoptosis assay

Cardiac apoptosis of six groups were quantified by TUNEL PITC Apoptosis Dectection Kit (Nuoweizan Biological Technology Development Co., Nanjing, China). Cardiac tissue sections by paraffine were soaked twice with xylene and steeped by ethyl alcohol for pretreatment. Then sections were added 100 μl 20 μg/ml Proteinase K for 20 min and washed by cold phosphate buffer saline (PBS) for 2-3 times. The samples were cultured by adding 100 μl Equilibration Buffer for 10-30 min. After samples were stained by PI, they were assessed by a confocal laser scanning microscope at 520 nm emission.

Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from cardiac muscle tissues of six groups respectively using Trizol reagent (Life Technologies, Gaithersburg, USA). For reverse transcription, complementary DNA (cDNA) was synthesized from 1 μg RNA using the RevertAid First strand cDNA Synthesis Kit (00064198; Fermentas, Glen Burnie, USA). Primers were generated by the miScript Primer Assays (Qiagen, Dusseldorf, Germany) and β-actin as internal control. The primer sequences were: calpain forward primer, 5' GATCGAGACGGTAATGGGAAAC'3, reverse primer, 5'CCAGGTC GGGCTCTGAGTAG'3; calpatatin forward primer, 5'AGTTTCCCAGGTGCCTGCT C'3, reverse primer, 5'CTCGCTATGCTCTGCTTTGATTT'3; β-actin forward primer, 5'GGAGATTACTGCCCTGGCTCCTA'3, reverse primer, 5'GACTCATCGTACTC CTGCTTGCTG'3. The PCR reactions were as follows: pre-denaturing at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, then a final step of 72°C for 8 min.

Western blot

Cardiac muscle tissues of control group, sham group, CLP group and three PF groups (30, 60, 120 mg/kg), respectively, were used for western blot assay. Every 20 mg tissues were mixed with 250 μl RIPA buffer containing 1 mg Phenylmethanesulfonyl fluoride (PMSF; Biyuntian Biological Technology Research Institute, Shanghai, China). We constructed the Bio-Rad Bis-Tris Gel western blotting system, in which primary antibodies calpain (ab133625), calpastatin (ab154568), caspase-3 (ab123784), caspase-8 (ab151707), B-cell lymphoma-2 (Bcl-2; ab164325), Bid (ab170784) and the internal control α-Tubulin were from Abcam (Cambridge, United Kingdom). Equivalent amounts of protein were separated on by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Biyuntian Biological Technology Research Institute, Shanghai, China) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Kelian Biological Co., Hangzhou, China). Next, membranes were incubated with primary antibodies (1:2000) at 4°C overnight. Goat anti-rabbit IgG secondary antibodies (1:2000; Biosharp, St. Louis, USA) were blotted by horseradish peroxidase for 2 h at 37°C and membranes were washed with Tween-20 (TBST; Biosharp, St. Louis, USA) for 10 min repeating 3 times. The ECL detection kit (2526151; Kelian Biological Co., Hangzhou, China) was used to visualize bands.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical differences were evaluated by software SPSS 19.0 (SPSS, Chicago, USA). The analysis of continuous variables in multiple groups was performed using One-way ANOVA. $P < 0.05$ was considered as statistically significant.

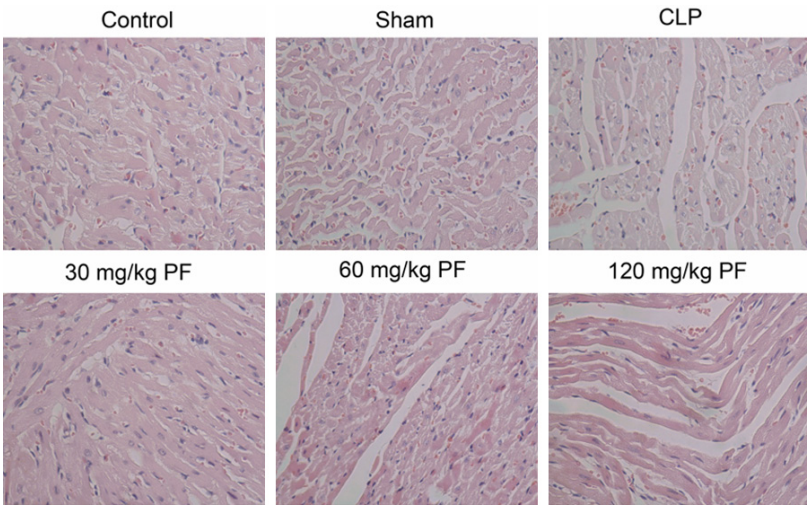

Figure 2. Representative pathological images in the myocardial sections stained by Hematoxylin-eosin (magnification × 400). In control group and the sham group, the structures of cardiocytes and cardiac muscle fibers were normal. In the CLP model group, cardiocytes appeared edemas and necroses with a lot of inflammatory cell infiltration. In 30 mg/kg PF group, a small amount of cardiocyte appears necroses and most fibers appeared to be arranged wavily. In 60 and 120 mg/kg PF groups, the myocardial injury was slightly alleviated. CLP, Cecal ligation and puncture; PF, paeoniflorin.

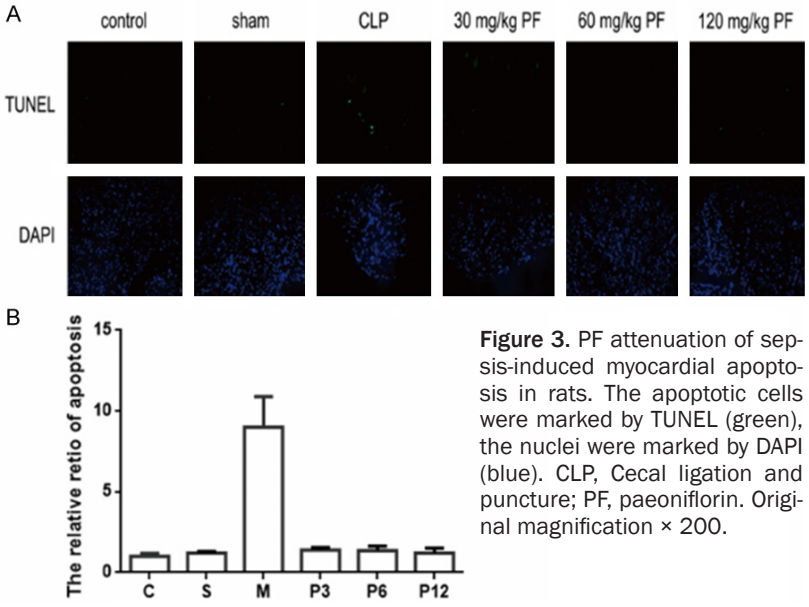

Figure 3. PF attenuation of sepsis-induced myocardial apoptosis in rats. The apoptotic cells were marked by TUNEL (green), the nuclei were marked by DAPI (blue). CLP, Cecal ligation and puncture; PF, paeoniflorin. Original magnification × 200.

Results

PF alleviated myocardial injury in CLP models by serum indicators

After 24 h post operation, the severity of myocardial injury in control group, sham group, CLP model and PF-treated groups were evaluated by measuring CK-MB and cTNI levels in rat serum. As shown in **Figure 1A**, compared with the sham group, the CLP model had increased CK-MB ($P < 0.05$). CK-MB levels were decreased by adding PF compared with CLP group, especially at 120 mg/kg PF ($P < 0.05$). In a concentration-dependent manner, the effect of PF was elevated. Additionally, CLP rats exhibited slightly higher cTNI levels than the sham group (**Figure 1B**). After adding PF, cTNI levels were decreased, especially at 120 mg/kg PF ($P < 0.05$). These results claimed that there was myocardial injury in rat CLP models and 120 mg/kg PF could alleviate it.

PF attenuated myocardial injury as indicated by HE staining

Cardiac muscle sections were stained with HE to assess CLP damage and PF effects to the myocardium. In the sham group, cardiocytes were normal and intact, and regularly arranged cardiac muscle fibers were observed with clear cross striations and normal structures (**Figure 2**). In the CLP model group, there were observable cardiocyte edemas, significant focal hemorrhages and necroses with a lot of inflammatory cell infiltration. With 30 mg/kg PF administration, there was a small amount of cardiocyte necroses, most fibers appeared to be arranged wavily. It suggested that PF could attenuate myocardial injury. In 60 and 120 mg/kg PF groups, the myocardial injury was slightly alleviated compared with CLP group, but was more serious than 30 mg/kg PF group. Accordingly, 30 mg/kg PF could improve pathological and inflammatory responses.

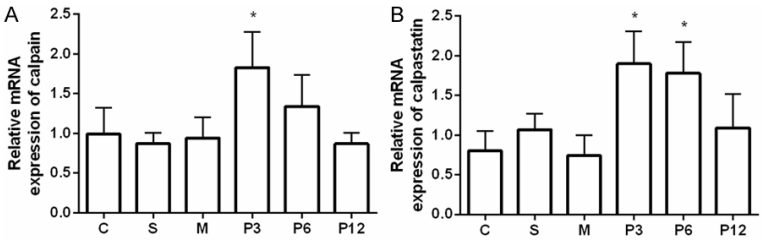

Figure 4. Effect of PF on mRNA expression of calpain and calpastatin. (A) Effect of PF on mRNA expression of calpain; (B) Effect of PF on mRNA expression of calpastatin; C, control group; S, sham group; M, Cecal ligation and puncture model group; P3, 30 mg/kg PF group; P6, 60 mg/kg PF group; P12, 120 mg/kg PF group; PF, paeoniflorin; *, $P < 0.05$.

PF alleviated myocardial apoptosis in septic rats

TUNEL was carried out to assess cell apoptosis in control group, sham group, CLP model and PF-treated groups, respectively. CLP models existed significant myocardial apoptosis in septic rats compared with the sham group (**Figure 3**). In PF treatment groups, 30 and 60 mg/kg PF group had no apoptotic cells, which was less than 120 mg/kg PF group. Particularly, PF treatment potently reduced the apoptotic index in the 30, 60 and 120 mg/kg PF groups (versus CLP group), revealing PF possessed protective effects on myocardial apoptosis in septic rats.

Effect of PF on mRNA expression of calpain and calpastatin

In CLP model, mRNA expression of calpain was slightly higher than sham group with no significant difference ($P > 0.05$; **Figure 4A**). After low concentration of PF treatment, mRNA expression of calpain was significantly increased compared with CLP group ($P < 0.05$). The effect of PF on calpain was reduced in a concentration-dependent manner. Moreover, mRNA expression of calpastatin was lower than sham group (**Figure 4B**). After low and middle concentrations of PF treatment, mRNA expression of calpastatin was significantly increased compared with CLP group ($P < 0.05$). Similarly, the effect of PF on calpastatin was reduced in a concentration-dependent manner.

Effect of PF on calpain related apoptotic proteins

Calpain, calpastatin, caspase-3, caspase-8, Bcl-2 and Bid proteins in control group, sham group, CLP model and PF-treated groups were measured by western blot. From **Figure 5**, calpain-1, caspase-3, caspase-8 and Bid proteins were increased, meanwhile, calpastatin and Bcl-2 proteins were down-regulated in CLP models compared with the sham group. With PF administration, calpain-1, caspase-3, caspase-8 and Bid proteins were drastically weakened at 30 mg/kg PF group. It was concluded that the cell apoptosis in rat CLP model might result from the activation of calpain inducing increase of caspase-3 and 30 mg/kg PF treatment could remarkably inhibit apoptosis.

Discussion

The current study made a primary research on protective effects of PF on myocardial injury induced by sepsis. We constructed sepsis-induced myocardial injury model in rats by CLP method. Next, the degree of myocardial injury in CLP model and effects of PF were measured in serum, pathological and apoptotic alters. CLP model had higher CK-MB and cTNI levels than the sham group. Observable cardiocyte edemas, significant focal necroses with a lot of inflammatory cell infiltration were investigated in paraffin sections. Then, mRNA expression of calpain was higher and calpastatin was lower in CLP model than sham group. Furthermore, cell apoptosis in rat CLP model might result from the activation of calpain inducing increase of caspase-3. Low concentration of PF remarkably improved cardiac functions, decreased inflammation responses and mitigated myocardial apoptosis associated with sepsis. We found above protective effects of PF was closely related to calpain. Taken together, this study lays the ground for dissecting the PF-mediated mechanism of myocardial injury, which provides potential targets for clinical therapy of sepsis.

Emerging reports serve as the basis for the treatment of PF on sepsis. Jiang *et al.* demonstrated that PF had anti-inflammatory effects on sepsis and the underlying mechanism might suppress activation of the NF-kB pathway in RAW264.7 cells [17].

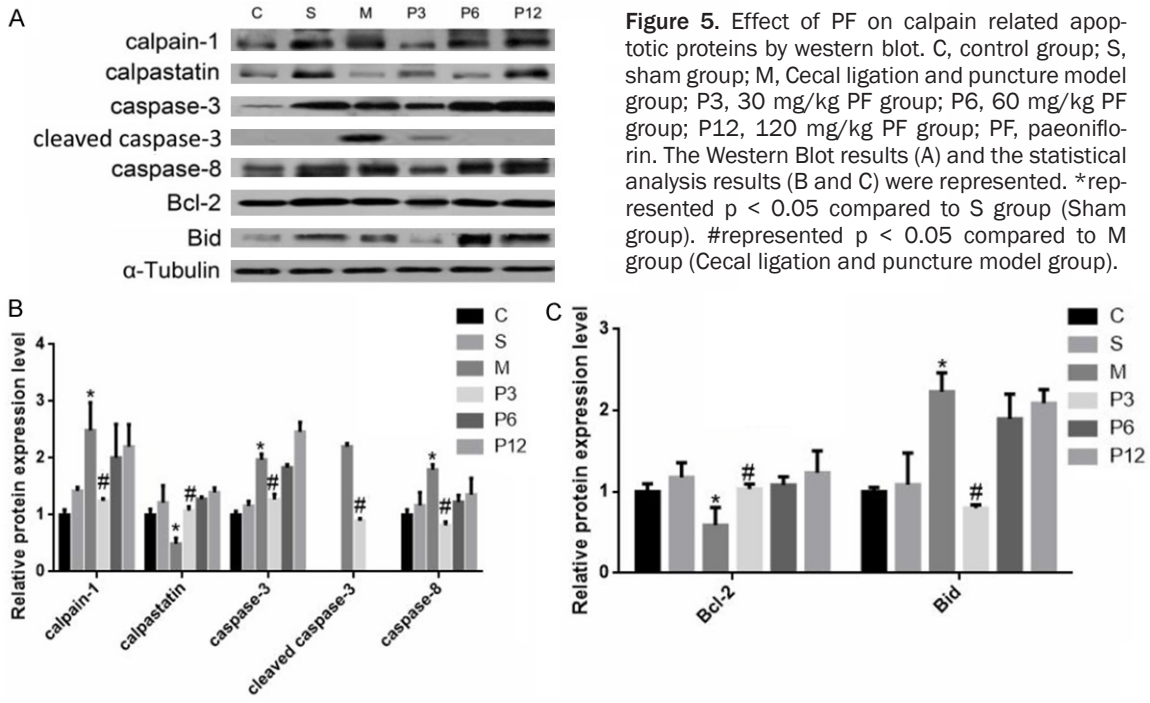

Figure 5. Effect of PF on calpain related apoptotic proteins by western blot. C, control group; S, sham group; M, Cecal ligation and puncture model group; P3, 30 mg/kg PF group; P6, 60 mg/kg PF group; P12, 120 mg/kg PF group; PF, paeoniflorin. The Western Blot results (A) and the statistical analysis results (B and C) were represented. *represented $p < 0.05$ compared to S group (Sham group). #represented $p < 0.05$ compared to M group (Cecal ligation and puncture model group).

Further, the similar anti-inflammatory mechanism of inhibiting NF-kB signal transduction was claimed in sepsis induced by bacterial lipoprotein (BLP) [18]. Zhai *et al.* proved that PF could protect mice from lipopolysaccharide (LPS)-triggered cardiac dysfunction via the activation of PI3K/Akt pathways [19]. It seems that most previous reports with respect to PF treatment on sepsis focused on its effect on anti-inflammatory responses but restraint of apoptosis. To our knowledge, the current study provided the first insight into the mechanism of PF on calpain pathway in myocardial injury induced by sepsis. It raised potential possibility of PF as a promising target to sepsis treatment and more PF research on animal and clinical experiments are wanted in the future.

Calpains have been proved to be involved in myocardial injury and their activation leads to cardiac injury and heart failure [16, 20]. Calpain inhibitor PD150606 restrained myocardial apoptosis caused by ischemia-reperfusion (I/R), which inferred that calpain activation might play an important role in the development of myocardial I/R [21]. Recently, deletion of *capns1* in cardiomyocyte reduced myocardial caspase-3 activity, causing attenuation of myocardial dysfunction, which suggested that calpain activation mediated lipotoxicity-induced cardiac injury [22]. It is well known that caspase-8, an apoptosis initiation factor, and caspase-3, the crucial executioner, are involved in the intrinsic signaling cascade of apoptosis. Thereby, activation of caspase-3 and caspase-8 were suggestive of biochemical hallmarks of apoptosis [23]. In addition, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bid have considerable implications for mediating the apoptotic response [24]. Recently, Vahidinia *et al.* reported that calpain-1 was a Ca^{2+}-dependent neutral protease which contributed to the activation of caspase-3 [25]. Given the fact that the attenuation of cardiac injury directly resulted from calpain disruption [22], in our study, we believed that PF suppressed cardiomyocyte apoptosis via attenuating activity of calpain-1, which needs further study for clarification. Consequently, one possible apoptotic mechanism in sepsis is CLP models contributed to calpain-1 activation. Calpain-1 mediated, sepsis-induced the activation of caspase-8, then causing the activation of caspase-3 in cardiomyocytes. The western-blot results pointed to calpain-dependent reduction of Bcl-2, which was in line with [26], demonstrating calpain-dependent apoptosis by cleavage of Bcl-2. Additionally, the expression of Bcl-2 protein was negatively correlated with Bid, which was consistent with the previous report [27]. Accor-

dingly, these results illustrate a promising mode for the cell apoptosis underlying myocardial injury induced by sepsis.

In summary, PF has a significant protective effect in scavenging apoptosis by inhibiting calpain pathways in CLP models. This study reveals that PF is praised to be a novel therapeutic indicator for myocardial injury protection and provides new insight improving limitations and efficacy of sepsis therapy.

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Disclosure of conflict of interest

None.

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