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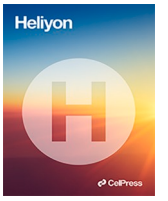

Research article

Comparative efficacy of sweated and non-sweated *Salvia miltiorrhiza* Bge. extracts on acute myocardial ischemia via regulating the PPARα/RXRα/NF-κB signaling pathway

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ABSTRACT

Salvia miltiorrhiza Bge. (*S. miltiorrhiza*) is a well-known traditional Chinese medicine for the treatment of cardiovascular diseases. The processing of *S. miltiorrhiza* requires the raw herbs to sweat first and then dry. The aim of this study was to investigate the anti-acute myocardial ischemia (AMI) of *S. miltiorrhiza* extracts (including tanshinones and phenolic acids) before and after sweating, and to further explore whether the "sweating" primary processing affected the efficacy of *S. miltiorrhiza.* The AMI animal model was established by subcutaneous injection of isoprenaline hydrochloride (ISO). After treatment, the cardiac function of rats was evaluated by electrocardiogram (ECG), biochemical, and histochemical analysis. Moreover, the regulation of *S. miltiorrhiza* extracts on the peroxisome proliferator-activated receptor α (PPARα)/retinoid X receptor α (RXRα)/nuclear transcription factor-kappa B (NF-κB) signaling pathway of rats was assessed by the Western blotting. The results showed that sweated and non-sweated *S. miltiorrhiza* extracts including tanshinones and phenolic acids significantly reduced ST-segment elevation in ECG and the myocardial infarction area in varying degrees. Meanwhile, sweated and non-sweated *S. miltiorrhiza* reversed the activities of aspartate transaminase (AST), lactic dehydrogenase (LDH), creatine kinase-MB (CK-MB), and superoxide dismutase (SOD), as well as the levels of interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) in AMI rats. Concurrently, the results of Western blotting revealed that *S. miltiorrhiza* extracts regulated the PPARα/RXRα/NF-κB signaling pathway to exert an anti-inflammatory effect. Most importantly, sweated *S. miltiorrhiza* tanshinones extracts are more effective than the non-sweated *S. miltiorrhiza*, and the anti-inflammatory efficacy of tanshinones extract was also better than

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that of phenolic acid extract. Although phenolic acid extracts before and after sweating were effective in anti-AMI, there was no significant difference between them. In conclusion, both tanshinones and phenolic acids extracts of sweated and non-sweated *S. miltiorrhiza* promote anti-oxidative stress and anti-inflammatory against AMI via regulating the PPARα/RXRα/NF-κB signaling pathway. Further, the comparations between sweated and non-sweated *S. miltiorrhiza* extracts indicate that sweated *S. miltiorrhiza* tanshinones extracts have better therapeutic effects on AMI.

1. Introduction

Salvia miltiorrhiza Bge. (*S. miltiorrhiza*) has been widely applied in clinic for a long history to treat various cardiovascular diseases [1]. The active components of *S. miltiorrhiza* are mainly including diterpenoids and phenolic acids, which possess several therapeutic effects, such as improving microcirculation, anti-atherosclerosis, anti-inflammation, and anti-tumor [2]. The quality of Traditional Chinese Medicine (TCM) lies in the production of Chinese herbal medicine. The first link of Chinese herbal medicine is standardized cultivation, followed by standardized processing at the source. The quality of *S. miltiorrhiza* is closely related to its origin and processing, which requires the herbs to sweat and then dry. Sweating is a common technique in the primary processing of Chinese herbs, which involves piling up fresh herbs, or baking them over a light fire until they are semi-dry or slightly steamed and then piling them up until their internal moisture overflows and hangs on the surface, which looks like the human body is "sweating" [3,4]. From the appearance, the inner core of the roots becomes purplish red, meaning that the *S. miltiorrhiza* is sweated. Generally, the processing of TCM will lead to changes in the components of herbs [4–6]. Our previous study has confirmed that the sweating method can increase the content of tanshinones in *S. miltiorrhiza* [6] and the ethanol extract of sweated and non-sweated *S. miltiorrhiza* had different effects on the expression and activity of hepatic UDP-glucuronosyltransferases (UGTs) enzyme [7]. As the connotation of sweated *S. miltiorrhiza* has not been thoroughly investigated, there are no reports on whether there are any differences in the medicinal effects between sweated and non-sweated *S. miltiorrhiza*.

The etiology of ischemic heart disease includes reduced local blood supply due to vascular stenosis, atherosclerosis, and infarction. Its complex pathological process involves multiple mechanisms, such as oxidative stress, calcium overload, and inflammation [8,9]. TCM believes that the pathogenesis of the ischemic cardiovascular disease is mainly caused by internal obstruction of blood stasis and insufficiency of blood flow [10]. *S. miltiorrhiza*, as a widely used TCM for invigorating blood circulation and removing blood stasis, is often used to treat acute myocardial ischemia (AMI). Studies have reported that both diterpenoids and phenolic acids of *S. miltiorrhiza* could exert anti-myocardial ischemic effects [11,12]. Few studies in recent years have linked the processing methods to drug efficacy, although our preliminary study has shown that sweating enhances the antioxidant activity of *S. miltiorrhiza* [13]. Hence, it remains unknown which component is better for AMI between sweated and non-sweated *S. miltiorrhiza*.

Myocardial ischemia leads to an oxidative/antioxidative imbalance in the body, which generates oxidative stress and affects the metabolic homeostasis of the myocardium, resulting in myocardial cell death and tissue damage [14]. Nuclear receptors (NRs) are ligand-regulated transcription factors that play different roles in cell differentiation, development, proliferation and metabolism, and an imbalance in nuclear receptor signalling may lead to an overall metabolic disorder in the body and may induce the onset and development of many diseases [15]. It has been found that nuclear receptors are extensively involved in gene transcription [16]. For example, the peroxisome proliferator-activated receptor (PPAR) γ forms a heterodimer with the retinoid X receptor (RXR), which interferes with nuclear transcription factor-kappa B (NF-κB) and activator protein-1 (AP-1) transcriptional activity and inhibits the production of inflammatory mediators [17]. However, it is still unknown whether *S. miltiorrhiza* extracts regulate the PPARα/RXRα/NF-κB signaling pathway to exert an anti-inflammatory effect.

Herein, we hypothesized that "sweating" method enhanced the anti-AMI effect of tanshinone extracts and phenolic acid extracts of *S. miltiorrhiza*. Therefore, we studied the anti-AMI effects and the mechanism of *S. miltiorrhiza* in rats, and further investigated whether the "sweating" method of primary processing affected the efficacy of *S. miltiorrhiza*. Our study revealed the potential therapeutic effects of sweated and non-sweated *S. miltiorrhiza* against AMI and pharmacodynamic differences between them. This study's exploration of the scientific content of sweating of *S. miltiorrhiza* also provides a basis for assessing sweating as a primary processing method.

2. Materials and methods

2.1. Materials

Sweated and non-sweated *S. miltiorrhiza*, identified by professor Nianjun Yu of Anhui University of Chinese Medicine, were purchased from ZhongDa ShenNong Biotechnology Co. (Hanshan County, Anhui Province, China). 2, 3, 5-Triphenyltetrazolium chloride (TTC) (cat. no. 320121) was obtained from BioFroxx Co. (Berlin, Germany). Isoprenaline hydrochloride (ISO) (cat. no. II0200) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Nitrocellulose filter (NC) and TBS powder were bought from Biosharp (Shanghai, China). Prestained Color Protein Marker (10–180 kDa) (cat. no. EC0019) was obtained from SparkJade (Shandong, China). The antibodies used in this research including rabbit anti-PPARα (cat. no. ab120832) was obtained from Abcam; rabbit anti-RXRα (cat. no. 21218-1-AP), anti–NF–κB p65 (cat. no. 380172), and anti-p–NF–κB p65 (cat. no. 3033) were purchased Proteintech Group, Inc. (Chicago, USA). Rabbit anti-β-actin (cat. no. TA-09) was purchased from Beijing Zhongshan Golgen

Bridge Biological Technology Co., Ltd (Beijing, China); anti-rabbit (cat. no. A21020) and anti-mouse (cat. no. 226700711) horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary antibodies, and enhanced chemiluminescence (ECL) (cat. no. ED0015-B) were purchased from SparkJade (Shandong, China). Ethanol (cat. no. 20210143) and Tween-20 (cat. no. 20210918) were purchased from Richjoint (China).

2.2. Preparation of sweated and non-sweated Salvia miltiorrhiza extracts

According to our previous study [18,19], the extracts of *S. miltiorrhiza* were divided into two categories: total phenolic acid extracts and tanshinones extracts (diterpenoids). Sweated and non-sweated *S. miltiorrhiza* were cut into small pieces (100 g), respectively, and soaked in 12 times the amount of water for 1.5 h. After that, the herbs were extracted twice at 80 °C for 1.5 h each time. The two extraction solutions of *S. miltiorrhiza* were combined, and rotary evaporated at 60 °C under reduced pressure. Then, ethanol was added to the extracts to make the alcohol content reach 70 %. The supernatant was taken after 12 h and the extracts were concentrated to a thick paste and then dried to powders, the sweated *Salvia miltiorrhiza* phenolic acid extracts (SSPA) and non-sweated *S. miltiorrhiza* phenolic acid extracts (NSPA) were obtained. The high-performance liquid chromatography (HPLC) characteristic chromatograms and quantitative analysis of NSPA and SSPA were shown in Supplementary materials.

Similarly, sweated *S. miltiorrhiza* tanshinones extracts (SST) and non-sweated *S. miltiorrhiza* tanshinones extracts (NST) were prepared as follows. Firstly, two herbs were crushed into powder (100 g), respectively. The extraction of the two herbs was carried out by heating and refluxing. After recovering ethanol by rotary evaporation under reduced pressure, the extracts were washed with ultrapure water and finally dried into powder. The extraction process of the NSPA, SSPA, SST, and NST were shown in Fig. 1 (A). The HPLC characteristic chromatograms and quantitative analysis of the extracts were shown in our previous study [6].

2.3. Animals and experimental design

Rats were obtained from the Pizhou Dongfang Breeding Co. and acclimatized for 7 days in facilities. The ninety-six rats were randomized into 12 groups: (1) control (0.5 % w/v aqueous Carboxyl methyl cellulose sodium, CMC-Na); (2) control (0.9 % w/v saline); (3) AMI group (subcutaneous injection of ISO only); (4) positive drug group: rats were administered with Isosorbide mononitrate sustained-release tablets (IMST) for 7 days after ISO injection (6.3 mg/kg); (5–8) drug administration groups: each group was

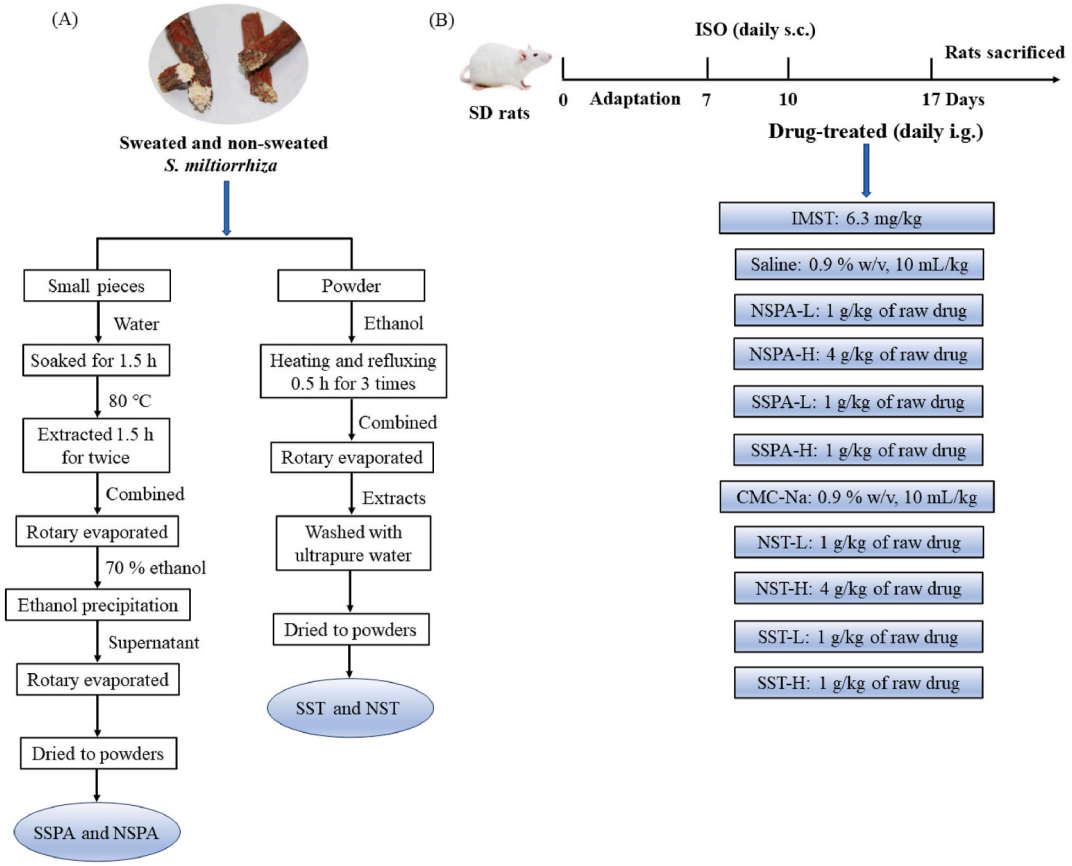

Fig. 1. The extraction process of the NSPA, SSPA, SST, NST (A) and the illustration of the animal experiments protocol (B).

intragastrically administered with low and high doses of SSPA and NSPA (SSPA-L, SSPA-H, NSPA-L and NSPA-H) for 7 days after ISO injection (converted to 1 g/kg and 4 g/kg of raw drug, respectively.); (9–12) drug administration group: each group was intragastrically administered with low and high doses of SST and NST (SST-L, SST-H, NST-L and NST-H) for 7 days after ISO injection (converted to 1 g/kg and 4 g/kg of raw drug, respectively) (n = 8 in each group). Animals were treated with ISO (30 mg/kg) to induce experimental AMI once a day for three days [20], and then administered drugs once a day according to groups. The illustration of the animal experiments was shown in Fig. 1 (B), and the dosing protocol of rats was shown in Table 1. The rats were anesthetized after the last injection of ISO and a lead II electrocardiogram (ECG) was recorded to confirm the model was built successfully. Rats were sacrificed after the last dose and electrocardiograms were measured.

2.4. Electrocardiogram recording

The ECG of the rats was recorded by the BL-420S biosignal acquisition and analysis system (Chengdu Tymon Technology Co., Chengdu, China). The operation process was described as our previous study [6]. ST-segment was calculated by the vertical distance between the point J and the TP section.

2.5. TTC staining

Rats were sacrificed, and their hearts were dissected. The heart was cut into serial sections after placing at −20 °C for 30 min. The sections were stained with 1 % TTC solution for 30 min in 37 °C, which was performed in dark conditions. The size of the cardiac area and infarct size were measured by Image J Software. The infarct size was calculated as a ratio of infarct area to cardiac area for assessing the myocardial infarct degree.

2.6. Determination of aspartate transaminase (AST), lactic dehydrogenase (LDH), superoxide dismutase (SOD) and creatine kinase-MB (CK-MB) release into serum

The myocardial injury was evaluated by the measurement of AST, LDH, SOD, and CK-MB activities or levels in serum. The activities of AST, LDH, and SOD in serum were assessed by commercially available kits (Nanjing Jiancheng Biological Engineering Research Institute, China). The levels of CK-MB were measured by ELISA kit. All procedures were following the manufacturer's instructions.

2.7. Measurement of inflammation-related cytokines by ELISA

To detect the release of inflammation-related cytokines in the serum of rats with myocardial ischemia. The expression of interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α) in the serum were measured using ELISA kits (Xiamen Lun Changshuo Biotechnology Co. China) according to the manufacturers' instruction.

2.8. Hematoxylin and eosin (H&E) staining

The heart tissues were subsequently embedded in paraffin and sectioned into 4 μm slices. These specimens were then subjected to H&E staining, alcohol dehydration, and xylene transparency before being sealed with neutral glue. The structural pathologically changes of heart tissue were observed under a light microscope (IX51, Olympus, Japan).

2.9. Western blotting analysis

Heart tissue was ground in liquid nitrogen and lysed in RIPA buffer (1 % SDS and 10 mM tris buffer pH 7.4), and the supernatant was collected by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was mixed with protein loading buffer at a volume of

Table 1
The dosing protocol of rats.

Groups	ISO (s.c.)	Dose of drugs (i.g.)
Control	–	0.5 % CMC-Na (10 mL/kg/d)
Control	–	0.9 % saline (10 mL/kg/d)
AMI	+	–
IMST	+	IMST (6.3 mg/kg/d)
NSPA-L	+	NSPA (1 g/kg/d of raw drug)
NSPA-H	+	NSPA (4 g/kg/d of raw drug)
SSPA-L	+	SSPA (1 g/kg/d of raw drug)
SSPA-H	+	SSPA (4 g/kg/d of raw drug)
NST-L	+	NST (1 g/kg/d of raw drug)
NST-H	+	NST (4 g/kg/d of raw drug)
SST-L	+	SST (1 g/kg/d of raw drug)
SST-H	+	SST (4 g/kg/d of raw drug)

4: 1 and boiled at 100 °C for 7 min. A BCA protein kit was used to quantify the supernatant. Subsequently, the proteins were then electrophoretically separated on polyacrylamide gels with 10 % sodium dodecyl sulfate (SDS-PAGE) and transferred onto nitrocellulose filter (NC) membrane for 90 min at 200 mA. After blocking with 5 % defatted milk for 2 h, the membrane was incubated overnight with specific primary antibodies (PPARα, 1: 1000; RXRα, 1: 1000; NF-κB p65, 1: 5000; p–NF–κB p65: 1: 1000; β-actin, 1: 1000) at 4 °C. After incubated overnight, the membrane was washed and incubated for 2 h with anti-rabbit/mouse IgG antibodies (1: 10000). Eventually, the protein bands were detected with enhanced chemiluminescence (ECL) in Digital images (GelView 6000 Plus, China). Image J was used to obtain the grey-scale value of signals. The expressions of all proteins were normalized to β-actin, and the Western blotting experiments were performed in triplicate.

2.10. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 software, and statistical comparisons were performed by one-way ANOVA followed by Bonferroni's post hoc test. Before ANOVA analysis, normal distribution test was carried out firstly. Data are expressed as mean ± SD, with p-values <0.05 considered statistically significant.

3. Results

3.1. Sweated and non-sweated S. miltiorrhiza extracts changed the ST-segment elevation in AMI rats

After 7 days of oral administration of *S. miltiorrhiza* extracts, ECG was recorded for each group of rats. The results in Fig. 2 (A-N) showed that there was a significant elevation of the ST-segment in the AMI group compared to the saline and CMC-Na groups ($p <$

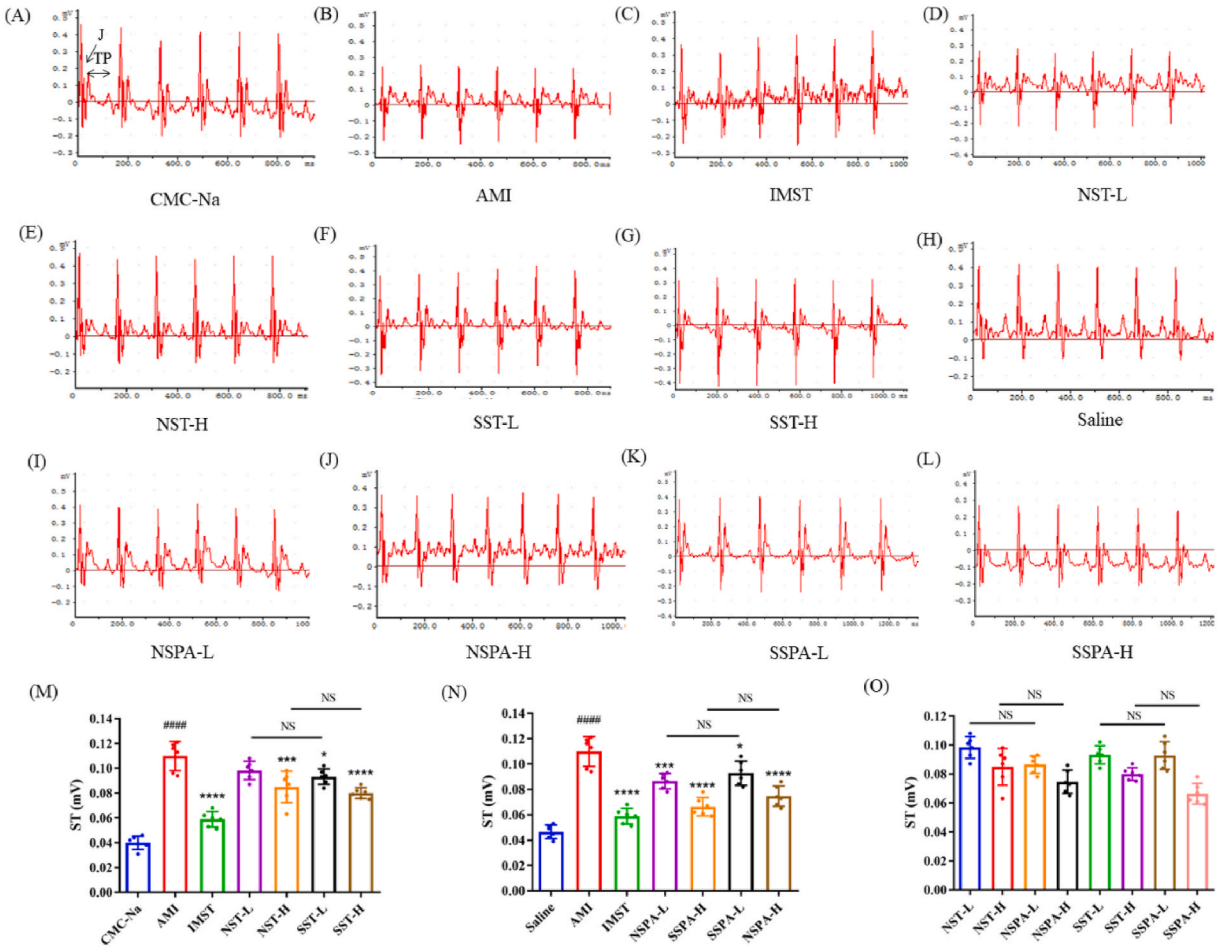

Fig. 2. The ECG images and ST-segment value in each group (n = 6) (A–G) The ECG of rats in the CMC-Na, model, IMST, NST-L, NST-H, SST-L, and SST-H groups. (H–L) The ECG of rats in the saline, NSPA-L, NSPA-H, SSPA-L and SSPA-H groups. (M – O) The value of ST-segment in groups. Compared with the saline group: $^{\#\#\#\#}p < 0.001$; compared with the AMI group: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; the tanshinones extracts groups compared with the phenolic acid extracts groups: NS: no significant difference.

0.0001). Compared with the AMI group, both sweated and non-sweated tanshinones extracts and phenolic acid extracts of *S. miltiorrhiza* all reduced ST-segment elevation in the ECG ($p < 0.0001, p < 0.001, p < 0.01, p < 0.05$) as well as the positive drug. But there was no significant difference between SST-L and NST-L, SST-H and NST-H, as well as the SSPA-L and NSPA-L, and SSPA-H and NSPA-H. Further, two extracts of sweated and non-sweated of *S. miltiorrhiza* were compared together, as shown in Fig. 2 (O). The results reveled that there was no significant difference between the two extracts before and after sweating.

3.2. Sweated and non-sweated S. miltiorrhiza extracts reduced the cardiac infarct area in AMI rats

As shown in Fig. 3 (A and C), the whole heart tissues of CMC-Na group and saline group showed no infarction, while the AMI group showed extensive infarction ($p < 0.001$). Compared to the AMI group, both the positive drug and *S. miltiorrhiza* extracts reduced the infarcted area of the myocardium to varying degrees, and with dose-dependent manner. In the comparison of sweated and non-sweated *Salvia miltiorrhiza* tanshinones extracts, the SST-L/SST-H groups were more significant than the NST-L/NST-H groups ($p < 0.05$). Fig. 3 (B and D) demonstrated that both sweated and non-sweated phenolic acid extracts remarkably reduced the infarcted area of the myocardium ($p < 0.01, p < 0.05$), and no significant difference were found between the SSPA-L/SSPA-H and NSPA-L/SSPA-H. However, in the comparison of sweated and non-sweated tanshinones and phenolic acid extracts (Fig. 3 (E)), there was no significant difference between the two extracts. Clearly, sweating enhances the effect of *Salvia miltiorrhiza* tanshinones extracts in protecting the heart.

3.3. Sweated and non-sweated S. miltiorrhiza extracts influenced the release of AST, LDH, SOD, and CK-MB into serum

The serum contents of AST, LDH, SOD, and CK-MB were detected in each group. Fig. 4 (A-L) showed that induction of ISO increased the activities of AST, LDH, and the level of CK-MB, but decreased the activity of SOD in AMI model rats. Compared with the AMI group, AST and LDH activities with the level CK-MB were decreased in IMST group and all the *S. miltiorrhiza* extracts groups, while the activity of SOD was increased. In the comparison of sweated and non-sweated tanshinones extracts, there was a significant difference in the

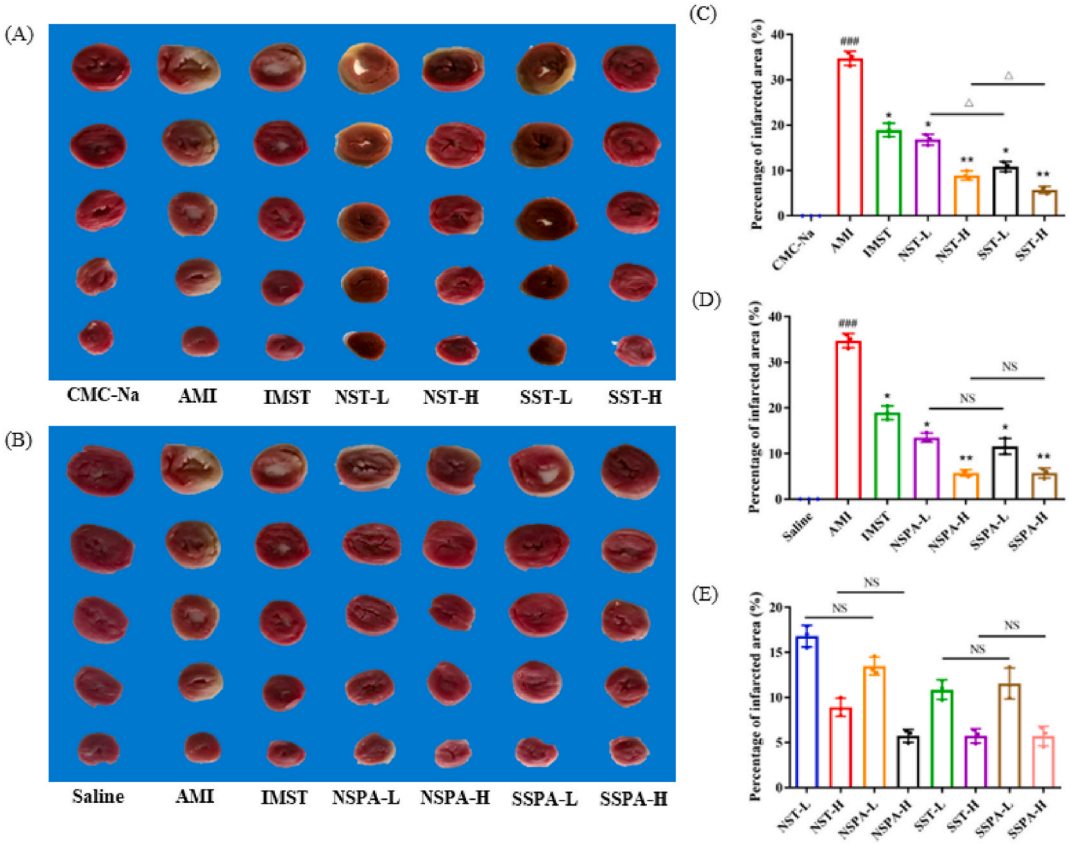

Fig. 3. The percentage of infarcted area in each group (n = 3) (A) TTC staining images of rat hearts in NST and SST groups. (B) TTC staining images of rat hearts in NSPA and SSPA groups. (C) The percentage of infarcted area in NST and SST groups. (D) The percentage of infarcted area in NSPA and SSPA groups. (E) Comparison of the percentage of infarcted area in the tanshinones extracts and phenolic acid extracts groups. Compared with the CMC-Na or saline group: $^{###}p < 0.001$; compared with the AMI group: $^{**}p < 0.01$, $^{*}p < 0.05$; the SST groups compared with NST groups: $^{\triangle}p < 0.05$; the tanshinones extracts groups compared with the phenolic acid extracts groups: NS: no significant difference.

reduction of AST, LDH, and CK-MB between NST-H and SST-H ($p < 0.05$), with SST exhibiting a stronger inhibitory effect on AST activity. Moreover, both sweated and non-sweated phenolic acid extracts were significantly reverse the AST, LDH, SOD, and CK-MB levels ($p < 0.01$, $p < 0.05$). However, there was no significant difference between the SSPA-L/SSPA-H and NSPA-L/NSPA-H.

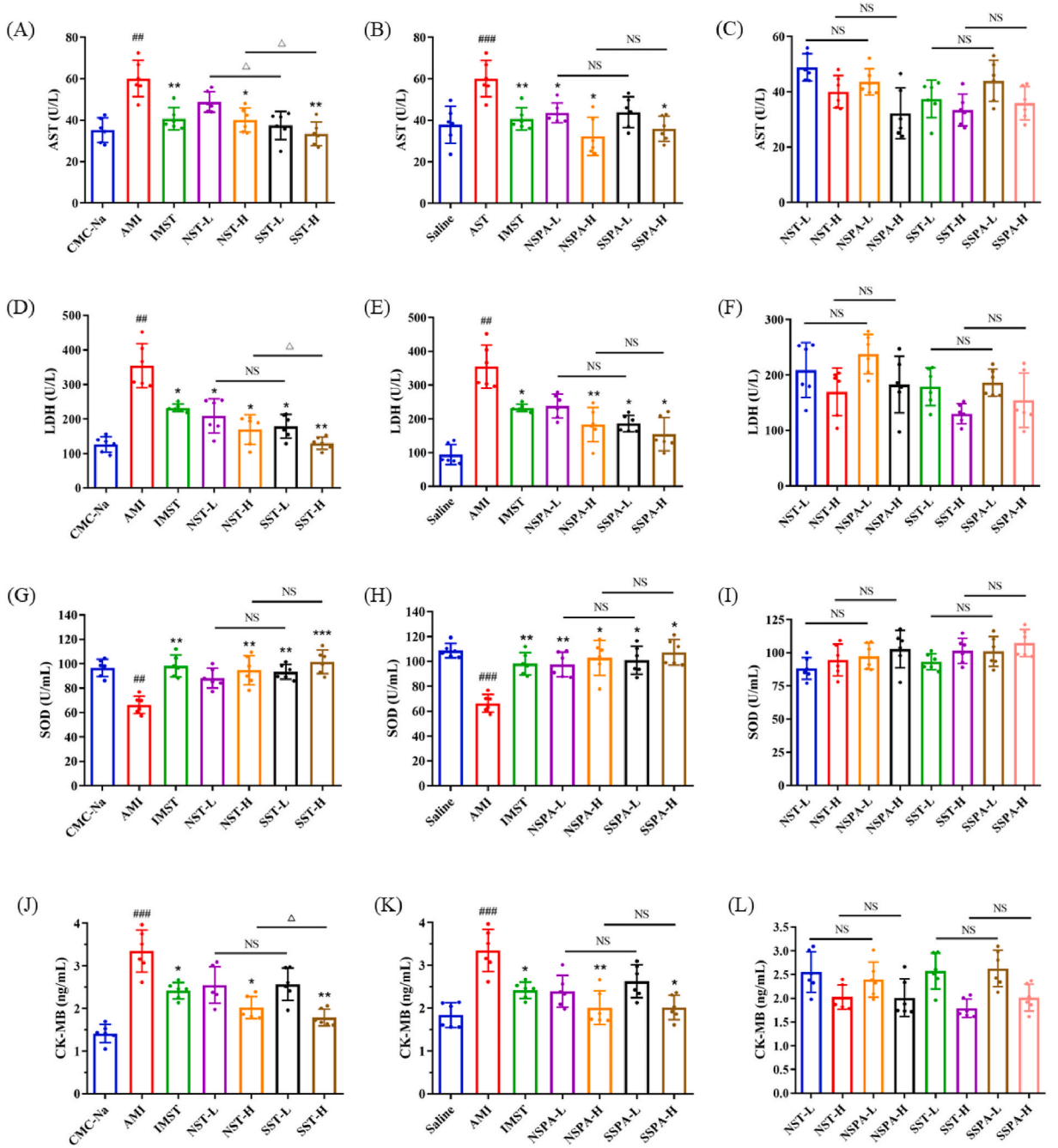

Fig. 4. Activities or levels of AST, LDH, SOD, and CK-MB in rats' serum (n = 6) (A–B) Activities of AST in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (C) Comparison of the AST activities between the tanshinones extracts and phenolic acid extracts groups; (D–E) The activities of LDH in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (F) Comparison of the LDH activities between the tanshinones extracts and phenolic acid extracts groups; (G–H) Activities of SOD in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (I) Comparison of the SOD activities between the tanshinones extracts and phenolic acid extracts groups; (J–K) The levels of CK-MB in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (L) Comparison of the SOD activities between the tanshinones extracts and phenolic acid extracts groups. Compared with the CMC-Na or saline group: $^{###}p < 0.001$, $^{##}p < 0.01$; compared with the AMI group: $^{**}p < 0.01$, $^{*}p < 0.05$; the SST groups compared with NST groups: $^{\triangle}p < 0.05$; NS: no significant difference.

Additionally, there was no significant difference between the two extracts before and after sweating.

3.4. Sweated and non-sweated S. miltiorrhiza extracts reversed the level of IL-6, IL-10, and TNF-α in the serum

IL-6 and TNF-α are promptly and transiently produced in response to infections and tissue injuries. The IL-6 and TNF-α play a pathological effect on chronic inflammation and autoimmunity [21]. In contrast, IL-10 is recognized as an anti-inflammatory and immunosuppressive factor [22]. Therefore, the inflammation-related cytokines IL-6, IL-10, and TNF-α in the serum of all groups were measured. Fig. 5 (A-I) demonstrated that compared with the CMC-Na group or saline group, there are significantly higher levels of inflammatory factors and lower level of IL-10 in the AMI group ($p < 0.01$; $p < 0.001$; $p < 0.0001$). Compared with the AMI group, the IMST, sweated and non-sweated *S. miltiorrhiza* extracts significantly decreased the levels of IL-6 and TNF-α, and increased the level of IL-10. Interestingly, there were significant differences in the level of IL-6, IL-10, and TNF-α between NST and SST groups ($p < 0.05$). There were no significant differences between SSPA-L/SSPA-H and NSPA-L/SSPA-H groups, although each group could significantly reduce inflammatory factor and increase anti-inflammatory factor ($p < 0.0001$; $p < 0.001$; $p < 0.01$; $p < 0.05$). In the comparison of two extracts before and after sweating, NST-L has a significant advantage in reducing IL-6 compared to NSPA-L ($p < 0.05$), and SST-L has a significant advantage in increasing IL-10 compared to SSPA-L ($p < 0.05$). These results demonstrated that the sweated tanshinones extracts and the phenolic acid extract all improve the biochemical indexes of AMI.

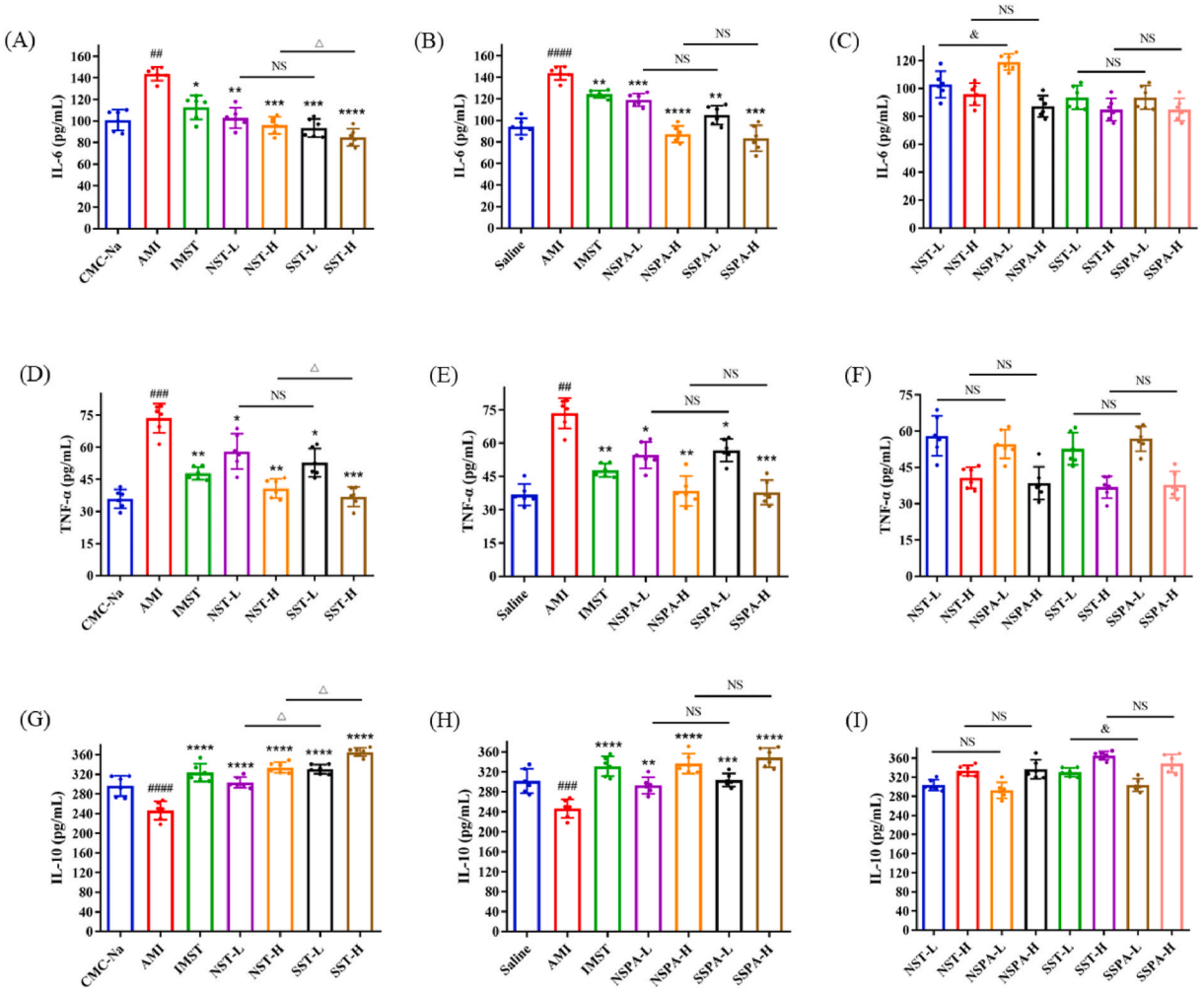

Fig. 5. The levels of IL-6 and TNF-α in rats' serum (n = 6) (A–B) The levels of IL-6 in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (C) The comparison of the IL-6 levels in the tanshinones extracts and phenolic acid extracts groups; (D–E) The levels of TNF-α in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (F) The comparison of the TNF-α levels in the tanshinones extracts and phenolic acid extracts groups. (G–H) The levels of IL-10 in rats' serum in sweated and non-sweated *S. miltiorrhiza* extracts groups; (I) The comparison of the IL-10 levels in the tanshinones extracts and phenolic acid extracts groups. Compared with the CMC-Na or saline group: $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$; compared with the AMI group: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; the SST-H group compared with NST-H group: $^{\triangle}p < 0.05$; the tanshinones extracts groups compared with the phenolic acid extracts groups: $^{\&}p < 0.05$, NS: no significant difference.

3.5. H&E staining

The results in Fig. 6 showed that the myocardial tissue morphology of the rats in the saline and CMC-Na groups was not change significantly, with neatly arranged myocardial fibers and no inflammatory cell infiltration or myocardial swelling and necrosis. Whereas the myocardial fibers in the AMI model group were disordered, with blurred myofibrillar gaps, unevenly sized nuclei, and irregularly broken myocardial cells. It also has obvious inflammatory cell infiltration [23]. The myocardial pathology of the rats in each administration group were improved in different degrees. In all *S. miltiorrhiza* extracts groups, the myocardial fibers became neatly arranged, and small focal myocardial necrosis changed to visible scattered red spots. Meanwhile, the inflammatory cell infiltration was significantly reduced.

3.6. Sweated and non-sweated S. miltiorrhiza extracts modulated the PPARα/RXRα/NF-κB pathway to reduce inflammation

To investigate whether *S miltiorrhiza* extracts exert anti-inflammatory effects by activating the nuclear receptors PPARα and RXRα and thus regulating the NF-κB pathway, their proteins were detected by the Western blotting. Fig. 7 (A-D) showed that the level of PPARα and RXRα were decreased in the AMI group compared to the CMC-Na group ($p < 0.05$). In the NST and SST administered groups, two proteins expressions were significantly increased ($p < 0.05$, $p < 0.01$). In the AMI model group, the expression of p–NF–κB p65/NF-κB p65 was significantly higher, indicating that there was serious inflammation in the rat heart during acute myocardial ischemia. Fortunately, the expression of the p–NF–κB p65/NF-κB p65 was reduced in different degrees in the NST and SST administered group. There was a significant difference in the expressions of p–NF–κB p65/NF-κB p65 between NST-H and SST-H ($p < 0.05$), indicating SST had a stronger modulation on the NF-κB signaling pathway.

Similarly, Fig. 8 (A-D) present that the expressions of PPARα and RXRα were decreased in the AMI group ($p < 0.05$). Correspondingly, these two proteins expressions were significantly increased in the NSPA and SSPA administered groups ($p < 0.05$, $p < 0.01$). In the IMST group, there was no significant difference in the expressions of PPARα and RXRα, suggesting that the improvement of IMST on AMI may not be mediated by this pathway. In the NSPA and SSPA administered group, the expression of the p–NF–κB p65/NF-κB p65 was reduced in different degrees. Obviously, the results in Fig. 8 (E-G) showed that the modulation of the NF-κB signaling pathway of the tanshinones extracts were stronger ($p < 0.01$, $p < 0.05$). The above results confirmed our previous hypothesis that "sweating" enhances the anti-AMI effect of tanshinones extracts, and the efficacy of tanshinones extracts was also better than that of phenolic acid extract. Although phenolic acid extracts were effective in anti-AMI, there was no significant difference before and after sweating, which is worth further investigation.

4. Discussion

AMI is a pathological condition in which myocardial energy metabolism is abnormal due to reduced perfusion of oxygenated blood to the heart, and normal physiological work cannot be performed due to reduced perfusion of blood and oxygen to the heart. When

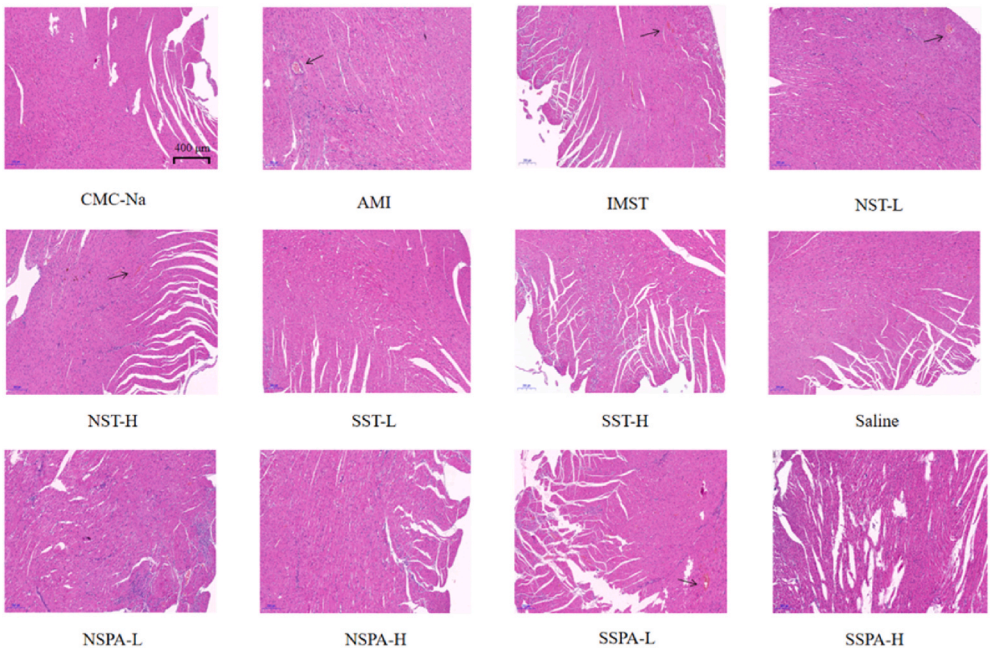

Fig. 6. The H&E staining of myocardial tissue of rats in each group (5 × , n = 3).

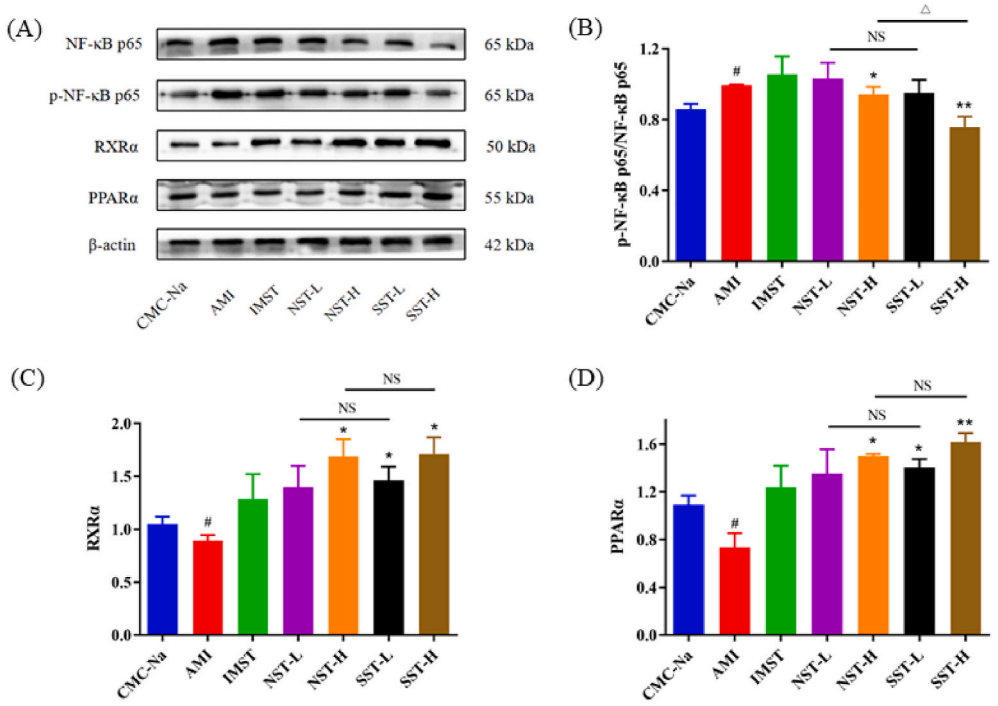

Fig. 7. The effects of NST and SST on PPARα and RXRα and inflammatory proteins expression (n = 3) (A) Western blotting analysis of the proteins; (B–D) Relative protein densities of p–NF–κB p65/NF-κB p65, RXRα, and PPARα, respectively. The full images of the proteins in (A) were shown in the supplementary data (Figs. S2–S6). Compared with the CMC-Na group: $^{\#}p < 0.05$; compared with the AMI group: $^{**}p < 0.01$, $^{*}p < 0.05$; the SST-H group compared with NST-H group: $^{\triangle}p < 0.05$; NS: no significant difference.

coronary blood flow is reduced, myocardial oxygen demand is not supplied, and metabolites are not removed promptly. It would result in myocardial cell damage or infarction, and cause disturbances in cardiac electrophysiological activity [24,25]. Study has been found that ISO could mimic the changes of AMI by causing a dramatic production and accumulation of large amounts of free radicals through autooxidation, thus leading to myocardial ischemia and hypoxia [26]. The ISO myocardial injury model is a classical model for anti-myocardial ischemia studies [27]. Therefore, ISO was intraperitoneally injected to establish a rat model of AMI in this study. The main sign of successful AMI modeling is the change of ST-segment in EGG [28]. In the ECG results, ST-segment in our experiment was remarkably shifted, suggesting that the model of AMI was successfully replicated.

There are numerous specific enzymes in myocardium, once myocardium is damaged or necrotic, these enzymes are released into serum. Thus, it is appropriate to measure the activities of these enzymes in the serum to diagnose and monitor AMI [29]. Wang et al. has reported that the levels of AST, LDH, and CK-MB were increased in the ISO-induced rats, and the activity of SOD was diminished. Fortunately, the danshen-honghua herb pair could reverse these indicators and play a beneficial role in AMI [30]. Likewise, studies have confirmed that the levels of IL-6, and TNF-α were increased while IL-10 was decreased in AMI [31,32]. There are many active components in *S. miltiorrhiza* are known to improve the inflammatory response in AMI such as tanshinone IIA [33] and salvianolic acid B [34]. In our study, the activities of AST, LDH, and CK-MB in the serum were significantly increased, and the activity of SOD was decreased in ISO-treated rats as well. Rats treated with sweated and non-sweated *S. miltiorrhiza* extracts could decrease the activities of AST, LDH, and CK-MB, and also could increase the activity of SOD. Notably, SST was more effective in changing the activity of these enzymes in serum. Both NSPA and SSPA were also effective, but there was no significant difference between them by side-to-side comparison. Further, we observed that *S. miltiorrhiza* extracts reversed the levels of IL-6, IL-10 and TNF-α in the serum of rats. Likewise, there were significant difference between NST and SST groups. SSPA-L, SSPA-H, NSPA-L and SSPA-H could significantly reverse the levels of inflammatory factor and increase anti-inflammatory factor. However, no significant differences were found between SSPA-L/SSPA-H and NSPA-L/SSPA-H groups. These results suggest that the sweated tanshinones extracts and the phenolic acid extract all improve the biochemical indexes of AMI.

PPAR α, β and γ play an important role in cardiovascular diseases and are expected to be new drug targets for the treatment of myocardial ischaemic injury. It has been reported that paeoniflorin activates PPARα and inhibits downstream NF-κB activation to reduce the release of inflammatory factors, thereby protecting against myocardial ischemia-reperfusion injury [35]. Intriguingly, the upstream region of the peroxisome proliferator response element, where the activated peroxisome proliferator-activated receptors (PPARs) form a dimer with the RXR, binds to the peroxisome proliferator response element and promotes the expression of the target enzyme. To further investigate the possible role of sweated and non-sweated *S. miltiorrhiza* extracts in ISO-induced AMI rats, protein expression levels of PPARα, RXRα, NF-κB p65, and p–NF–κB p65 were measured. Our results suggested that the effect of *S. miltiorrhiza* extracts may be related to inhibiting the phosphorylation of NF-κB downstream target proteins associated with activation of PPARα

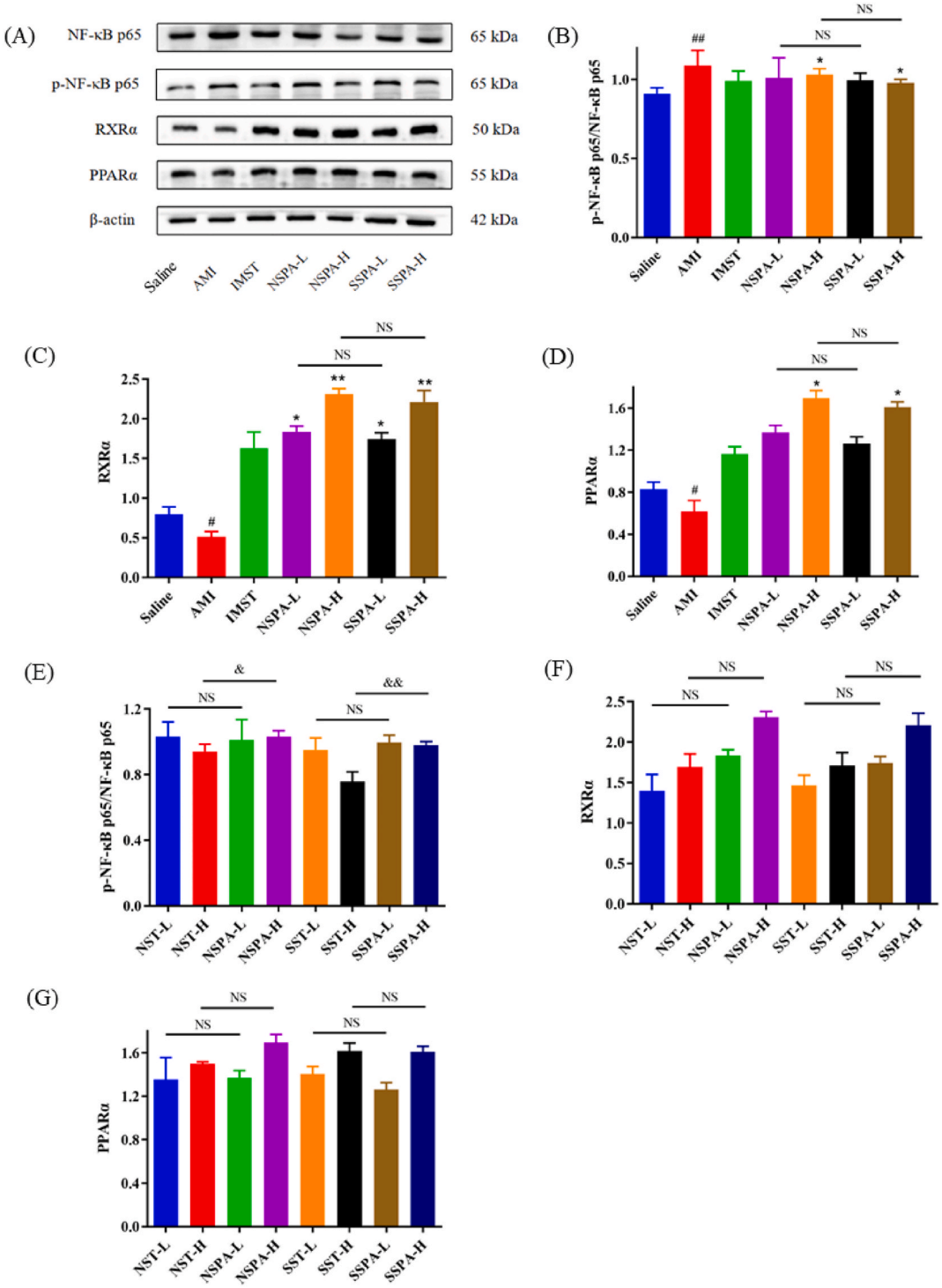

Fig. 8. The effects of NSPA and SSPA on PPARα and RXRα and inflammatory proteins expression (n = 3) (A) Western blotting analysis of the proteins; (B–D) Relative protein densities of p–NF–κB p65/NF-κB p65, RXRα, and PPARα, respectively; (E–G) The comparison of the protein's levels in the tanshinones extracts and phenolic acid extracts groups. The full images of the proteins in (A) were shown in the supplementary data (Figs. S7–S11). Compared with the saline group: $^{\#}p < 0.05$; compared with the AMI group: $^{**}p < 0.01$, $^{*}p < 0.05$; the tanshinones extracts groups compared with the phenolic acid extracts groups: $^{\&\&}p < 0.01$, $^{\&}p < 0.05$, NS: no significant difference.

and RXRα [36,37]. Deeply, the anti-AMI effect of tanshinone extract after sweating was more significant than before sweating. There was a significant difference in the expression of NF-κB p65 between NSPA-L and SSPA-L, indicating SSPA had a stronger modulation on NF-κB signaling pathway. These results demonstrated that sweating enhanced the anti-inflammatory effects of the two extracts. Additionally, the efficacy of tanshinone extract was also better than that of phenolic acid extract. To sum up, the sweated tanshinones extracts had a strong modulation on the PPARα/RXRα/NF-κB signaling pathway.

S. miltiorrhiza has been widely used clinically to treat cardiovascular diseases. Traditionally, sweated *S. miltiorrhiza* was thought to be more effective than non-sweated *S. miltiorrhiza*, but it is not clear which group of components plays the main role in this process. During the process of sweating, the internal temperature of the herbs gradually rises, and a series of enzymatic reactions occur, resulting in the chemical composition transformation of the herbs. As the sweating process continues, the increased internal temperature of the herbs will activate the plant's resistance to heat stress, resulting in the production of secondary metabolites that enhance the content of the active ingredients. Cao et al. found that sweating has significant effects on metabolites composition and content of *S. miltiorrhiza* [38]. As a unique origin processing method, sweating facilitates the drying of the herbs and the formation of high-quality herbs. Generally, the quality of the herbs after sweating is better than that of the non-sweated herbs. The 2020 edition of the Chinese Pharmacopoeia recorded that *S. miltiorrhiza* has a brownish-red bark and a woody, greyish-yellow, or purplish-brown part. It is the characteristic trait of sweated *S. miltiorrhiza* [39]. In this study, *in vivo* results showed that both sweated and non-sweated *S. miltiorrhiza* extracts could be against AMI. In comparison, SST could be more effective, especially in the protein expressions of PPARα/RXRα/NF-κB pathway. This may be associated to the significantly increased content of cryptotanshinone and tanshinone IIA after sweating [40]. Phenolic acid extracts were effective on anti-AMI, whereas there was no significant difference before and after sweating. This may be due to the shorter *in vivo* half-life and lower bioavailability of salvianolic acids, such as salvianolic acid B [41]. At the same time, we also compared the effects of tanshinones extracts with those of phenolic acid extracts. Take all the results together, both the tanshinones and phenolic acid extracts have a good protective effect on the heart, and the effect of tanshinones extract is better than that of phenolic acid extract. Notably, tanshinones extracts have a significant advantage in reversing the inflammation-related indicators such as IL-6, IL-10, and NF-κB/p–NF–κB protein expressions, suggesting that we can further explore this aspect in the future.

The efficacy of *S. miltiorrhiza* before and after sweating processing were compared in this study. Due to the complex factors in the process of sweating and the multi-component and multi-target effects of TCM, it is difficult to fully explain the scientific connotations of sweating from the perspective of component changes and pharmacodynamics. Next, we will further explore the scientific connotations of sweating *S. miltiorrhiza* from the perspective of inflammation and pharmacokinetics.

5. Conclusion

In summary, *S. miltiorrhiza* extracts could effectively treat AMI in an ISO-induced rats' model in this study, and its therapeutic mechanism may be regulation of the PPARα/RXRα/NF-κB expression. Specifically, the differences between sweated and non-sweated *S. miltiorrhiza* extracts indicate that the sweating processing improves the efficacy of *S. miltiorrhiza*, and the components responsible for this difference are mainly tanshinones extracts.

Ethics statement

This study was reviewed and approved by the Animal Experimental Ethics Committee of Anhui University of Chinese Medicine with the approval number: AHUCM-rats-2021073, dated: September 13th, 2021.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Data availability statement

All data used during this study are included in the submitted article.

CRediT authorship contribution statement

Xiaoxiao Shan: Writing – original draft, Validation, Supervision, Software, Methodology. **Junying Li:** Writing – review & editing, Visualization, Validation, Supervision, Data curation. **Bangzhen Hong:** Methodology, Formal analysis, Data curation, Conceptualization. **Huihui Yin:** Visualization, Methodology, Investigation, Conceptualization. **Ziyi Lu:** Methodology, Investigation, Formal analysis, Data curation. **Guokai Wang:** Resources, Investigation, Funding acquisition. **Nianjun Yu:** Formal analysis, Data curation, Conceptualization. **Daiyin Peng:** Project administration, Methodology, Funding acquisition, Conceptualization. **Lei Wang:** Writing – review & editing, Visualization, Funding acquisition. **Caiyun Zhang:** Writing – review & editing, Visualization, Resources, Funding acquisition. **Weidong Chen:** Software, Resources, Project administration, Methodology, 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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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31923.

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