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Original Article

Leonurine alleviates doxorubicin-induced myocarditis in mice via MAPK/ERK pathway inhibition

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Abstract: Objective: To investigate the effects of naturally derived leonurine (Leo) on doxorubicin (Dox)-induced myocarditis and analyze its potential mechanisms. Methods: Dox was intraperitoneally injected to establish a myocardial injury model in mice. The effect of Leo on inflammatory cytokine levels in myocardial tissue was assessed by ELISA. Pathological changes in myocardial tissue and apoptosis in myocardial cells were observed using hematoxylin-eosin (HE) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Protein levels were analyzed by Western blot (WB). Mouse myocardial H9c2 cells were divided into control group, Dox group, Leo (10 μmol/L) + Dox group, and Leo (20 μmol/L) + Dox group. Cell viability was assessed using Cell Counting Kit-8 (CCK8), and the levels of inflammatory cytokines were measured. The oxidation level and protein levels in H9c2 cells were also detected. Results: Leo significantly reduced the levels of inflammatory cytokines in both serum and cell culture supernatant. Additionally, Leo also decreased the levels of inflammatory cytokines in cardiac tissue. Moreover, Leo suppressed Dox-induced myocardial cell apoptosis by modulating the BCL2 signaling pathway. In vitro studies revealed that both inflammatory cytokines and oxidative stress markers were decreased after treatment with Leo. Conclusion: Leo exerts significant cardioprotective effects through anti-inflammatory mechanisms, likely mitigating Dox-induced myocardial inflammation by inhibiting the activation of MAPK/ERK pathways. These findings highlight Leo's potential as a promising cardioprotective agent, underscoring its therapeutic promise.

Keywords: Leonurine, doxorubicin, myocarditis, apoptosis, MAPK/ERK

Introduction

Myocarditis is an inflammatory heart disease triggered by pathogenic infections, including bacteria, viruses, and chlamydia, as well as toxins and allergic reactions [1, 2]. Clinical manifestations of myocarditis vary, with mild cases often showing no obvious symptoms, while severe cases can lead to significant heart failure, marked by myocardial cell edema, degeneration, and necrosis [3]. Current research suggests that oxidative stress-induced inflammatory myocardial cell necrosis and apoptosis may be a common pathway leading to myocardial cell damage, myocardial remodeling, and ultimately resulting in reduced cardiac function and heart failure [4]. Extensive epidemiological investigations indicate that drug-induced cardiotoxicity is one of the primary triggers of myocarditis. This occurs when drugs, either directly or indirectly, cause myocardial damage [5, 6].

Doxorubicin (Dox) (**Figure 1**), a secondary metabolite derived from Streptomyces peucetius var. caesius, is a highly effective broad-spectrum anti-tumor drug commonly used to treat various tumors in both adults and children [7]. However, as an anthracycline drug, Dox exhibits dose-dependent cardiotoxicity that can lead to drug-induced cardiomyopathy, dilated cardiomyopathy, and heart failure [8]. This significantly limits its widespread clinical application [9]. Wang et al. injected mice with Dox and observed typical signs of heart failure [10], and similar myocardial injury models have been successfully established by researchers both domestically and abroad.

For various types of myocardial diseases, implementing effective measures to protect myocardial cells and reverse myocardial remodeling is crucial for treating myocardial damage [11]. However, current treatment methods such as

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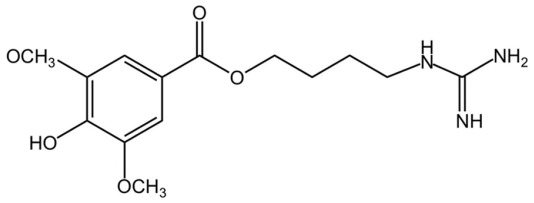

Figure 1. The chemical structure of Leonurine (Leo).

triple therapy, immunomodulation, and cell transplantation, while showing some effectiveness, are insufficient to alleviate myocarditis caused by drug toxicity during the treatment [12, 13]. Therefore, further exploration of optimal and safe immunomodulatory drugs for treating myocarditis is warranted.

Leonurine (Leo), a single active compound in traditional Chinese medicine, exhibits various biological activities such as immunomodulation, antioxidation, anti-inflammation, and cardiac protection [14]. These properties have attracted significant attention to Leo in the field of traditional Chinese medicine, leading to extensive research and application. Traditional medicine has confirmed that Leo can treat cardiovascular diseases by combating atherosclerosis, reducing myocardial infarction, and providing cardiac protection. However, these beneficial effects are primarily attributed to its anti-inflammatory and antioxidative properties [15, 16]. A study indicates that Leo significantly reduces serum TNF-α and IL-6 in mice [17], suggesting that Leo may regulate its immune response by activating the P38/NF-KB and JNK/MAPK signaling pathways [18]. Recent studies have demonstrated that Leo can alleviate cardiac damage and dysfunction induced by angiotensin II through the NF-kB and MAPK pathways [19]. This inhibition subsequently suppresses myocardial remodeling in rats with heart failure, improving cardiac function. Moreover, no significant adverse reactions of Leo were observed in toxicity experiments conducted on animals [20]. However, whether Leo is beneficial in Dox-induced myocardial damage remains unclear.

Some studies have found that properly inhibiting the MAPK signaling pathway helps suppress the development of inflammation and alleviate the progression of myocarditis [21]. MAPK is an important signal transduction system present in most eukaryotic cells, serving as a vital mechanism for transducing extracellular signals into cellular responses [22]. Many studies have indicated a close correlation between MAPK signaling pathways and inflammation, as well as the production of inflammatory cytokines. Among these pathways, ERK is one of the earliest discovered, and activated by various growth factors, inflammation, and stress, contributing to the occurrence and development of various diseases. Research on the relationship between ERK and cardiovascular system diseases has shown that ERK activation is associated with myocardial damage, heart failure, and myocardial cell apoptosis induced by drugs or various factors [19].

Therefore, we aimed to investigate the preventive and protective effects of Leo on Dox-induced myocarditis and explore its underlying mechanisms. Firstly, we induced myocarditis in mice using Dox and simultaneously administered Leo treatment. Indicators associated with myocardial inflammation and cell apoptosis were assessed to preliminarily evaluate the therapeutic effect of Leo on myocarditis. Additionally, H9c2 cells were pre-treated with Dox *in vitro* to simulate a myocarditis model, and cell viability as well as the protein expression levels of relevant pathways were assessed.

Materials and methods

Experimental reagents

Dox (CAS: 2869-27-4) and Leo (CAS: 24697-74-3) were both sourced from Sigma-Aldrich (St. Louis, MO, US). Trizol reagent and Bouin's tissue fixative were obtained from Shanghai United Biological Co., Ltd. (Shanghai, China). Assay kits for CK-MB, LDH, cTnT, IL-1β, IL-6, and TNF-α were purchased from Beijing Solaibao Biotechnology Co., Ltd. (Beijing, China). Hematoxylin-eosin (HE) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining kits were obtained from Shanghai Fusheng Industrial Co., Ltd. (Shanghai, China). Penicillin, streptomycin, fetal bovine serum, DMEM culture medium, and Cell Counting Kit-8 (CCK8) were all purchased from Beijing Solaibao Technology Co., Ltd. (Beijing, China).

Experimental animals and grouping

A total of 32 SPF C57BL/6J male mice were provided by Zhejiang Weitonglihua Experimental Animal Co., Ltd. (Zhejiang, China). The mice were housed in a controlled environment with a

temperature of 23 ± 2°C, humidity between 45-55%, and a 12-hour light-dark cycle. After one week of acclimatization, the mice were divided into four groups: control group, Dox group, Dox + low-dose Leo group (10 mg/kg/day), and Dox + high-dose Leo group (20 mg/kg/day). Except for the control group, all groups were administered Dox (3 mg/kg) via intraperitoneal injection to establish the myocardial injury model. The treatment groups were concurrently administered low and high doses of Leo via gavage once daily for 21 consecutive days. The control group received an equivalent volume of physiological saline via intraperitoneal injection. The dosage and administration route of Leo were determined based on a previous study [23]. After the final administration, mice were anesthetized using pentobarbital sodium (0.1 mL/10 g), and blood was collected from the eyeballs. Serum was separated by centrifugation and stored at -80°C. Subsequently, mouse heart tissues were harvested, with a portion fixed in 4% paraformaldehyde for HE and TUNEL staining experiments. All animal experiments were conducted in accordance with ethical guidelines and approved by the Animal Experimentation Committee of the Wenzhou Research Institute of UCAS (WIUCAS24092601).

Measurement of serum CK-MB, LDH, and cTnT levels

Cardiac damage caused by myocarditis was assessed by measuring cardiac injury markers in the serum [24]. Previously frozen serum samples were retrieved for analysis. The levels of CK-MB, LDH, and cTnT in the serum were measured using the corresponding assay kits according to the manufacturer's instructions.

Measurement of inflammatory cytokines related to myocardial injury

After centrifugation of the myocardial tissue homogenate, the supernatant was collected as described earlier [25]. In short, standard samples and serum samples were incubated in a 96-well plate with antibodies against luteinizing hormone or follicle-stimulating hormone. After washing, peroxidase-conjugated avidin was added, followed by washing to remove unbound components. Then, chromogenic substrate (TMB) and stop solution were added. Within 15 min, the OD values of each well were measured using an ELISA reader. The IL-1β, IL-6, and TNF-α contents were calculated based on the standard curve.

Preparation of cardiac tissue sections

The myocardial tissues fixed in 4% paraformaldehyde were removed and placed in a tissue embedding box, dehydrated in alcohol, transparented in xylene, and soaked in soft and hard wax. Once completely dehydrated, the myocardial tissue was embedded in paraffin wax. Tissue slices of 2-3 μm were prepared for HE staining and TUNEL assay.

HE staining

The prepared paraffin sections were placed in a 60°C oven for 2 h. The sections were immersed in xylene, 100% ethanol, 95% ethanol, 85% ethanol, and 75% ethanol. Then, after washing three times with distilled water, the sections were stained with hematoxylin, differentiated with 1% hydrochloric ethanol, rinsed with running water, and stained with eosin. Finally, the sections were dehydrated, made transparent, and mounted with neutral gum. HE staining was used to observe the infiltration of inflammatory cells in cardiac tissue under microscope.

TUNEL staining

TUNEL assay kit was used to stain the heart tissue sections and observe apoptosis in mouse myocardial cells [26]. In short, after routine dewaxing and PBS washing, the slices were sequentially treated with proteinase K solution followed by 3% hydrogen peroxide (H_2O_2) solution. Let the reaction stand for 5 min, then wash with PBS. Next, TUNEL reaction solution (at 37°C for 60 min), reaction termination solution (at room temperature for 20 min), horseradish peroxidase (HRP)-conjugated anti-Streptavidin antibody, and DAB chromogenic solution were sequentially added. Finally, after washing, dehydration, and transparency, the samples were sealed with 50% glycerol. Apoptosis of myocardial cells was observed under an optical microscope. 5 fields of view were randomly selected to calculate the apoptosis rate of myocardial cells.

H9c2 myocardial cell culture and grouping

H9c2 cardiomyocytes were routinely resuscitated and cultured in medium containing

DMEM/F12, 1% penicillin-streptomycin, and 10% fetal bovine serum in a cell incubator at 37°C. The establishment of a Dox-induced H9c2 cell model refered to a previous study [27]. Logarithmic growth phase H9c2 myocardial cells were selected and grouped as following: control group, Dox group, Leo (10 μmol/L) + Dox group, and Leo (20 μmol/L) + Dox group. In the control group, cells received no special treatment. In the Dox group, cells were intervened with 300 μmol/L of Dox.

CCK8

CCK8 assay kit was used to detect the cell viability of each group. In brief, untreated cell suspensions were collected and seeded at a density of 4000 cells per well in a 96-well plate. After 24 h of incubation, H9c2 myocardial cells were treated with Leo and incubated for 24 h. Finally, CCK8 solution was added to each well, and the OD value at 450 nm was measured.

Measurement of intracellular MDA, SOD, CAT, and GSH levels

The H9c2 cardiomyocytes were divided into experimental groups and treated accordingly. Cells from each group were collected and lysed. After extracting intracellular proteins using cell lysis buffer, the MDA, SOD, CAT, and GSH levels were measured using biochemical kits according to the manufacturer's instructions. Quantitative analysis was performed using the standard curve method.

Western blot

WB was performed as described previously [28]. Both heart tissue and cell samples were fully homogenized with RIPA lysis buffer, completely triturated, and allowed to stand on ice for 30 min for lysis, followed by centrifugation at -4°C for 15 min. Protein concentration was determined using the bicinchoninic acid (BCA) assay kit, followed by quantification using loading buffer. The proteins were then boiled at 100°C for 10 min for denature. Equal amounts of protein were separated by 8-12% SDS-PAGE gel electrophoresis, and then transferred to a PVDF membrane. The membrane was blocked with 5% skim milk and incubated with corresponding primary antibodies (BAX, BCl2, CASP3, CASP8, p38, p-p38, ERK, p-ERK, JNK, p-JNK, GAPDH, 1:1000) overnight at 4°C. The next day, the membrane was incubated with appropriately diluted secondary antibody at room temperature for 1 hour. Finally, a chromogenic agent was added, and the gel was exposed using an imaging system. Quantitative analysis was performed using Image J software (NIH, MD, USA). The relative level of target protein expression was calculated by determining the ratio of the intensity values between the target band and GAPDH.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7.0 software (GraphPad Software Inc., USA). All experimental data were presented as Mean ± SEM. Differences among groups were initially compared using one-way analysis of variance (ANOVA) or repeated measures ANOVA, followed by Dunnett's multiple comparison test. Significance levels were denoted as $P < 0.05$.

Results

Mouse general condition

Dox-induced myocarditis mice were treated with Leo (10 mg/kg/d and 20 mg/kg/d) to evaluate its effects on Dox-induced myocardial tissue damage. Upon various interventions, mice in the control group exhibited good mental activity, normal eating habits, and steady weight gain without any deaths. Mice in the Dox group showed a decrease in body weight after the 6th day, reduced activity, and occasionally exhibited symptoms of diarrhea and abdominal distention. Upon dissection, a small amount of fluid accumulation was observed in the abdominal cavity. The mental status of mice in two Leo treatment groups was better than that of the Dox group. After the experiment, it was found that the body weight, heart weight, and relative heart weight of mice from both the Dox group and the Dox + low Leo (10 mg/kg) treatment group were significantly decreased (**Figure 2**).

Leo alleviated Dox-induced pathological changes in mouse myocardial tissue

Under light microscopy (**Figure 3A**), the myocardial tissue structure of the control group exhibited clear hierarchical organization, normal intercellular spaces, no edema, no myocardial fiber damage, and intact membranes. In

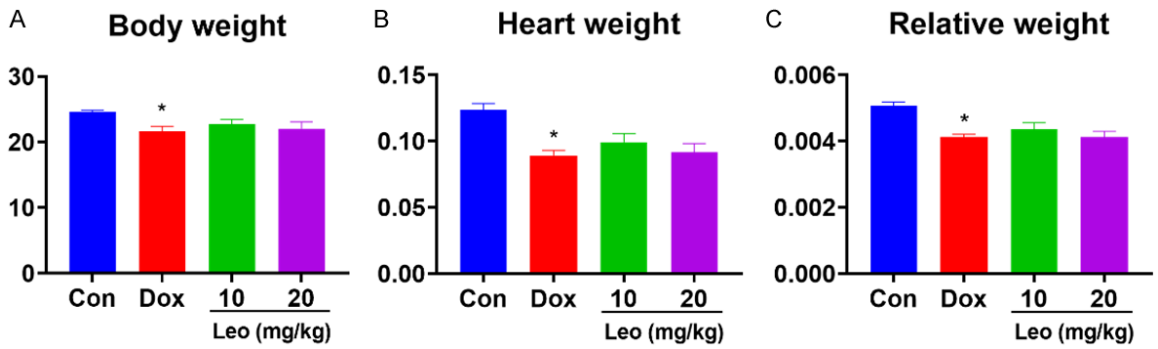

Figure 2. Effect of Leo on body weight and cardiac index in mice. A. Body weight (BW); B. Heart weight (HW); C. Changes in HW/BW. Mean ± SEM, n = 8. *$P < 0.05$ indicates significant differences vs the control. Dox: Doxorubicin; Leo: Leonurine.

the Dox group, myocardial cells showed large vacuoles and fiber rupture. In the low-dose Leo group, there were some fiber ruptures. However, in the high-dose Leo group, myocardial cells did not exhibit obvious pathological changes.

Leo reduced the levels of myocardial injury markers in mouse serum

As shown in **Figure 3B-D**, compared to the control group, the Dox group showed a 1.2-fold increase in serum CK-MB, LDH, and cTnT levels. In comparison to the Dox group, the levels of LDH decreased by 0.85-fold and 0.68-fold in the Leo groups (10 mg/kg, 20 mg/kg), while CK-MB and cTnT levels decreased by 0.34-fold and 0.26-fold respectively. These results indicate that Leo has a protective effect on the cardiac tissue of Dox-induced myocarditis mice [29].

Leo reduced myocardial tissue inflammation factor levels

As shown in **Figure 3E-G**, compared to the control group, the levels of inflammation factors (IL-1β, IL-6, and TNF-α) in Dox group were significantly increased. However, the content of inflammatory cytokines in myocardial tissue of Leo groups (10 mg/kg, 20 mg/kg) was significantly decreased compared with that of the Dox group. These findings indicate that Leo can decrease the levels of inflammatory cytokines in myocardial tissue.

Leo inhibited Dox-induced myocardial cell apoptosis by modulating the BCL2 signaling pathway

To further investigate whether Leo ameliorates apoptosis in myocardial cells, TUNEL staining was performed, and the results showed that myocardial cells in the control group remained in a normal state, whereas the number of apoptotic cells significantly increased in the Dox group. However, in the Leo-treated groups, myocardial cell apoptosis was significantly reduced (**Figure 4**). Next, WB was conducted to examine the expression of the BCL2 family-related proteins. The analysis results indicated that compared to the control group, the protein expression levels of BAX, CASP3, and CASP8 in the Dox group were significantly increased, while the expression level of BCL2 was decreased (**Figure 5**). Compared to the Dox group, the expression levels of BAX, CASP3, and CASP8 significantly decreased, while the expression level of BCL2 increased. These findings suggest that Leo can alleviate Dox-induced myocardial cell apoptosis, at least in part, through modulation of the BCL2 signaling pathway.

Leo increased cell viability

To further demonstrate the effect of Leo on myocarditis, in vitro experiments were conducted using the mouse myocardial cell line H9c2. Viability of H9c2 cells incubated with different doses of Leo was detected (**Figure 6A**, **6B**). The results showed that the cell viability in H9c2 cells treated with Dox was only 58% (**Figure 6B**). However, the cell viability in the Leo groups increased incrementally with increased drug concentration, without any adverse effects on cell activity. Furthermore, Leo neutralized DOX-induced changes in MDA, SOD, CAT, and GSH levels (**Figure 6C-F**). ELISA results showed that the levels of IL-1β, IL-6, and TNF-α in the cell supernatant of the Dox group were significantly increased, while the levels of inflammatory

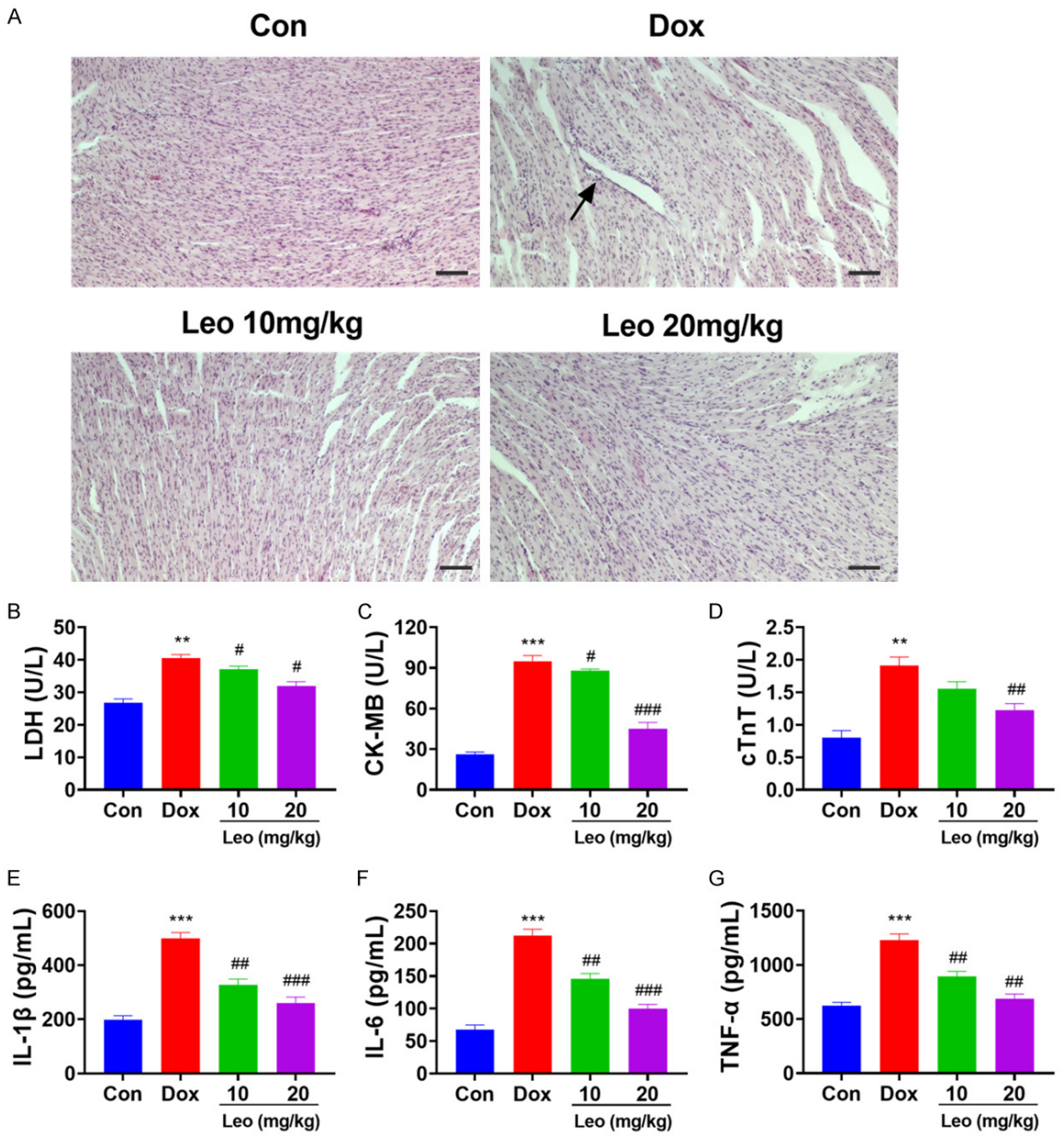

Figure 3. Effects of Leo on myocardial tissue and inflammatory cytokine levels in mice. A. HE staining of myocardial tissue. Scale bar, 100 μm, n = 8 (random samples); B. LDH levels; C. CK-MB levels; D. cTnT levels; E. IL-1β levels; F. IL-6 levels; G. TNF-α levels. Black arrows indicate inflammation cell infiltration and myocardial injury. Mean ± SEM, n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the Dox group. Dox: Doxorubicin; Leo: Leonurine.

cytokines in the Leo group were significantly decreased (**Figure 6G-I**).

Leo inhibited inflammation by inhibiting the MAPK/ERK signaling pathway

Previous research has shown that TNF-α can trigger various intracellular signaling pathways, thereby activating the MAPK pathway [19]. Another study indicated that [23] Leo improved post-myocardial infarction function by inhibiting the activation of ERK in cardiac fibroblasts induced by angiotensin-converting enzyme II. In our study, WB analysis revealed that the protein levels of p38, ERK, and JNK in the MAPK pathway increased in the Dox group. However, the MAPK/ERK pathway was significantly inhibited in the Leo groups, and the phosphorylation of p38, ERK, and JNK was alleviated (**Figure 7**). These results suggest that Leo can

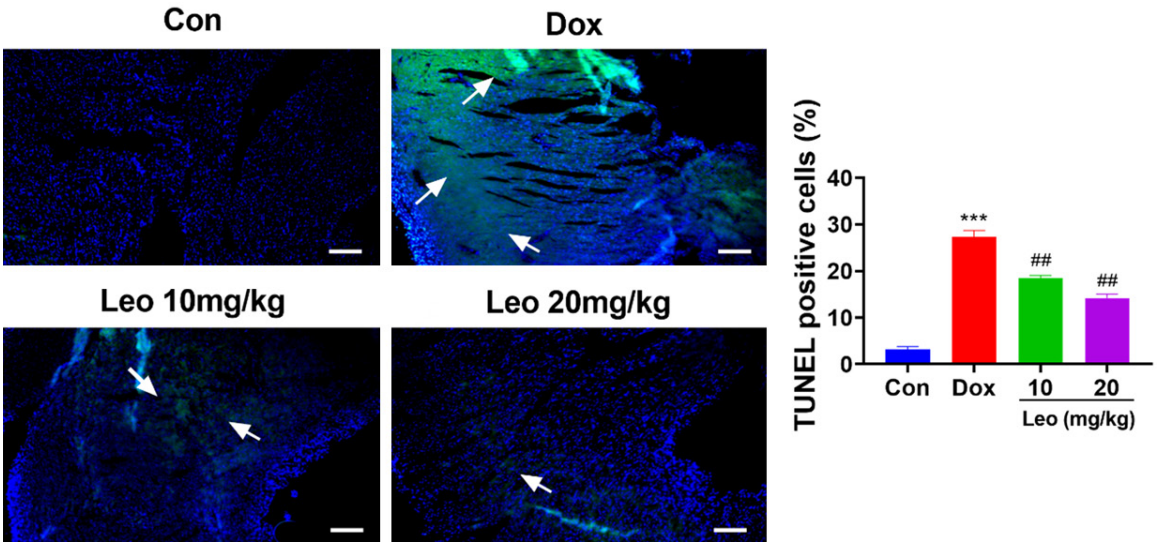

Figure 4. Effects of Leo on myocardial cell apoptosis in mice. TUNEL staining images of myocardial tissue (white arrows represent myocardial cell apoptosis, original magnification ×400). Bar = 10 μm. ***P < 0.001 vs the control. ##P < 0.01 vs the Dox group. Dox: Doxorubicin; Leo: Leonurine.

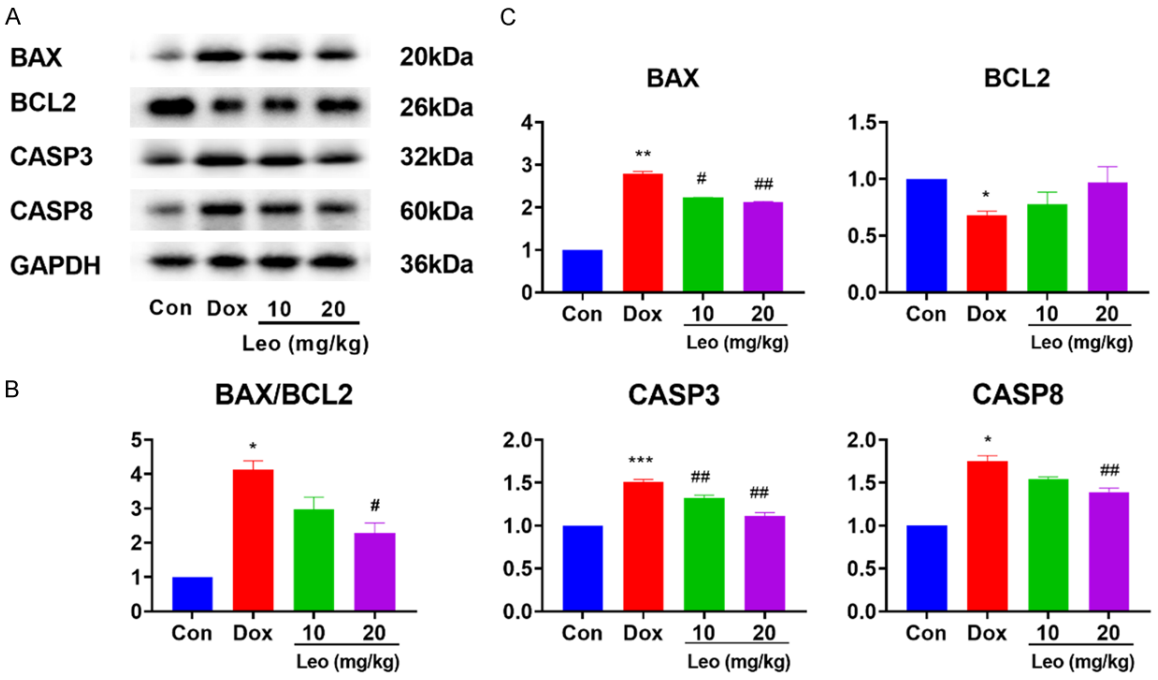

Figure 5. Effects of Leo on BCL2 pathway. A. WB images; B. BAX and BCL2 ratios; C. Quantitative analysis of BAX, BCL2, CASP3, CASP8 proteins in each group. Mean ± SEM, n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group. #P < 0.05, ##P < 0.01 vs the Dox group. Dox: Doxorubicin; Leo: Leonurine.

suppress inflammation by modulating the MAPK/ERK signaling pathway.

Discussion

Anthracycline drugs are a major cause of chemotherapy-induced cardiotoxicity. Dox, widely used as an anthracycline drug, exhibits potent anticancer effects and plays an irreplaceable role in the treatment of various cancers [30]. However, Dox-induced cardiotoxicity can damage myocardial cells, leading to pathological cardiomyopathy, which significantly limits its clinical use [31, 32]. Currently, there are no

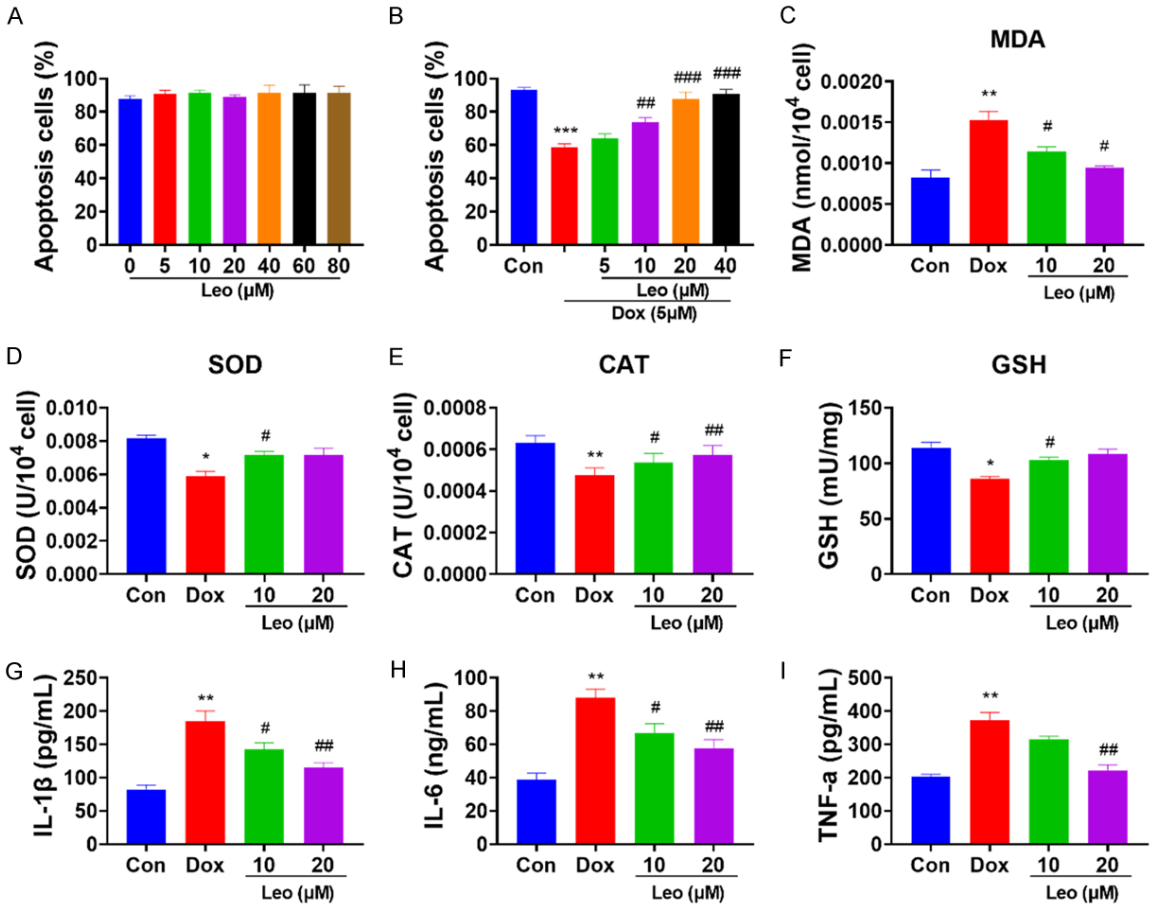

Figure 6. Leo enhanced the viability of Dox-induced H9c2 cells and suppressed inflammatory response. A. Effect of Leo treatment on the viability of normal H9c2 cells after 24 h; B. Effect of Leo on survival rate of Dox-induced H9c2 cells; C. MDA levels; D. SOD levels; E. CAT levels; F. GSH levels; G. IL-1β levels; H. IL-6 levels; I. TNF-α levels. Mean ± SEM, n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the Dox group. Dox: Doxorubicin; Leo: Leonurine.

effective strategies for preventing and treating Dox-induced cardiotoxicity, making the search for safe and effective preventive and therapeutic medications extremely urgent.

Recent research has highlighted that oxidative stress and cell apoptosis are important in the development of Dox-induced cardiotoxicity, providing new directions for its prevention and treatment. Gao et al. found that adding Leo can effectively enhance the viability of hypoxia-induced H9c2 cells, thereby enhancing the heart's antioxidant capacity and offering protection [33]. Several studies currently suggest that Leo enhances the activity of endogenous antioxidant enzymes and inhibits myocardial inflammatory factor expression, contributing to vasodilation and vasoconstriction effects [34, 35]. However, its protective effect against Dox-induced myocarditis remains unclear.

To investigate whether Leo can improve myocardial injury, we successfully established an experimental model of dox-induced myocarditis. In this experiment, apart from the control group, all groups were treated with Dox, while the treatment groups were given different doses of Leo. The results showed that the mice in the Dox group showed weight loss, gray hair, and significantly decreased activity. Although mice in the treatment group experienced weight loss, their general condition was better compared to the Dox group.

CTnT is a myocardial-specific regulatory protein, and its levels significantly increased when myocardial cells were damaged, demonstrating high specificity for myocardial injury [36]. Therefore, we assessed cardiac injury markers in serum and levels of inflammatory cytokines in cardiac tissue. We found that the levels of

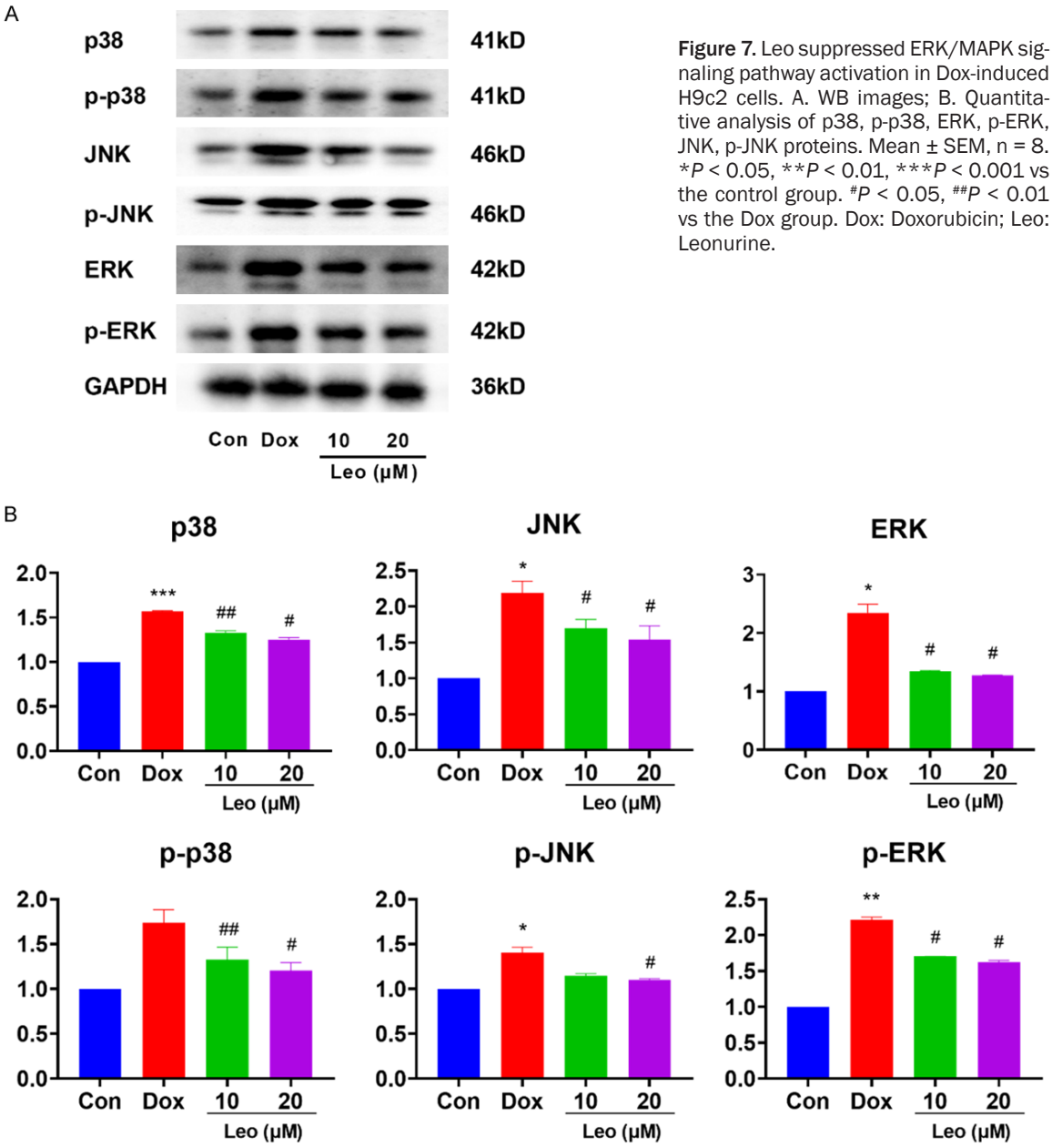

Figure 7. Leo suppressed ERK/MAPK signaling pathway activation in Dox-induced H9c2 cells. A. WB images; B. Quantitative analysis of p38, p-p38, ERK, p-ERK, JNK, p-JNK proteins. Mean ± SEM, n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the Dox group. Dox: Doxorubicin; Leo: Leonurine.

these markers in the Dox group were significantly increased, indicating myocardial cell damage and inflammatory responses. Interestingly, the Leo treatment group exhibited relatively lower levels of cardiac injury markers and inflammatory cytokines, suggesting alleviated myocardial cell damage.

Cell death resulting from the activation of pre-existing intracellular death programs by external factors is termed apoptosis [37]. In drug-induced cardiomyopathy caused by Dox, apoptosis of myocardial cells plays a crucial role. Related studies have shown that myocardial cells exposed to low concentrations of Dox undergo apoptosis. While some researchers have found that Leo can inhibit apoptosis caused by viral myocarditis, there are no reports regarding whether Leo has an inhibitory effect on myocardial cell apoptosis in Dox-induced myocardial injury [38]. To further investigate its effects, we examined myocardial cell apoptosis using TUNEL staining and found a significant increase in the apoptosis rate in the Dox group. However, after treatment with Leo, the apoptosis rate of myocardial cells markedly

decreased. WB analysis revealed that compared to the Dox group, the Leo group exhibited a significant decrease in the protein levels of BAX, CASP3, and CASP8, while the expression level of BCL2 protein significantly increased. Through HE staining, we observed that in the Dox group, myocardial cells exhibited disorganized arrangement, with loose and disordered myocardial fibers and increased intercellular space. However, after treatment with Leo, myocardial cell arrangement became more orderly, staining became uniform, and cell boundaries were clear. From this, it can be inferred that Leo may improve myocardial cell damage and provide a certain preventive and protective effect on Dox-induced myocardial injury.

To explore the potential mechanism of Leo, we successfully induced an inflammatory state in H9c2 cells by treating them with Dox. It was observed that after Dox treatment, myocardial cells exhibited decreased cell viability, increased levels of oxidative stress marker MDA, decreased levels of antioxidant enzymes SOD and GSH, and elevated levels of inflammatory cytokines in the cell suspension. However, after treatment with Leo, both inflammatory cytokines and oxidative stress markers showed a decrease. These results suggest that Leo can alleviate inflammatory damage to myocardial cells to some extent.

The MAPK signaling pathway is primarily involved in cell growth, differentiation, and development [39]. ERK, a key member of the mitogen-activated protein kinases (MAPKs) family, mediates pathways through phosphorylation cascade reactions and plays an indispensable role in regulating various biological activities [40]. The MAPK signaling transduction pathway is associated with myocardial cell apoptosis. External stimuli binding to the cell membrane surface transmit signals into the cell membrane, triggering MAPK cascade reactions [21]. Currently, the ERK signaling pathway, one of the most studied MAPK-related pathways, participates in the regulation of various biological processes, including the survival, growth, and death of various cells, as well as inflammation-related immune responses [41]. In this study, we observed that in Dox-induced *in vitro* myocardial cell injury, compared to the Dox group, the expression levels of key proteins such as p38, p-p38, ERK, p-ERK, JNK, and p-JNK in the ERK/MAPK signaling pathway were significantly reduced in the Leo group. This indicates that Leo may protect cardiac function by inhibiting the ERK/MAPK signaling pathway. Additionally, research has reported that downstream molecule ERK in the ERK/MAPK signaling pathway can, under stimulation, transmit upstream cascade signals to the cell nucleus, ultimately regulating the expression of various apoptosis-related genes (BCL2, BAX, CASP3, CASP8) in the nucleus [42, 43].

In summary, Leo can reduce the apoptosis rate of myocardial cells induced by Dox, decrease the area of myocardial cell damage, alleviate myocardial tissue injury, and thereby exert a cardioprotective effect. Its protective mechanism may be related to the inhibition of ERK/MAPK signaling pathway. This study provides a comprehensive solution for the treatment of Dox-induced myocarditis. However, there are still some notable limitations. The relatively small sample size may affect the statistical power and generalizability of the findings. Additionally, since the research was conducted solely in a mouse model, the direct applicability of these results to human patients may be limited. Future studies with larger, diverse populations and clinical trials will be essential to validate these findings and explore their relevance in a clinical context.

Conclusions

This study found that Leo significantly improved Dox-induced myocardial injury in mice, reduced inflammation occurrence, and inhibited the activation of CASP3. Additionally, Leo was able to regulate the BCL2 signaling pathway to inhibit Dox-induced myocardial cell apoptosis. Interestingly, in vitro cell studies showed that Leo could significantly suppress Dox-induced apoptosis of H9c2 cells in a concentration-dependent manner. The mechanism of action is primarily achieved through inhibiting the activation of MAPK/ERK signal pathway.

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Disclosure of conflict of interest

None.

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