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Original Article

Sini decoction alleviates inflammation injury after myocardial infarction through regulating arachidonic acid metabolism

Cuiping Long [a,b,1], Qian Zhou [c,1], Min Xu [b,d], Xin Ding [b], Xingxing Zhang [a,b], Ya Zhang [b], Yuping Tang [a,*], Guangguo Tan [b,*]

[a] School of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang 712046, China
[b] Department of Pharmacy, Air Force Medical University, Xi'an 710032, China
[c] Department of Traditional Chinese Medicine, Xijing Hospital, Air Force Medical University, Xi'an 710032, China
[d] The Third Stationed Outpatient Department, General Hospital of Central Theater Command of PLA, Wuhan 430070, China

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ABSTRACT

Objective: Myocardial inflammation during myocardial infarction (MI) could be inhibited by regulating arachidonic acid (AA) metabolism. Recent studies demonstrated that Sini Decoction (SND) was identified to be an effective prescription for treating heart failure (HF) caused by MI. But the anti-inflammatory mechanism of SND remained unclear. The work was designed to investigate the anti-inflammatory mechanism of SND through the AA metabolism pathway *in vitro* and *in vivo* experiments.

Methods: An inflammatory injury model of H9c2 cells was established by lipopolysaccharide (LPS)-stimulated macrophage-conditioned medium (CM). The MI model was built by the ligation of left anterior descending (LAD) branch of coronary artery in rat. Meanwhile, the rats were divided into five groups: sham group, MI group, MI + Celecoxib group, MI + low-dose SND group (SND-L) and MI + high-dose SND group (SND-H). Cardiac function, histopathological changes and serum cytokines were examined four weeks later. Western blot analysis was conducted to verify the key enzymes levels in the AA metabolic pathway, including phospholipase A2 (PLA2), cyclooxygenases (COXs) and lipoxygenases (LOXs).

Results: These *in vivo* results demonstrated that SND could improve the cardiac function and pathological changes of rats with MI, and regulate the key inflammatory molecules in the AA metabolism pathway, including sPLA2, COX-1, COX-2, 5-LOX and 15-LOX. *In vitro*, SND could decrease the release of pro-inflammatory cytokines including TNF-α and IL-6 and inhibit cell apoptosis in CM-induced H9c2 cells. Moreover, SND could protect H9c2 cells from the damage of CM by regulating nuclear factor kappa-B (NF-κB) signal pathway and the expression of COX-2.

Conclusion: SND may be a drug candidate for anti-inflammatory treatment during MI by regulating the multiple targets in the AA metabolism pathway.

1. Introduction

Myocardial infarction (MI), mainly resulting from myocardial damage relied on blood flow obstruction and myocardial ischemia, is one of the leading causes of cardiovascular death and morbidity, which ultimately evolves into heart failure (HF) (Bahit, Kochar, & Granger, 2018; Benjamin et al., 2019; Ho & Wang, 2021; Asiwe et al., 2024). Despite the therapeutic approaches of MI have made a great progress, there is increasing evidence that the risk of cardiovascular death in patients with MI remains unacceptably high (Nägele & Flammer, 2022; Weir & McMurray, 2006). Current treatment regimens still lack one or more key pathological mechanisms. Therefore, it is urgently needed to develop an alternative and complementary treatment for MI.

The intense inflammatory reaction is triggered by MI, which results in the remodeling of the left heart chamber and myocardial injury (Mezzaroma et al., 2011). At this stage, the arachidonic acid (AA) metabolism pathway is related to the inflammatory response post-MI (Sonnweber, Pizzini, Nairz, & Weiss, 2018). The AA metabolism is involved in three metabolic pathways, including the cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 monooxygenases (CYPs) pathways (Levick,

* Corresponding authors.
E-mail addresses: yupingtang@sntcm.edu.cn (Y. Tang), guangguotan@gmail.com (G. Tan).
[1] These authors contributed equally to this work.

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Loch, Taylor, & Janicki, 2007). The key metabolic enzymes and the eicosanoid metabolites are of significance in the adjustment of myocardial inflammation (Jenkins, Cedars, & Gross, 2009). Accordingly, the critical molecules in the AA metabolism pathway may represent potential targets to develop novel drugs for the treatment of MI.

Up to now, there are no approved anti-inflammatory agents to prevent HF after MI. Sini Decoction (SND), a representative traditional Chinese medicine (TCM) prescription, consisting of *Aconiti Lateralis Radix Praeparat* (Fuzi in Chinese, the lateral roots of *Aconitum carmichaelii* Debeaux), *Zingiberis Rhizoma* (Ganjiang in Chinese, the rhizome of *Zingiber officinale* Roscoe), and *Glycyrrhizae Radix* et *Rhizoma* (Gancao in Chinese, the radix and rhizoma of *Glycyrrhiza uralensis* Fisch.) (Zhou, Meng, Wang, Dong, & Tan, 2019; Liu et al., 2023). SND has been used over the last few centuries to treat coronary heart disease, and the therapeutic effect has been widely recognized in laboratory and clinical studies (Zhu et al., 2018; Zhang, Chen, Wan, & Liu, 2023). Based on the holistic and synergistic theory, *Aconiti Lateralis Radix Praeparata* and *Aconiti Lateralis Radix Praeparata*-based formulas were prescribed to improve circulation in patients with cold extremities, weak pulse and general weakness (Singhuber, Zhu, Prinz, & Kopp, 2009; Wang et al., 2023). *Zingiberis Rhizoma* is a beneficial traditional Chinese medicinal herb for the management of cardiovascular diseases because of its anti-inflammatory and antioxidative stress activities (Roudsari et al., 2021). *Glycyrrhizae Radix* et *Rhizoma* is the most frequently used TCM owing to its various pharmacological effects. Moreover, *Glycyrrhizae Radix* et *Rhizoma* can enhance the efficacy and reduce the toxicity of other TCMs (Jiang et al., 2020). Our and other research groups previously analyzed the chemical and metabolic profiling of SND and its therapeutic effect and compatibility mechanism on MI, demonstrating that SND could inhibit the inflammatory response to MI and improve post-MI cardiac remodeling (Liu et al., 2014; Tan et al., 2014; Tan et al., 2018; Tan et al., 2011; Zhou et al., 2020). Nevertheless, the underlying mechanism by which SND ameliorates inflammation in myocardial tissue remains unclear.

In this study, we hypothesized that the anti-inflammatory effect of SND was promoted through regulating the AA metabolism. Therefore, we performed *in vitro* and *in vivo* experiments to verify our hypothesis. Firstly, an H9c2 cell injury model was induced following treatment with the conditioned-medium (CM) from lipopolysaccharide (LPS)-stimulated macrophages, which was established to investigate the protective effects of SND on the inflammatory injury of H9c2 cells. Moreover, the anti-inflammatory mechanism of SND was further explored in rat model of MI produced by the left coronary artery ligation. Our extensive *in vitro* and *in vivo* experiments focused on the regulation of the AA metabolic pathway will help in deciphering the potential targets of SND in inflammatory pathways and providing a new therapeutic direction for MI.

2. Materials and methods

2.1. Animals

Fifty adult male Sprague-Dawley (SD) rats [(200 ± 10) g] were obtained from the Animal Laboratory Center of Air Force Medical University (Xi'an, China). We made the rats live in a room temperature with 12 h light/dark cycle and (55 ± 10)% humidity. All animal experimental procedures were carried out in accordance with Administrative Committee on the Care and Use of Laboratory Animals in Air Force Medical University (Licence No. KY20194116).

2.2. Establishment of MI model in rats and grouping

The rat model of MI was established by permanent ligature of the left anterior descending (LAD) coronary artery according to our previous work with minor modifications (Tan et al., 2012). In short, the rats were anesthetized with 10% chloral hydrate (350 mg/kg) and connected with a small animal ventilator and electrocardiograph, in which electrocardiogram was used to assess whether the operation was successful. And then, the rats were performed to make blunt cardiac thoracotomy between the third and fourth ribs of the left chest. The LAD of rat was ligated with 5-0 surgical suture with needle skillfully about 2–3 mm below the left auricle. Next, the chest was compressed for exhaust and the 3-0 surgical suture line was used for suturing the skin incision. Accordingly, the sham operation group was only threaded without ligation and other procedures were the same as the MI group. Six rats were died within 24 h in the MI group, whereas all rats did well in the sham-operated group. Finally, all rats were divided into five groups at random: the sham group (n = 7), the MI group (n = 11), the Celecoxib group (30 mg/kg, n = 8), the MI + low-dose SND (1 g/mL) group (SND-L, n = 9) and the MI + high-dose SND (3 g/mL) group (SND-H, n = 9). The dosage was set based on the previous study with minor modification (Tan et al., 2011; Zhou et al., 2017).

2.3. Reagents and materials

Three component herbs of SND, *Aconiti Lateralis Radix Praeparata* (Sichuan, China), *Zingiberis Rhizoma* (Sichuan, China) and *Glycyrrhizae Radix* et *Rhizoma* (Gansu, China) were bought from Beijing Tongrentang (Group) Co., Ltd. (Xi'an, China). The freeze-dried powder of SND for the cell experiments was made by our research group in the early stage. The voucher specimen was deposited in Department of Pharmacy of Air Force Medical University under accession No. LCP001. Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Celecoxib was provided by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Isoflurane was provided by Hebei Yipin Pharmaceutical Co., Ltd. (Shijiazhuang, China). Cell counting kit-8 (CCK-8) was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Nitric oxide (NO) assay kit and bicinchoninic acid (BCA) assay kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from Applygen Technologies Co., Ltd. (Beijing, China).

The primary antibodies including rabbit monoclonal antibody to 5 Lipoxygenase (ab169755, Abcam, Boston, MA, USA), rabbit monoclonal antibody to 15 Lipoxygenase (ab244205, Abcam, Boston, MA, USA), rabbit monoclonal antibody to NF-kB p65 (ab32536, Abcam, Boston, MA, USA), rabbit monoclonal antibody to COX-1 (ab109025, Abcam, Boston, MA, USA), rabbit monoclonal antibody to PLA2G2A (M02259-1, BOSTER, Wuhan, China), rabbit monoclonal antibody to COX-2 (66351-1-1 g, Proteintech, Wuhan, China), rabbit polyclonal antibody to Bax (50599-2-1 g, Proteintech, Wuhan, China), rabbit polyclonal antibody to Bcl-2 (26593-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody to Cleaved Caspase-3 (19677-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody to TNF alpha (17590-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody to IL-6 (DF6087, Affinity Biosciences, Cincinnati, Ohio, USA), rabbit polyclonal antibody to Beta-actin (GB11001, Servicebio, Wuhan, China). The secondary antibodies including HRP Conjugated AffiniPure Goat Anti-Rabbit IgG (BA1054, BOSTER, Wuhan, China) and Goat Anti-Mouse IgG (H + L) (peroxidase/HRP conjugated) (E-AB-1001, Elabscience, Wuhan, China).

2.4. Preparation of SND and phytochemical investigation

SND was prepared by water extraction and alcohol precipitation. Briefly, the crude drugs of *Aconiti Lateralis Radix Praeparata* 300 g, *Zingiberis Rhizoma* 200 g and *Glycyrrhizae Radix* et *Rhizoma* 300 g were mixed and soaked in 8 L of water overnight and decocted to boil for 3 h, and then the decoction was filtered through gauze. Next, the crude drugs were decocted again for 1.5 h with 4 L of water and filtered by the gauze. Afterwards, the successive SND were condensed to 1 200 mL and precipitated with adding 5-fold volume of 95% ethanol (6 000 mL) at 4 °C overnight. Finally, the supernatant was condensed to the concentrations of 1.0 g/mL and 3.0 g/mL (The concentration was expressed as raw materials). The chemical profile of SND was identified according to our previously published paper (Tan et al., 2011).

2.5. Biomedical and histological analysis

Approximately 3 mL of blood was collected from the pars abdominal aorta and centrifuged for 10 min at a speed of 4 000 r/min at 4 °C. Then the serum was utilized to detect myocardial enzyme markers including creatine kinase (CK), creatine kinase MB (CK-MB) and LDH with the automatic biochemistry analyzer (Chemray 240, Shenzhen, China).

For hematoxylin-eosin (H&E) staining, the heart was fixed 4% paraformaldehyde and dehydrated with gradient alcohol in accordance with the manufacturer's descriptions. Next the hearts were embedded in paraffin and cut into 4–5 μm tissue slices. Finally, the paraffin-embedded hearts were stained with hematoxylin and eosin in turn. In addition, Masson staining assay was performed using a Masson stain kit.

2.6. Echocardiography

On the 28 d post operation, cardiac function was evaluated using Vevo 3100LT echocardiography Imaging System (VisualSonics, FUJIFILM, Shanghai, China). The parameters of cardiac function were measured, including the left ventricular end-diastolic dimension (LVID; d), the left ventricular end-diastolic diameter (LVID; s), the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS).

2.7. Culture and treatment of RAW 264.7 cells and H9c2 cells

H9c2 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). RAW 264.7 macrophages were obtained from Pricella Biotechnology Co., Ltd. (Wuhan, China). RAW 264.7 macrophages and H9c2 cells were incubated in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (Zhejiang TianHang Biotechnology Co. Ltd., Zhejiang, China) and 1% penicillin–streptomycin at 37 °C incubator with 5% CO_2. To assess the effect of SND on the release of pro-inflammatory factors in LPS-stimulated RAW 264.7 cells, RAW 264.7 cells were divided into control group, LPS-treated group, SND-treated group and Celecoxib group, respectively. RAW 264.7 cells were pretreated for 2 h with SND (200, 400, 600, 800 and 1 000 μg/mL) and subsequently cultured for 24 h with LPS. The pretreatment of the positive drug (Celecoxib) on RAW 264.7 cells was consistent with the above method. Based on the above method, approximately 2×10^4 cells/well were seeded in 96-well plates for the cell viability test. About 6×10^5 cells/well were seeded in the 6-well plates treating with 1 μg/mL LPS for 24 h, and the cell supernatant was collected for further analysis. Moreover, for Western blot analysis, about 3×10^6 cells of RAW 264.7 were pretreated for 2 h with SND (200, 600 and 1 000 μg/mL) and following cultured for 24 h with LPS.

In order to induce inflammation, H9c2 cells were cultured into 96-well plates with LPS (10, 5, 1 μg/mL) for 24 h. And the cell supernatant was collected for further analyzing the nitrite concentration and LDH. Besides, LPS treatment alone had a weak inflammatory response in H9c2 cells. A novel cell injury model of H9c2 cells was established by CM. H9c2 cells was treated with CM for 24 h to generate inflammatory injury. To verify the anti-inflammatory effect of SND on CM-induced H9c2 cells, H9c2 cells were divided into control group, CM-treated group, SND-treated group and Celecoxib group, respectively. The concentration gradient of SND was consistent with the above description. H9c2 cells were pretreated with different concentrations of SND and Celecoxib for 6 h, followed by CM for 24 h to generate the cell injury model. The cell supernatant was collected for further analysis. For Western blot analysis, about 9×10^5 cells were pretreated with SND (200, 600 and 1 000 μg/mL) and Celecoxib, followed by CM for 24 h.

2.8. Determination of cell viability

A commercial CCK-8 assay kit was used to measure cell viability. In accordance with the manufacturer's descriptions, 10 μL of CCK-8 solution was added to each of the 96-well plates for 2 h in the incubator, and the absorbance was measured at 450 nm by the AMR-100 microplate reader (Hangzhou, China). Formula for the calculation of cell viability: Cell viability (%) = [(experimental well − blank well) / (control well − blank well)] × 100. All experiments were repeated in triplicate.

2.9. Measurement of NO and LDH

The release of NO and LDH were detected from the cell supernatant. The NO assay kit was utilized to measure the production of NO based on the Griess method, the LDH cytotoxicity assay kit was used to determine the release of LDH.

2.10. Western blot analysis

Western blot was used to profile the expression of targeted protein in cardiac tissues or cells. The protein lysate was added to the treated myocardial tissue or cells for 30 min on ice and subsequently centrifuged at 12 000 r/min at 4 °C for 30 min. The BCA protein concentration assay kit was used to measure the protein concentration from the collected supernatant (total protein solution).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to isolate the target protein. The target protein was transferred to the polyvinylidene fluoride (PVDF) membranes by the wet turning method. Then the membrane was incubated with the respective primary antibodies overnight and incubated with secondary antibodies for 1 h. Beta-actin was the internal control. Next, the target protein was clearly visualized by the ECL chemiluminescence kit (Billerica, USA) and performed using Image lab software (Bio-Rad, Hercules, CA, USA).

2.11. Statistical analysis

All data were shown as the mean ± standard deviation (SD). The GraphPad Prism Version 7.0 Software was used for statistical analysis. One-way ANOVA was used to verify between the differences of two or more groups followed by Tukey's multiple comparison tests. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. SND reduced release of inflammatory factors on LPS-activated RAW 264.7 cells by alleviating phosphorylation of NF-κB and expression of COX-2

As shown in Fig. 1A and B, SND was no cytotoxic to RAW 264.7 cells and could reduce the release of NO when the LPS-induced RAW 264.7 cells were pretreated with 200–1 000 μg/mL SND, which demonstrated that SND had a significant anti-inflammatory role. Furthermore, SND could inhibit the release of pro-inflammatory cytokines by Western blot analysis, including IL-6 and TNF-α (Fig. 1C). SND could also effectively down-regulate the expression of COX-2 and phosphorylated NF-κB (p-NF-κB) (Fig. 1D). Therefore, we speculated that SND may exert anti-inflammatory effects by down-regulating the levels of COX-2 and regulating NF-κB signal pathway.

3.2. SND suppressed inflammation of H9c2 cells inducedby CM through inhibiting NF-κB pathway and expression of COX-2

The above results confirmed SND had an anti-inflammatory effect on LPS-stimulated RAW 264.7 cells. Thus, the effect of SND on inflammation in H9c2 cells was further explored. It was shown that LPS with the concentration of 0.5–10 μg/mL had no significant cytotoxic effect on H9c2 cells and made no difference to the release of NO and LDH (Fig. 2A–C), which indicated LPS had no significant effect on H9c2 cells to induce inflammatory injury. Therefore, an inflammatory H9c2 cells model was further established using macrophage-CM. As shown in Fig. 2D, the maximum release of NO was obtained when the concentration of LPS was 1 μg/mL. Therefore, RAW 264.7 cells were treated with 1 μg/mL LPS to produce conditioned medium. The expression of TNF-α and COX-2 in the CM-stimulated H9c2 cells were higher than those in LPS-stimulated H9c2 cells (Fig. 2E). Based on the above results, we further explored the anti-inflammatory effect of SND on CM-treated H9c2 cells. As shown in Fig. 3, SND could alleviate the CM-induced inflammatory injury and reduce the release of NO and LDH and the expression of IL-6 and TNF-α in H9c2 cells induced by CM. Western blot analysis shown that the expressions of Bax and Cleaved Caspase-3 were increased and the expression of Bcl-2 was decreased in CM-treated H9c2 cells, whereas SND could reverse their expressions (Fig. 4A), which demonstrated that SND could reduce the apoptosis of H9c2 cells induced by CM. Furthermore, SND could inhibit the expressions of p-NF-κB and COX-2 (Fig. 4B). The results indicated that SND alleviated CM-induced inflammation in H9c2 cells through regulating NF-κB signal pathway and suppressing the expression of COX-2.

3.3. SND significantly protected cardiac function of rats with MI

The cardio-protective effect of SND *in vivo* was assessed in rat of MI induced by ligation of the LAD. The representative images of LVID; d and LVID; s indicated that the cardiac chamber was significantly expanded in the model group. Moreover, the parameters of LVEF and LVFS, reflecting left ventricular function in rat, were significantly decreased in the model group. As expected, SND treatment could reverse the cardiac functional indices in rat with MI (Fig. 5A and B).

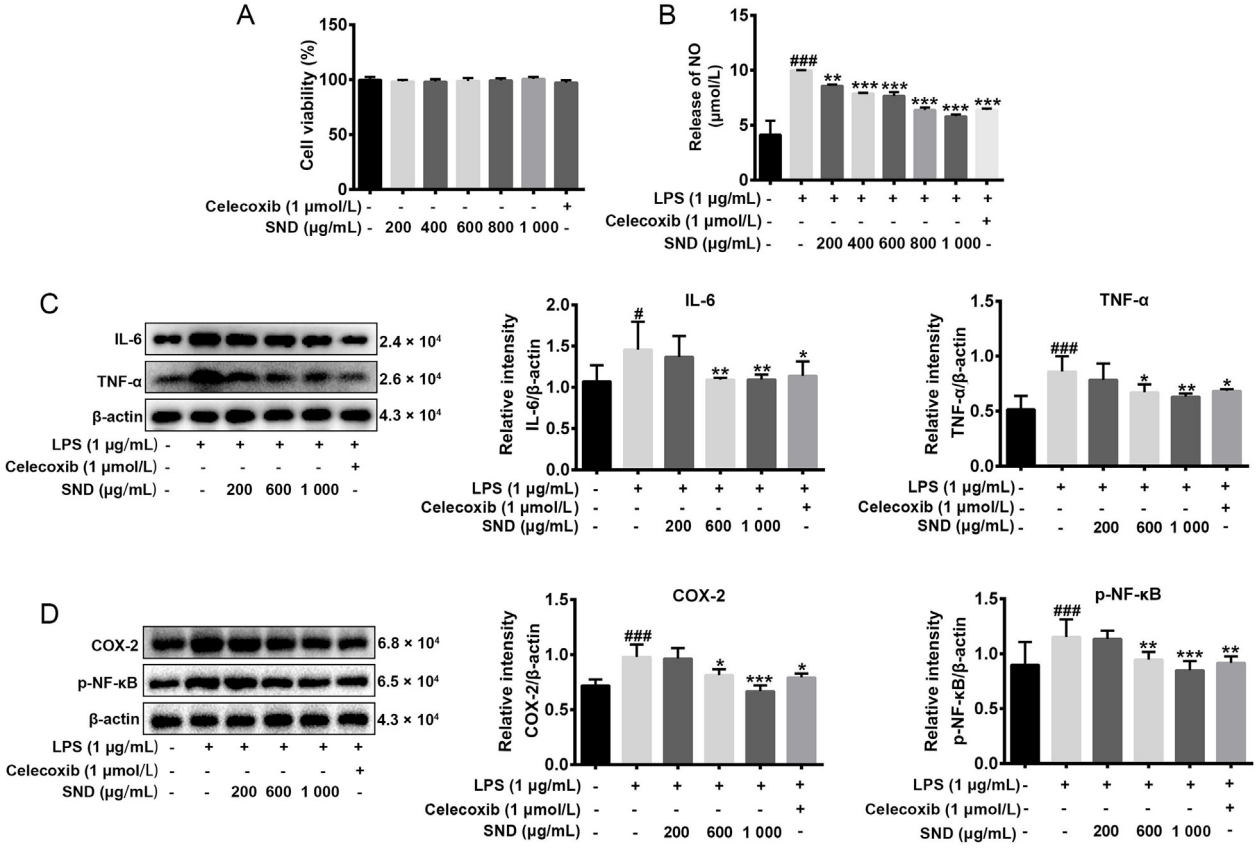

Fig. 1. SND reduced release of inflammatory mediators in LPS-induced RAW264.7 cells by inhibiting expression of p-NF-κB and COX-2. (A) Cell viability was measured by CCK-8 assay (mean ± SD, $n = 4$). (B) Release of NO in LPS-stimulated RAW 264.7 was detected by Griess method (mean ± SD, $n = 4$). (C) Expression of TNF-α and IL-6 in LPS-stimulated RAW 264.7 was detected by Western blot (mean ± SD, $n = 4$). (D) Western blot detected expression of COX-2 and p-NF-κB (mean ± SD, $n = 5$). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* control group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* model group.

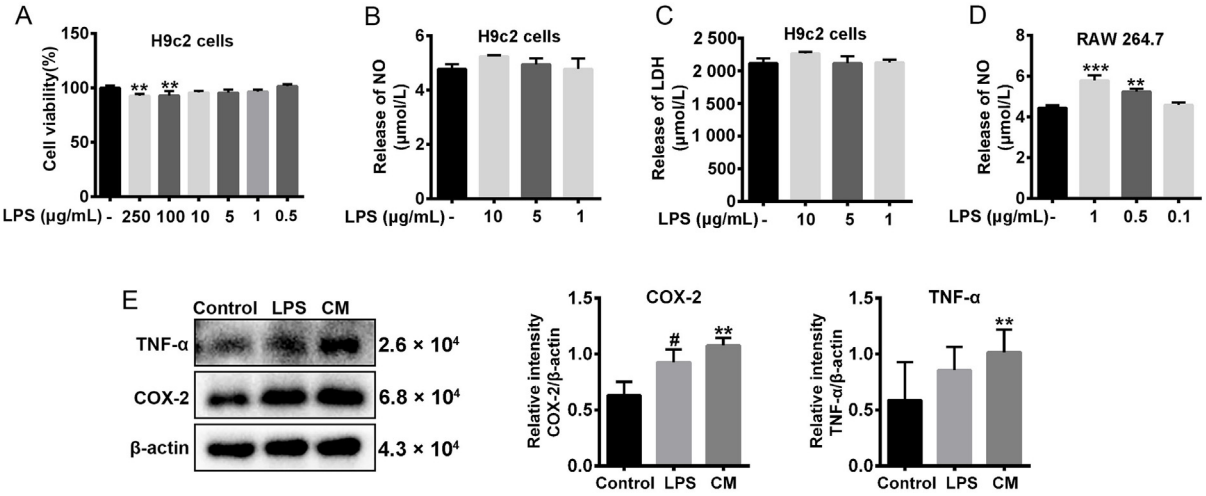

Fig. 2. Comparison of CM-induced inflammation and LPS-induced inflammation. (A) Cell viability was measured on H9c2 cells treated with 0.5–250 μg/mL LPS (mean ± SD, $n = 5$). (B) Release of NO was detected by Griess method (mean ± SD, $n = 4$). (C) LDH cytotoxicity assay (mean ± SD, $n = 3$). (D) Release of NO of RAW 264.7 cells treated with LPS (0.1, 0.5, 1 μg/mL) (mean ± SD, $n = 3$). (E) Expression of COX-2 and TNF-α by Western blot in CM-induced H9c2 cells and LPS-induced H9c2 cells (mean ± SD, $n = 4$). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control group in A–D. $^{\#}P < 0.05$ *vs* control group, $^{**}P < 0.01$ *vs* LPS group in E.

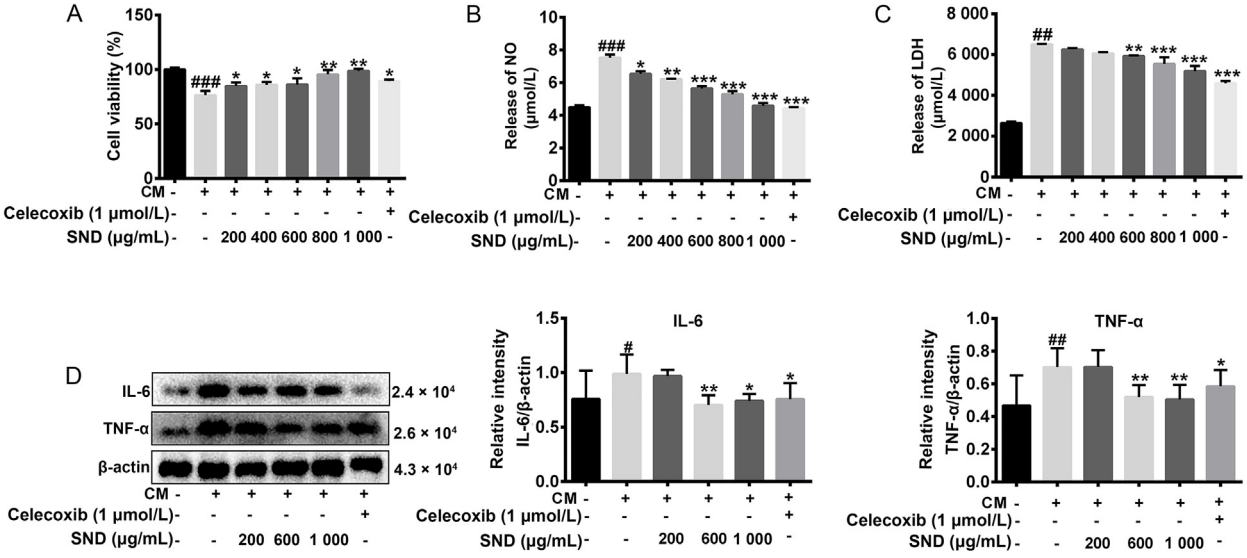

Fig. 3. SND alleviated CM-induced inflammatory injury. (A) Cell viability measured in CM-induced H9c2 cells with treatment of SND (mean ± SD, $n = 5$). (B–C) NO and LDH were detected in CM-induced H9c2 cells (mean ± SD, $n = 3$). (D) Expressions of IL-6 and TNF-α were detected by Western blot (mean ± SD, $n = 4$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* control group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* model group.

The results from the histopathological analysis demonstrated that the myocardial tissue of rats with MI showed the massive necrosis and irregular arrangement, as demonstrated by H&E staining (Fig. 5C), and the increased amounts of collagen deposition, as demonstrated by Masson staining (Fig. 5D). Similarly, SND treatment could effectively alleviate the histopathological changes and inflammatory infiltration in rats with MI. Additionally, the activities of CK, CK-MB and LDH were significantly increased in the MI group. As expected, SND treatment reversed the levels of these abnormal enzymatic in rat with MI (Fig. 5E).

3.4. SND alleviated myocardial inflammation in rat with MI through modulating key enzymes in AA-COXs/LOXs metabolism

The effect of SND on the key enzymes related to the process of inflammatory response in the AA-COXs/LOXs metabolism pathway was further investigated, including sPLA2, COX-1, COX-2, 5-LOX and 15-LOX. Western blot analysis showed that the expressions of sPLA2, COX-1, COX-2, 5-LOX and 15-LOX were obviously increased in the MI group, whereas SND could decrease the expressions of these crucial enzymes in the AA-COXs/LOXs metabolism pathway (Fig. 6). Taken together, SND could attenuate myocardial injury in rat with MI through regulating the key enzymes in the AA-COXs/LOXs metabolism.

4. Discussion

Inflammation is an important cause of cardiovascular disease (Soysal, Arik, Smith, Jackson, & Isik, 2020). AA and its metabolites (eicosanoids) play an important role in the process of inflammation. Our previous metabolomics studies demonstrated that the AA metabolic pathway was involved in the treatment of SND for HF (Zhou et al., 2020). In this study, we further explored the

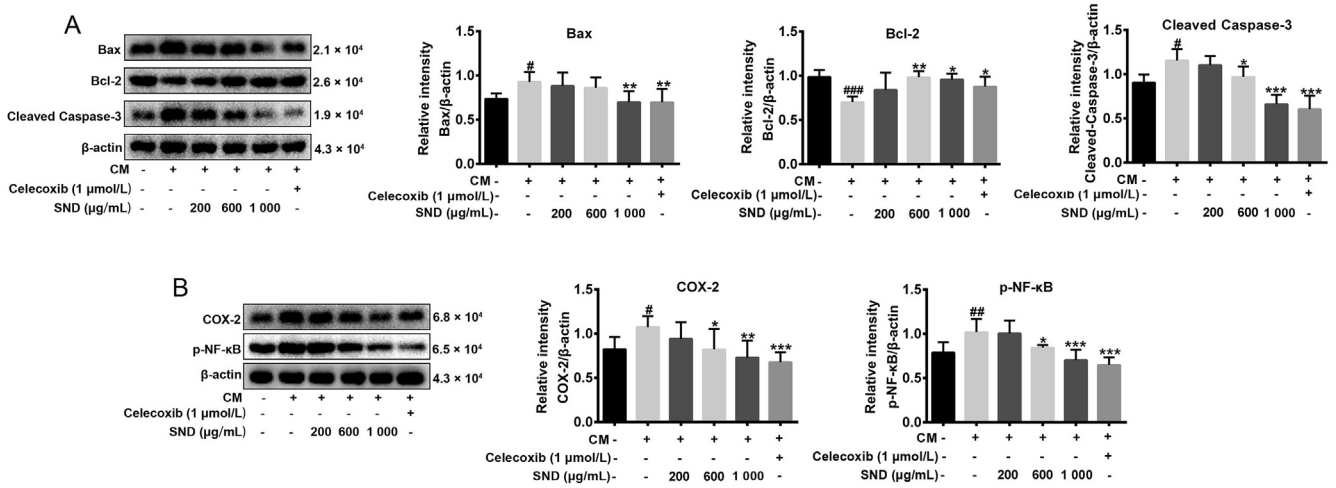

Fig. 4. SND suppressed apoptosis of CM-induced H9c2 cells and expression of p-NF-κB and COX-2. (A) SND regulated expression of Bax, Bcl-2 and Cleaved Caspase-3 after CM-induced inflammation of H9c2 cells (mean ± SD, n = 6). (B) Expression of COX-2 and p-NF-κB was detected by Western blot (mean ± SD, n = 5). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group.

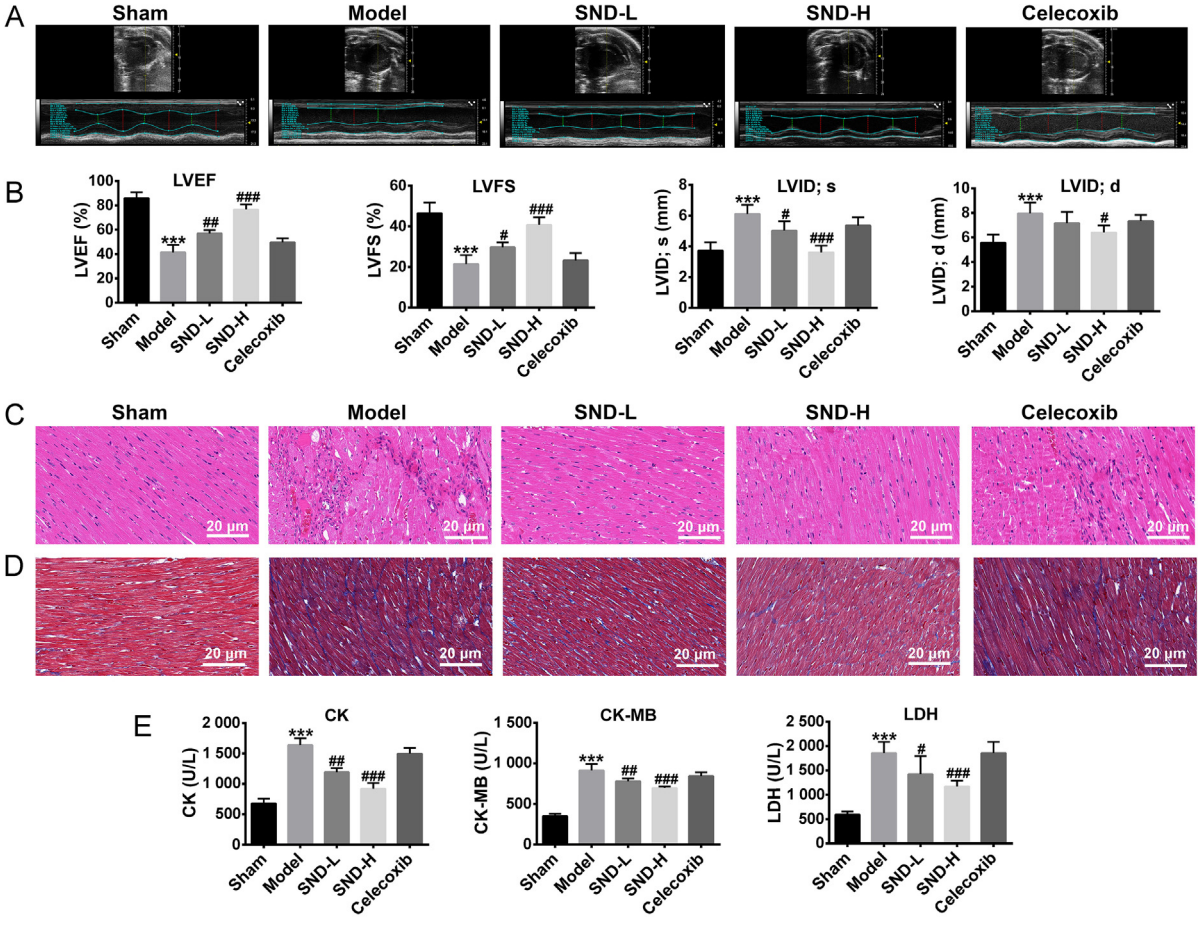

Fig. 5. SND significantly protected cardiac function of rats with MI (mean ± SD, n = 5). (A–B) Cardiac function was tested by echocardiography in each group. Summary echocardiogram data of LVEF, LVFS, LVID; s and LVID; d. (C) Representative images of H&E staining. Scale bars, 20 μm (× 400). (D) Representative images of Masson staining. Scale bars, 20 μm (× 400). (E) Contents of CK, CK-MB and LDH in serum. $^{***}P < 0.001$ *vs* sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* model group.

anti-inflammatory mechanism of SND in treating MI by extensive *in vitro* and *in vivo* experiments focused on the AA metabolism. The main findings suggest that the anti-inflammatory effect of SND could be produced by the regulation of PLA2-COXs/LOXs pathway.

The AA on the cell membrane, a precursor for eicosanoids, could be hydrolyzed by PLA2. AA is mainly metabolized by three kinds of monooxygenase pathway, including COXs pathway, LOXs pathway and CYP450 pathway (Wang, Fu, & Chen, 2019). In the COXs pathway, COXs are mainly composed of two key enzymes including

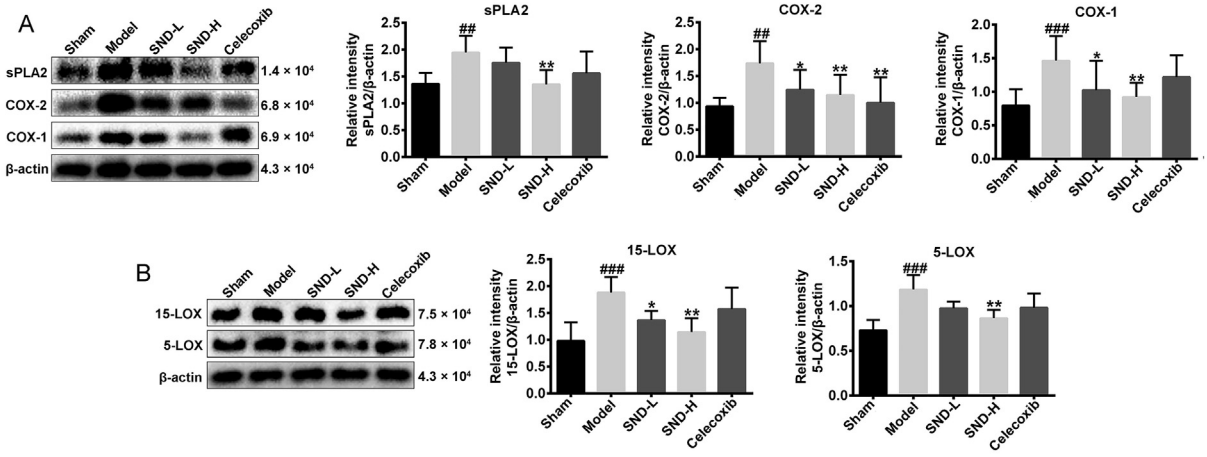

Fig. 6. SND alleviated myocardial inflammation caused by MI through modulating AA metabolism pathway (mean ± SD, $n = 5$). (A) Expressions of sPLA2, COX-1 and COX-2 in cardiac tissue were measured by Western blot. (B) Expressions of 15-LOX and 5-LOX were measured by Western blot. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs* sham group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group.

COX-1 and COX-2. COX-1 is a constitutive expression protein that exists in the healthy tissues and maintains the stability of physiological functions of tissues and organs. COX-2 is an inducible enzyme which is related to inflammation and can be induced by the inflammatory stimuli. COX-2 is regularly considered to be a mediator of the inflammatory response, which can result in myocardial injury (Pannunzio & Coluccia, 2018; Zidar et al., 2007). In the LOXs pathway, 5-LOX is an important rate-limiting enzyme and can catalyze the conversion of AA to leukotrienes (LTs), which perform a significant function in the inflammatory development. 15-LOX can catalyze the conversion of AA to hydroxyeicosatetraenoic acids (HETEs) and promote the expressions of pro-inflammatory factors (Kayama et al., 2009; Mashima & Okuyama, 2015). Interestingly, there is currently evidence that 15-LOX and 5-LOX are associated with cardiovascular diseases (Song et al., 2022; Tourki, Black, Kain, & Halade, 2021). Taken together, targeting the key enzymes in the AA-COXs/LOXs signaling pathway to inhibit myocardial inflammation is an effective strategy for the treatment of cardiovascular diseases. However, the positive drug (Celecoxib), the selective COX-2 inhibitor, can lead to serious adverse drug reactions such as gastrointestinal bleedings/perforations and obstructions (Lehmann & Beglinger, 2005). Therefore, targeting the multiple targets in the inflammatory pathways could be an attractive alternative in the development of new drugs for the treatment of cardiovascular diseases.

It has been proved that SND has a significant anti-inflammatory effect on the treatment for cardiovascular diseases (Liu et al., 2014). Our results *in vitro* suggested that SND treatment could inhibit the release of pro-inflammatory cytokines including TNF-α and IL-6 in LPS-stimulated macrophages. Moreover, the level of NF-κB phosphorylation can be inhibited by SND in macrophages, in which the activation of NF-κB could induce gene transcription of pro-inflammatory factors (Tak & Firestein, 2001). These results demonstrated that SND can mitigate the activation of macrophages induced by LPS. Afterwards, the anti-inflammatory effect of SND on H9c2 cells was further investigated in this study, where the inflammatory injury model of H9c2 cells was established by LPS-stimulated macrophage-CM. To our knowledge, the CM system was successfully performed in the studies of neurodegenerative diseases (Marcotorchino et al., 2012; Tseng, Hsu, Shih, & Lo, 2012), but it was rarely applied to cardiovascular diseases. Our results indicated that the CM was able to induce the inflammatory injury of H9c2 cells. However, SND could alleviate the apoptosis and cytotoxicity of H9c2 cells induced by CM. Furthermore, we found that SND could significantly decrease the levels of COX-2 and the phosphorylation of NF-κB, which demonstrated that the anti-inflammatory effect of SND on CM-induced H9c2 cells could be achieved through regulating the PLA2-COXs and NF-κB pathways.

Besides these *in vitro* results, we further explored the *in vivo* anti-inflammatory effects of SND in rat with MI. The results showed that SND treatment remarkably improved the cardiac function and decreased inflammatory injury in rats with MI. Moreover, SND could decrease the expressions of sPLA2, COX-1, COX-2 in the PLA2-COXs pathway and 5-LOX and 15-LOX in the PLA2-LOXs pathway. In contrast, Celecoxib is the selective COX-2 inhibitor, which has no significant effect on other enzymes in PLA2-COXs/LOXs pathways. Moreover, the findings of recent evidence-based medicine indicate a potential association between the excess use of Celecoxib and an increased risk of HF (Chen et al., 2021). These results suggest that SND performs synergistic cardioprotective function and anti-inflammatory effect through regulating the multiple targets of the inflammatory pathways. The work provides an innovative insight into the anti-inflammatory pharmacological mechanism of SND in suppressing the progression of MI.

5. Conclusion

In conclusion, the anti-inflammatory mechanism of SND in treating MI can be mediated by suppression of PLA2-COXs and PLA2-LOXs pathways. The work demonstrates that the regulation of the multiple targets from the inflammatory pathways can produce the synergistic effects on the treatment of MI and give insights into an alternative therapeutic approach for MI.

CRediT authorship contribution statement

Cuiping Long: Validation, Methodology, Formal analysis, Writing – original draft. **Qian Zhou:** Formal analysis, Data curation. **Min Xu:** Formal analysis, Methodology. **Xin Ding:** Methodology, Formal analysis, Data curation. **Xingxing Zhang:** Formal analysis. **Ya Zhang:** Formal analysis, Methodology. **Yuping Tang:** Formal analysis, Methodology, Validation. **Guangguo Tan:** Conceptualization, Validation, Methodology, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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