Protective effect of puerarin against burn-induced heart injury in rats

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Abstract. The present study evaluated the potential protective effects of puerarin and its associated mechanism on burn-induced myocardial damage. A total of 40 healthy adult Wistar rats were randomly divided into four groups: i) Sham; ii) burn; iii) burn + puerarin; and iv) puerarin. Serum levels of interleukin (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 were measured using ELISA. Myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels were determined in myocardial homogenates using a commercial assay kit. TUNEL staining and western blot analysis of cleaved and pro-caspase-3 were also performed to assess apoptosis. Activation of p38-MAPK, ERK, JNK and AKT were measured using western blot analysis. Left ventricular systolic pressure, maximum rates of increase in left ventricular pressure, creatine kinase MB activity and cardiac troponin T levels were found to be altered in the burn group 12 h after burn, which were reversed by puerarin treatment. Injection of puerarin following burn injury also reduced heart water content. Serum levels of IL-1β, TNF-α and IL-6 were significantly higher in the burn group compared with those in the sham group. Puerarin treatment reduced serum levels of IL-1β, TNF-α and IL-6, in addition to reducing MPO activity and MDA levels in myocardial tissues. Puerarin inhibited the activation of caspase-3, p38, ERK and JNK following severe burn, but elevated Akt activation following severe burn. In conclusion, puerarin improved cardiac function in rats following severe burn injury, which may be due to reduced myocardial injury, inhibition of cardiomyocyte apoptosis and reduced oxidative inflammatory stress; the MAPK and AKT signaling pathways are proposed to be the underlying mechanism of these findings.

Introduction

Severe burns can be fatal and are associated with complex and long-term pathological effects known as ‘burn disease’, which can occur within hours of the incident in question (1). Clinical studies have previously demonstrated that myocardial damage occurs in the initial stages of severe burns, with burn areas that cover >30% of the total body surface area (TBSA) (2,3). In the absence of immediate medical intervention after severe burn, myocardial injury can lead to cardiac dysfunction, where potential burn shock can occur, resulting in impaired circulatory function and possible mortality (4). Although the precise mechanism of burn-induced myocardial damage have not been fully elucidated, accumulating evidence have indicated that myocardial damage involves a number of processes, including hypoxia, inflammation, calcium signaling, apoptosis, sepsis and oxidative stress (5-7). Previous research efforts have focused on the development of novel therapeutic agents to effectively treat burn injury and reduce life-threatening burn-induced complications (8).

Radix Puerariae lobatae, also known as Gegen in Chinese, is the dry root of P. lobata (Willd.) Ohwi. Radix Puerariae lobatae has been applied therapeutically as a Traditional Chinese Herbal Medicine or as food for general consumption in East and Southeast Asian countries, particularly in ancient China (9). As a result of the abundant pharmacological properties and mild side effects exhibited by this plant, Radix Puerariae lobatae has been extensively used to treat diarrhea, diabetes, cardiac dysfunction, liver injury, weight loss and toxicosis (10). Puerarin (4'-7-dihydroxy-8-b-D-glucosylisoflavone) is a major isoflavone compound that can be found in the root of Radix Puerariae lobatae (11). Previous studies have reported puerarin to be beneficial for the treatment of a number of conditions, including cardiovascular diseases (12), neurological dysfunction (13), diabetes, liver injury (14), osteoporosis (15) and rheumatoid arthritis (16). In addition, puerarin has also been observed to confer protective effects against inflammation, hyperlipidemia, metabolic disorders and oxidative damage (10,11,17). Puerarin can be administered alone or as an adjuvant in combination with other pharmacological agents (17), in the form of an injectable, tablet or capsule. In particular, a puerarin injection has been approved by the State Food and Drug Administration in China for clinical treatment (e.g., angina pectoris and coronary heart disease) (18,19).

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Puerarin has been previously demonstrated to exert substantial therapeutic effects against cardiovascular diseases, including widening of the coronary artery, preservation of arterial endothelial integrity and myocardial ultrastructure, reduction of myocardial oxygen consumption, relief of myocardial ischemia and protection against myocardial ischemic-reperfusion injury (20-23). Mechanistic studies in animals and clinical settings have revealed that puerarin may exert cardioprotective effects by inhibiting the production and release of inflammatory cytokines, preventing oxidative stress in addition to regulating cardiomyocyte apoptosis and calcium signaling (24-27).

Although a number of studies have examined the mechanism underlying the therapeutic effects of puerarin in cardiovascular diseases, the protective effects of puerarin against severe burn-induced myocardial injury remain poorly understood. Therefore, in the present study, in vivo experiments were performed to investigate the potential protective effects of puerarin on severe burn-induced myocardial injury and to characterize the potential mechanisms underlying these effects.

Materials and methods

Animals. In total, 40 adult male Wistar rats (age, 6-8 weeks; weight, 210-250 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experimental procedures were approved by the Ethics Committee of Gansu Provincial Hospital (Lanzhou, China), according to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (28). The rats were housed at 2-3 rats per cage, at 23±1°C with 50% humidity on a 12 h light/dark cycle for ≥7 days prior to experimental procedures. All the rats were provided standard food and water ad libitum.

Experimental procedures. Rats were assigned into the following four groups (n=10 for each group): i) Sham; ii) burn; iii) burn + puerarin (10 mg/kg; Aladdin Scientific Ltd.), and iv) puerarin (10 mg/kg). All the rats were anesthetized using pentobarbital (40 mg/kg, intraperitoneal), following which the fur on the back and upper sides of the body was removed. The burn model was established in accordance with that described in a previous study (29). Briefly, the rats were restrained on a template device, where their naked skin was exposed to water (100°C) for 12 sec to produce 30% TBSA, whereas the BP and P groups were injected with 5% ketamine (4 ml/kg, intraperitoneal), xylazine (32), following which blood samples were collected for analysis using a chemistry autoanalyzer (VITROS® 750; Johnson & Johnson), while cTnT levels were measured using a rat cTnT ELISA kit (CSB-E16443r; CUSABIO) according to manufacturer's protocols.

Evaluation of myocardial injury. Serum samples were obtained at 12 h following the burn procedure and subsequently centrifuged at 2,000 x g for 15 min at 4°C. The supernatants were collected and stored at 4°C until further use. Myocardial injury was assessed by measuring cardiac troponin T (cTnT) levels and creatine kinase MB fraction (CK-MB) activity in the serum. Serum CK-MB activity was analyzed using a chemistry autoanalyzer (VITROS® 750; Johnson & Johnson), while cTnT levels were measured using a rat cTnT ELISA kit (CSB-E16443r; CUSABIO) according to manufacturer's protocols.

Determination of moisture content in myocardial tissues. Myocardial moisture content was measured using the dry/wet weight method. Appropriate amounts (200 mg) of myocardial tissue were dried using filter paper and weighed, which would be designated as the wet weight. The tissue samples were then incubated at 100°C for 48 h in an electric oven, and weighed using an electronic balance, which is designated as the dry weight. Moisture content was then measured using the formula: Moisture content = (wet weight - dry weight)/wet weight x100%.

Quantification of IL-1β, TNF-α, and IL-6 level in the serum. Serum samples were obtained at 12 h following the burn procedure and subsequently centrifuged at 700 x g for 30 min at 4°C, following which the supernatants were collected for analysis using a chemistry autoanalyzer (VITROS® 750; Johnson & Johnson), while cTnT levels were measured using a rat cTnT ELISA kit (CSB-E16443r; CUSABIO) according to manufacturer's protocols.

Measurement of myeloperoxidase (MPO) activity and heart malondialdehyde (MDA) concentration. An appropriate amount of myocardial tissue (50 mg) was homogenized in reagent II of the myeloperoxidase assay kit (cat. no. A044-1-1; weight:volume ratio, 1:19). Myocardial MPO activity was measured according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute). One unit of MPO (U/g) was defined as the amount of MPO required to degrade 1 µM peroxide/g wet heart tissue at 37°C.

MDA content was determined using the thiobarbituric method in myocardial tissue homogenates. The myocardial tissue was homogenized in reagent II at a weight: volume ratio of myocardial tissue and reagent II of 1:9. Tissue homogenates were centrifuged at 700 x g for 30 min at 4°C, following which the supernatants were collected for analysis using a malondialdehyde (MDA) assay kit (cat. no. A003-1-2) according to manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute). Biuret method was used to minimize protein interference and correct for protein content. MDA content was reported as nmol/mg protein.

TUNEL apoptosis assay. Myocardial tissues (20 mg) from the right ventricle was first fixed with 4% paraformaldehyde for 12 h

Measurement of cardiac function parameters. The right common carotid arteries of the rats were first separated and exposed. A polyethylene catheter filled with 25 U/l heparin saline was then inserted through the right common carotid artery into the left ventricle. The end of the catheter was in turn connected to a physiological signal acquisition system (model no. RM6240B; Chengdu Instrument Factory) for the acquisition of data. Following 5 min of calibration, left ventricular systolic pressure (LVSP) and the maximum rates of increase/decrease in left ventricular pressure (±dp/dt max) were measured to determine cardiac function.

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at 4˚C, following which the tissue was embedded in paraffin wax and cut into 5 µm sections for plating. TUNEL staining was performed according to manufacturer’s protocols of the TUNEL Assay Kit-HRP-DAB (cat. no. ab206386; Abcam). The rehydration and specimen were performed with xylene and ethanol before the permeabilization with 1% proteinase K at room temperature for 20 min. The endogenous peroxidases were inactivated by incubating in 3% H₂O₂ at room temperature for 5 min. Then labeling reaction was performed by incubating with TdT Labeling Reaction Mix at 37˚C for 1.5 h after incubating the specimens in TdT Equilibration buffer at room temperature for 30 min. The specimens were detected by incubating with 1X Conjugate at room temperature for 30 min and developed by incubating with working DAB solution at room temperature for 15 min after termination of labeling reaction and blocking. All reaction mixes, buffers, conjugates and solutions were included in the TUNEL Assay kit. Finally, nuclei were stained with hematoxylin (cat. no. G1140; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 10 min and the specimen were mounted by neutral balsam (cat. no. 822941; MACKLIN) on glass slides. The number of total cardiomyocytes and apoptotic cardiomyocytes were counted from 10 randomly selected non-overlapping fields of view under a light microscopy (magnification, x400) per condition. The percentage of apoptotic cardiomyocytes was calculated using the formula: % apoptotic cardiomyocytes = number of apoptotic cardiomyocytes/total number of cardiomyocytes x100%.

Western blotting. Myocardial tissue was homogenized in RIPA buffer (cat. no. R0020; Solarbio) containing 1% PMSF (Solarbio), which were then centrifuged at 12,000 x g for 5 min at 4˚C. Protein concentration was quantified using bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) and was adjusted to 5 µg/µl using lysis buffer. A total of 10 µg protein was loaded per lane for SDS-PAGE, electrophoresis was then performed by 10% SDS-PAGE to separate the protein extracts. The separated proteins were subsequently transferred to PVDF membranes (EMD Millipore) and blocked for 1 h at room temperature in TBS supplemented with 0.05% Tween-20 (TBST) containing 5% non-fat milk. The membranes were then incubated with primary antibodies against cleaved caspase-3 (dilution: 1:1,000; cat. no. ab49822; Abcam), p38 (dilution: 1:5,000; cat. no. ab170099; Abcam), phosphorylated (p)-p38 (dilution: 1:1,000; cat. no. ab47363; Abcam), JNK (dilution: 1:5,000; cat. no. ab199380; Abcam), p-JNK (dilution: 1:2,000; cat. no. ab47337; Abcam), Akt (dilution: 1:10,000; cat. no. ab179463; Abcam), p-Akt (dilution: 1:1,000; cat. no. ab179463; Abcam), ERK (dilution: 1:1,000; cat. no. AF1576; R&D Systems, Inc.), p-ERK (dilution: 1:1,000; cat. no. AF1018; R&D Systems, Inc.) overnight at 4˚C. Following washing in TBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution: 1:1,000; cat. no. HAF008; R&D Systems, Inc.) at room temperature for 1 h. Protein bands were visualized using an ECL Western Blotting Detection Reagent (GE Healthcare

Figure 1. Puerarin attenuated severe burn-induced acute myocardial injury. All parameters were evaluated 12 h after burn injury. (A) Effects of puerarin treatment on LVSP, (B) +dP/dt max, and (C) -dP/dt max. (D) Effects of puerarin treatment on serum CK-MB levels and (E) cTnT levels. (F) Effects of puerarin treatment on myocardial water content (%). Values above the bars are the mean values. n=10 in each group. *P<0.01 and **P<0.05. LVSP, left ventricular systolic pressure; +dP/dt max, maximum rates of increase in left ventricular pressure; -dP/dt max, maximum rates of reduction in left ventricular pressure; CK-MB, creatin kinase MB; cTnT, cardiac troponin.
Life Sciences). Densitometric analysis was performed using ImageJ software, version 1.41 (National Institutes of Health), which was normalized further to the loading control β-actin.

Statistical analysis. All values are presented as the mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc.). Statistical differences between the groups were analyzed by one-way analysis of variance with Bonferroni’s correction or Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Puerarin relieves severe burn-induced myocardial injury. To evaluate the effects of puerarin treatment on burn-induced myocardial injury, the rats first underwent a 30% TBSA full-thickness burns, following which hemodynamic parameters and indices of myocardial injury were measured 12 h post-injury. LVSP, which represents left ventricle contractile function, was significantly lower in the burn group 12 h post-burn compared with that in the sham group (Fig. 1A). Similarly, the ±dp/dt max in the burn group were also significantly lower compared with those in the sham control (Fig. 1B and C). Puerarin treatment significantly reversed cardiac dysfunction in the burn + puerarin group, as evidenced by the significant higher LVSP and ±dp/dt max in the burn + puerarin group compared with those in the burn group (Fig. 1A-C).

CK-MB activity and serum cTnT levels were subsequently measured as indices of myocardial injury. In the burn group, serum CK-MB activity was significantly higher compared with that in the sham group (Fig. 1D). In addition, serum CK-MB activity in rats in the burn + puerarin group was significantly lower compared with that in the burn group 12 h following the burning procedure (Fig. 1D). Within the same timeframe, serum cTnT levels of rats in the burn group were significantly higher compared with that in the sham group (Fig. 1E). Rats in the burn + puerarin group exhibited significantly lower serum cTnT compared with that in the burn group (Fig. 1E).

Edema in myocardial tissue in the burn group was found to be significantly higher compared with that in the sham group (Fig. 1F), whereas that in the burn + puerarin group was significantly lower compared with that in the burn group (Fig. 1F).

Interestingly, no differences were observed in any of the aforementioned parameters between the sham and puerarin only groups, suggesting that puerarin treatment did not produce adverse effects in animals that did not receive severe burns.

Puerarin alleviates cardiac inflammation and oxidative stress caused by severe burn injury. Inflammatory cytokines have been previously demonstrated to contribute to the pathogenesis of severe burn-induced myocardial injury, which can be alleviated puerarin treatment (2,33-35). To investigate the effect of puerarin on burn-induced acute inflammation, serum levels of inflammatory cytokines IL-6, IL-1β and TNF-α were measured. Serum levels of IL-6, IL-1β and TNF-α in rats in the burn group were significantly higher compared with those in the sham group 12 h post-burn (Fig. 2A-C). Rats in the burn + puerarin group exhibited significantly lower serum levels of IL-6, IL-1β and TNF-α compared with those in the burn group (Fig. 2A-C).

MPO activity was subsequently measured to evaluate neutrophil accumulation in the heart tissues. Compared with the sham group, MPO activity in myocardial tissues from the burn group was found to be significantly higher 12 h post-burn compared with that in the Sham group (Fig. 2D). Myocardial tissues isolated from rats in the burn + puerarin group exhibited significantly lower MPO activity compared with those in the burn group (Fig. 2A-C).

Tissue MDA accumulation is considered an indicator of oxidative stress and lipid peroxidation (36). To determine the effects of puerarin on burn-induced oxidative stress, the concentration of MDA was measured in myocardial tissues isolated from rats from each treatment group. MDA levels
were found to be significantly higher in tissues isolated from rats in the burn group compared with those in the sham group 12 h after the burn procedure (Fig. 2E). By contrast, tissues from rats in the burn + puerarin group demonstrated significantly lower MDA levels compared with those in the burn group (Fig. 2E).

Puerarin attenuates severe burn-induced cardiomyocyte apoptosis. Cardiomyocyte apoptosis typically occurs within hours of burn injury, where previous studies have demonstrated that increased apoptotic rates may affect cardiac function (37). To evaluate the effect of puerarin on cardiac apoptosis, TUNEL staining was performed of myocardial tissue slices isolated from rats in each treatment group. Myocardial cell death was found to be significantly higher in the burn group compared with that in the sham group (Fig. 3A). Tissues isolated from rats in the burn + puerarin group demonstrated significantly reduced cardiomyocyte apoptosis compared with that in the burn group. Protein levels of cleaved caspase-3, a marker of apoptosis, was also revealed to be significantly increased in myocardial tissues isolated from rats in the burn group 12 h after burn injury compared with those in the Sham group (Fig. 3B). Cleaved caspase-3 levels were lower in the burn + puerarin group compared with that in the burn group (Fig. 3B).

Puerarin attenuates burn-induced MAPK and Akt activation. Although data presented in the present study provided evidence for the protective effects of puerarin against myocardial injury as a result of severe burn, the mechanism underlying the protective effects of puerarin remains poorly
characterized. Since previous studies have demonstrated that burn injury promoted apoptotic signaling through the inhibition of Akt and activation of p38-MAPK (38,39). The effects of puerarin treatment on MAPK and Akt activation following burn-induced cardiomyocyte injury was next evaluated. Phosphorylation levels of p38, ERK and JNK were found to be significantly higher in the burn group compared with those in the Sham group at 12 h post-injury, whilst the opposite was observed in terms of Akt phosphorylation (Fig. 4). p38, ERK and JNK phosphorylation were found to be significantly lower in the burn + puerarin group compared with those in the burn group; but the opposite was observed for Akt phosphorylation (Fig. 4).

Discussion

Recent clinical and animal studies have demonstrated that myocardial damage occurs in the early stages of severe burns (2,40). Myocardial injury results in cardiac dysfunction, which aggravates ischemic and hypoxic damage in other tissues and organs (3,4). Therefore, early intervention in preventing burn-induced myocardial injury has become a significant focus in previous studies. Puerarin, one of the major bioactive components found in Radix Puerariae lobatae, a traditional Chinese herb, has been historically applied therapeutically for cardiovascular disease (17,21). However, the potential therapeutic effects of puerarin on severe burn-induced myocardial damage have yet to be fully elucidated.

Although the present study and a previous study performed by Liu et al (31) investigated the therapeutic effects of puerarin on rat myocardial injury as a result of burn, differences exist. In addition to the sham, burn and burn + puerarin groups, a puerarin only group was included into the present study. The present study also included IL-1β and IL-6 measurements, which reflected myocardial inflammation. LVSP, ±dp/±dt max and water content of the heart were measured to analyze myocardial function. Finally, the protein levels of cleaved caspase-3, ERK, p-ERK, JNK, p-JNK, Akt and p-Akt, key components of the MAPK and Akt signal pathways, were analyzed further to elucidate the mechanism underlying myocardial injury formation. However, the full-thickness burn was not demonstrated in this study, which serves as a limitation of the present study.

LVSP and dp/dt max, which directly reflect cardiac function, were significantly reduced 12 h after severe burn, suggesting that rats suffered from reduced myocardial contractility, heart rate, left ventricular systolic and diastolic function. Puerarin treatment effectively reversed the aforementioned effects on cardiac function, suggesting that myocardial injury was mitigated. Serum CK-MB activity and cTnT levels, which served as indicators of myocardial damage in the present study, were found to be increased by severe burns, which was reversed by puerarin treatment. These findings suggest that puerarin alleviated acute myocardial damage induced by severe burns in rats.

MPO is a major producer of ROS which promotes endothelial dysfunction through oxidation of low-density lipoprotein (OxLDL) (41). Elevated circulating MPO levels are associated with coronary artery disease (CAD) (42). Puerarin has been previously reported to exhibit antioxidant effects, resulting in reduced neutrophil infiltration and MPO activity in the heart (31). MPO-induced OxLDL peroxidation can result in reduced nitric oxide (NO) bioavailability, thereby weakening vasodilation and promotion of a pro-inflammatory state (43). Previous studies have shown that high MPO levels are associated with increased risks of cardiovascular events (44). Additionally, elevation in MPO levels as a result of leukocyte activation was negatively correlated with the tissue availability and microvascular permeability index of constitutive nitric oxide synthase (cNOS) (45). In the present study, myocardial MPO activity was significantly increased following severe burn. This observation was associated positively with burn-induced changes in serum CK-MB activity and cTnT levels, which were reversed by puerarin treatment.

Severe burn has been previously demonstrated to substantially increase proinflammatory cytokine production (46-48). The present study showed that severe burn significantly increased levels of IL-1β, IL-6 and TNF-α in the serum. Consistent with previous studies, puerarin treatment reversed the burn-induced increases in IL-6, IL-1β and TNF-α production. TNF-α is a multifunctional cytokine in myocardial cells, which can inhibit myocardial cell contraction and inhibit cAMP signaling (49). In addition to its role in inflammation, TNF-α also serves a critical role in microvascular and cardiac damage by inducing the adherence of neutrophils to the endothelium (50). In this study, MPO activity directly reflects neutrophil sequestration in heart tissue, which was significantly increased in response to severe burn and reversed by puerarin treatment (Fig. 2F), same as in the previous study (51). These results suggested that the cardioprotective effects of puerarin following severe burns were associated with the inhibition of TNF-α production and neutrophil infiltration.

Burn injury results in the mitochondrial release of an apoptotic factors, which is associated with the pathogenesis of myocardial injury (52). Cleavage of caspase-3 is a key step in the regulation of apoptotic DNA fragmentation (53). Caspase-3 activation induces edema, which can be inhibited by puerarin (54). p38-MAPK has been previously shown to mediate oxidative stress-dependent apoptosis in neurons and cardiac cells (55,56). By contrast, the PI3K-Akt pathway is known to inhibit apoptosis in response to extracellular signals through transcriptional regulation or direct phosphorylation. Previous studies showed that burn injury promoted apoptosis by inhibiting of Akt activation while activating p38-MAPK signaling (38,39). Suppression of apoptosis through the inhibition of p38-MAPK signaling can reduce TNF-α expression and oxidative stress following burn injury in the current study. These findings suggest that puerarin can inhibit burn-induced oxidative stress, cardiac edema and apoptosis by modulating the MAPK and Akt signaling pathways.

In conclusion, puerarin was demonstrated to exert protective effects against burn-induced cardiac dysfunction by attenuating inflammation, oxidative stress and myocardial apoptosis. The protective effects of puerarin are likely to be mediated through Akt activation and concomitant p38-MAPK inhibition.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

JuL and JL made significant contributions to data acquisition, data analysis and the manuscript draft. MB and HW conducted data interpretation. JL conceived and designed the study and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Gansu Provincial Hospital (Lanzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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