Citri Reticulatae Pericarpium alleviates postmyocardial infarction heart failure by upregulating PPARγ expression

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Abstract
Heart failure after myocardial infarction (MI) is the leading cause of death worldwide. Citri Reticulatae Pericarpium (CRP) is a traditional Chinese herbal medicine that has been used in the clinic for centuries. In this study, we aimed to investigate the roles of CRP in cardiac remodelling and heart failure after MI, as well as the molecular mechanisms involved. Male C57BL/6 mice aged 8 weeks were subjected to coronary artery ligation to mimic the clinical situation in vivo. Echocardiography was used to assess the systolic function of the mouse heart. Masson trichrome staining and Wheat germ agglutinin (WGA) staining were utilised to determine the fibrotic area and cross-sectional area of the mouse heart, respectively. Cardiomyocytes and fibroblasts were isolated from neonatal rats aged 0–3 days in vitro using enzyme digestion. TUNEL staining and EdU staining were performed to evaluate apoptosis and proliferation, respectively. Gene expression changes were analysed by qRT-PCR, and protein expression changes were assessed by Western blotting. Our findings revealed that CRP attenuated cardiac hypertrophy, fibrosis and apoptosis and alleviated heart failure after MI in vivo. Furthermore, CRP mitigated cardiomyocyte apoptosis and fibroblast proliferation and differentiation into myofibroblasts. In addition, the PPARγ inhibitor T0070907 completely abolished the abovementioned beneficial effects of CRP, and the PPARγ activator rosiglitazone failed to further ameliorate cardiac apoptosis and fibrosis in vitro. CRP alleviates cardiac hypertrophy, fibrosis, and apoptosis and can ameliorate heart failure after MI via activation of PPARγ.

KEYWORDS
Citri Reticulatae Pericarpium, heart failure, myocardial infarction, PPARγ

1 | INTRODUCTION

Myocardial infarction (MI) induced by coronary artery occlusion is the leading cause of death globally.1 A series of pathological processes...
Post-MI adverse cardiac remodelling, Previous studies have revealed that activation of CRP is the dried fruit peel which places a heavy burden on society and individuals. Therefore, identifying novel therapeutics for post-MI heart failure is of great significance.

PPARs are nuclear hormone receptor superfamily transcription factors activated by ligands. PPARs, or peroxisome proliferator-activated receptors, were discovered in 1990, and mainly regulate fatty acid oxidation in fat and other tissues and glucose metabolism. There are three PPAR isoforms: PPARα, PPARβ/δ, and PPARγ. Accumulating evidence indicates that PPARγ plays key roles in controlling cardiac metabolism. Previous studies have revealed that activation of PPARγ can significantly ameliorate cardiac hypertrophy and cardiac fibrosis in mice with pressure overload-induced, angiotensin II (Ang II)-induced and isoproterenol (ISO)-induced pathological myocardial remodelling. In addition, upregulating PPARγ expression can increase cardiac output in rats with myocardial ischaemia-reperfusion (I/R) injury. Moreover, the PPARγ agonist pioglitazone can probably reduce the expression of inflammatory cytokines, including TNFα, TNFβ and MCP-1, and attenuate cardiac remodelling and heart failure in mice with MI. A recently published review systematically identified PPARγ as a novel therapeutic target for cardiac fibrosis. However, other studies have found that PPARγ activation can result in fluid retention, weight gain, osteoporosis and even cause heart failure. Thus, identifying potential PPARγ regulators that do not cause side effects may provide options for the clinical treatment of post-MI heart failure.

Traditional Chinese medicine (TCM) plays important roles in the clinical treatment of cardiovascular diseases. Citri Reticulatae Pericarpium (CRP) is a famous TCM that is used for the treatment of multiple diseases, such as cardiovascular diseases, digestive diseases, respiratory diseases and even tumours. CRP is the dried fruit peel of Citrus reticulata Blanco. The southeastern region of China is the main production area of CRP, such as Fujian, Guangdong and Zhejiang provinces. Phytochemical studies have identified more than one hundred chemical components of CRP, and its abundant bioactive compounds, including flavonoids, phenolic acids and limonoids, can exert positive effects on health. CRP has been described as a qi regulator for centuries and was registered in the first edition of the Chinese Pharmacopoeia in 1953. CRP has attracted increasing attention from researchers because it has multiple pharmacologic effects and rich resources with low toxicity and costs. Modern pharmacological investigations have revealed that CRP can fight against inflammation, oxidative stress, atherosclerosis, thrombus, liver injury and tumours. Our prior studies showed that CRP can alleviate cardiac hypertrophy and fibrosis induced by Ang II and ISO via activating PPARγ. However, whether CRP has a protective effect on post-MI heart failure is unclear.

In the current research, we demonstrated that CRP can protect against post-MI heart failure by activating PPARγ, suggesting the potential of CRP in the clinical treatment of heart failure after MI.

2 | RESULTS

2.1 | The quality of CRP was determined by high performance liquid chromatography (HPLC)

CRP compounds various protective flavonoids which were identified by comparing corresponding standards using HPLC. The results showed that hesperidin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone (HMF), tangeretin and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (PMF) are contained in CRP (Figure S1A-B).

2.2 | CRP attenuates cardiac injury after MI in vivo

Since our previous studies demonstrated that CRP protects against Ang II-induced and ISO-induced cardiac dysfunction, we further investigated the role of CRP in post-MI heart failure in the present study. C57BL/6 mice aged 8 weeks were subjected to LAD and then given CRP for 3 weeks. To determine the optimal dosage of CRP, we set three concentrations for treatment. As shown in Figure S2, the dosage of 0.5 and 1.0 g/kg/day could both improve cardiac systolic function after MI. We choose the administration of 0.5 g/kg/day for further investigation because the 1.0 g/kg/day dosage did not perform better. Then the results of echocardiography suggested again that CRP significantly increased the LVEF and LVFS (Figure 1A). In addition, WGA staining and qRT-PCR showed that CRP reduced cardiac hypertrophy (Figure 1B) and decreased the activation of fetal genes (Anp and Bnp) in the hearts of mice with MI (Figure 1C). Cardiac fibrosis is a pivotal hallmark of cardiac remodelling and heart failure after MI. Our data revealed that compared with vehicle, CRP obviously attenuated collagen deposition in the heart post-MI (Figure 2A-C). Cardiac apoptosis is another feature of cardiac remodelling and heart failure after MI. We found that the expression of the antiapoptotic protein Bcl2 was increased and that the expression of the proapoptotic protein Bax was decreased by CRP treatment (Figure 2D). Taken together, our results demonstrate that CRP can alleviate cardiac hypertrophy and cardiac fibrosis as well as cardiac apoptosis after MI injury and thus plays important roles in post-MI heart failure.

2.3 | CRP ameliorates OGD-induced cardiomyocyte apoptosis and TGFβ-induced cardiac fibroblast activation in vitro

To further investigate the protective effects and underlying mechanisms of CRP in vitro, neonatal rat cardiomyocytes (CMs) and cardiac
fibroblasts (CFs) were obtained by enzyme digestion. Similarly, three different concentrations were set to find the most suitable dosage of CRP in vitro. It was found that 0.5 and 1.0 μg/mL CRP administration could both ameliorate the cell apoptosis induced by oxygen–glucose deprivation (OGD), but there was no significant difference between the two concentrations (Figure S3). Therefore, we choose the 0.5 μg/mL dosage as the optimal concentration for further study in vitro. As shown in Figure 3A, B, CRP decreased the number of TUNEL-positive nuclei and reduced the Bax/Bcl2 ratio in OGD-treated CMs, providing evidence for the antiapoptotic function of CRP. In addition, prior to administering TGFβ to activate CFs, CRP was mixed in the culture medium for 24 h. Immunofluorescence analysis indicated that TGFβ enhanced CF proliferation and differentiation into myofibroblasts and that CRP administration reversed these effects (Figure 3C). We further examined the mRNA levels of Col1a1, Col3a1 and α-SMA by qRT–PCR, and the results illustrated that CRP may reduce the increase in the expression of fibrotic genes in TGFβ-stimulated CFs (Figure 3D). Collectively, these results showed that CRP can alleviate OGD-induced cardiomyocyte apoptosis and TGFβ-induced cardiac fibroblast activation in vitro.

2.4 | PPARγ is activated by CRP treatment both in vivo and in vitro

Metabolic disturbance is a crucial feature of cardiac remodelling and heart failure after MI, and PPARγ is a well-known regulator
Our Western blotting results showed that PPARγ expression was decreased in the hearts of mice with MI and in OGD-induced CMs and TGFβ-stimulated CFs. Interestingly, under the pathological conditions mentioned above, CRP dramatically upregulated the expression of PPARγ (Figure 4A–C). These data suggest that PPARγ activation contributes to the beneficial effects of CRP in protecting against post-MI heart failure.

2.5 CRP alleviates OGD-induced cardiomyocyte apoptosis and TGFβ-induced cardiac fibroblast activation by upregulating PPARγ expression

To study whether PPARγ is a pivotal downstream effector of CRP, rosiglitazone and T0070907 were applied. We found that T0070907 inhibited the beneficial effects of CRP on OGD-induced cardiomyocyte apoptosis, while rosiglitazone failed to
provide additional protection in the presence of CRP (Figure 5A, B). Similarly, the protective function of CRP in TGFβ-induced cardiac fibroblast activation was impaired by T0070907, and rosiglitazone did not further decrease CF proliferation and differentiation into myofibroblasts caused by TGFβ in combination with CRP (Figure 5C, D). Generally, these findings suggest that CRP attenuates OGD-induced cardiomyocyte apoptosis and TGFβ-induced cardiac fibroblast activation by maintaining the activation of PPARγ.

2.6 | CRP improves post-MI heart failure by activating PPARγ

Based on the data above, we explored whether PPARγ activation is involved in the protective effects of CRP in post-MI heart failure in vivo. Mice with MI were intraperitoneally injected with T0070907 intragastrically administered CRP. According to the echocardiography results, the LVEF and LVFS were increased by CRP treatment, while both were significantly decreased by T0070907 injection (Figure 6A).
The cardioprotective effect of CRP in cardiac hypertrophy after MI was also blocked by a PPARγ inhibitor (Figure 6B), as was the expression of fetal genes (ANP and BNP) (Figure 6C). Regarding cardiac fibrosis, the CRP-mediated reduction in the fibrotic area and downregulation of fibrotic molecule expression were reversed by T0070907 (Figure 7A–C). As shown in Figure 7D, T0070907 inhibited the ability of CRP to alleviate cardiac apoptosis after MI. Consequently, these results indicated that CRP exerts positive effects on post-MI heart failure by upregulating PPARγ expression.

3 | DISCUSSION

Reversing cardiac remodelling after MI is a major challenge worldwide. The efforts of scientists worldwide have led to a decrease in the death rate in the acute phase after MI. However, chronic injury after MI, pathological cardiac remodelling and heart failure result in high morbidity and mortality in MI patients. Currently, there are limited pharmacological therapies for heart failure after MI. Novel strategies are urgently needed.

CRP, a traditional Chinese herbal medicine used in the clinic for centuries, has anti-inflammatory, antioxidant, anticancer and other beneficial properties according to modern pharmacological studies. In addition, CRP may be effective for diseases affecting multiple systems, such as the digestive system, the respiratory system and particularly the cardiovascular system. Accumulating evidence suggests that CRP has cardioprotective effects. Our previous investigations have demonstrated that CRP can ameliorate cardiac hypertrophy and fibrosis induced by Ang II and ISO by upregulating PPARγ expression.

In this study, we found that CRP attenuated cardiac injury and improved cardiac function after MI by alleviating cardiac hypertrophy, fibrosis and apoptosis. In vitro, CRP not only decreased OGD-induced CM apoptosis but also inhibited TGF-β-stimulated CF proliferation and differentiation. Our findings suggested that CRP plays important roles in cardiac remodelling after MI and may be a new potential therapeutic target for post-MI heart failure.

Cardiac remodelling and heart failure after MI involve metabolic dysfunction, in which PPARγ is a pivotal regulator. PPARγ is a member of the PPAR nuclear receptor family and is expressed abundantly in multiple types of cells. Our results showed that PPARγ expression was downregulated in the hearts of mice with MI, OGD-induced CMs and TGF-β-stimulated CFs. Interestingly, PPARγ expression was upregulated by CRP treatment. In addition, T0070907, a known PPARγ inhibitor, completely blunted the inhibitory effect of CRP on cardiac dysfunction after MI, OGD-induced CM apoptosis and TGF-β-stimulated CF activation. However, rosiglitazone did not exert further protective effects in vitro in the presence of CRP, which indicated that rosiglitazone and CRP may work through the same pathway that is completely activated by either medicine alone. Therefore, CRP alleviates post-MI cardiac injury and heart failure via activation of PPARγ. Increasing evidence shows that activation of PPARγ can attenuate cardiac hypertrophy, alleviate cardiac fibrosis and decrease myocardial apoptosis, which is consistent with our findings. However, PPARγ activators have been found to have side effects, including fluid retention and oedema, as PPARγ was broadly used as a hypoglycaemic drug decades ago. No signs of oedema were observed in the mice treated with CRP, but whether CRP causes the other reported side effects of PPARγ activators needs to be elucidated in further studies.

CRP is a traditional herbal medicine with more than 140 ingredients mainly including hesperidin, nobiletin, tangeretin, 5,6,7,8,3′,4′-heptamethoxyflavone (HMF) and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (PMF). However, it is unclear which of these components play leading roles in the cardioprotective effects of CRP and whether these potential bioactive ingredients could activate PPARγ. Hesperidin is the most famous and effective ingredient among components in CRP. Accumulating evidence showed that hesperidin has positive effects on various diseases including acute liver injury, acute lung injury, tumours and diabetes. Moreover, hesperidin could protect against myocardial ischaemia, cardiac hypertrophy and myocardial toxicity by upregulating PPARγ expression, which is consistent with our findings (Figures S4 and S5). As shown in Figure S4B, C, hesperidin, nobiletin and tangeretin could alleviate CMs apoptosis induced and increase the expression of PPARγ. Furthermore, our results showed that hesperidin, nobiletin and tangeretin could preserve cardiac systolic function after MI and activate PPARγ (Figure S5A, B). Besides, one of our previous studies suggested that nobiletin attenuated pathological cardiac remodelling after MI via activating PPARγ which is identical to our findings here. As to tangeretin, several studies indicated that it played an important role in lipid metabolism by increasing PPARγ.
However, it is the first time that we identified that tangeretin could mitigate CMs apoptosis and heart failure after MI. Meanwhile, tangeretin could activate PPARγ both in vitro (Figure S4C) and in vivo (Figure S5B). Therefore, like hesperidin and nobiletin, tangeretin also contributed to cardiac benefits of CRP. Interestingly, HMF could obviously promote CMs proliferation (Figure S4A), but showed no effects on CMs apoptosis (Figure S4B). In a word, we believe that among the bioactive flavonoids in CRP, hesperidin, nobiletin and tangeretin play important roles in protection against CMs apoptosis and heart failure after MI. It is possible that these ingredients have cooperative effects with each other. Combination experiments of the three effective candidates are urgently needed in future studies.

In conclusion, we demonstrated that CRP attenuated cardiac injury after MI by activating PPARγ and provided a potential therapeutic way for patients who suffered from heart failure after MI.

4 | METHODS

4.1 | ETHICS STATEMENT

All procedures with animals of this study were in accordance with the Guidelines of Laboratory Animals for biomedical research published by National Institutes of Health (NIH publication, revised in 2011).
The experimental protocol was reviewed and approved by the ethical animal committees of Nanjing Medical University (licence number: IACUC-1903016).

### 4.2 Phytochemical analysis

To determine whether CRP used in our study contains bioactive flavonoids such as hesperidin, nobiletin, tangeretin, HMF and PMF, the sample was analysed by HPLC (Waters, Milford, MA, USA). HPLC condition: chromatographic column, Dima C18 (250 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile-0.1% formic acid; current speed, 1.0 mL/min; detection wavelength, 330 nm; gradient elution procedure, 0–15 min, 25–50% acetonitrile; 15–35 min, 50–60% acetonitrile; 35–40 min, 60–85% acetonitrile; column temperature, 35°C.

### 4.3 Animal models

Adult male C57BL/6 mice were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). The mice were randomly divided into groups. MI was established by ligation of the left anterior descending coronary artery (LAD) utilising a 7/0 silk thread. The sham group mice underwent the same process except...
FIGURE 7  Citri Reticulatae Pericarpium (CRP) attenuates cardiac fibrosis and apoptosis by upregulating PPARγ expression. (A) Masson trichrome staining ($n = 8,7,6,7$), (B) qRT–PCR ($n = 6$) and (C) Western blotting ($n = 6$) revealed that the increase in the fibrotic area, fibrotic gene expression and fibrotic protein expression in mice with myocardial infarction (MI) was attenuated by CRP administration, but the protective effects were reversed by T0070907. (D) Western blotting analysis showed that the increase in the Bax/Bcl2 ratio in the MI group declined after CRP treatment, but T0070907 inhibited the cardioprotective effect of CRP ($n = 6$). The data are presented as the mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Scale bar = 500 μm
LAD ligation. CRP was obtained from Shijiazhuang Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China). Hesperidin, nobiletin, tangeretin, HMF and PMF were purchased from Nanjing Ben Cao Co. Ltd. (Nanjing, China), and their purities were > 98%, as determined by HPLC. The animals were intragastrically administered CRP (0.25, 0.5 or 1.0 g/kg/day), hesperidin (0.3 g/kg/day), nobiletin (0.05 g/kg/day) or tangeretin (0.1 g/kg/day) for 3 weeks after MI. A PPARγ inhibitor (T0070907) was used to investigate the mechanism by which CRP protects against post-MI heart failure. CRP was administered after MI, and T0070907 was intraperitoneally injected (1 mg/kg/day) concurrently for 21 days.

4.4 | Echocardiography

At the experimental endpoint, left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were determined by echocardiography to assess the systolic function of the mouse heart. Isoflurane (1.5-2%) was applied to anaesthetise the mice, and transthoracic echocardiography was carried out by a Vevo 2100 instrument (Visual Sonics Inc, Toronto, Ontario, Canada) with a 30 MHz central frequency scan head.

4.5 | Masson trichrome staining

Heart tissues were fixed with 4% paraformaldehyde for 48 h and then embedded in paraffin. To assess the extent of fibrosis, heart sections were stained with Masson trichrome following a standard procedure. Images were captured by computer-assisted video densitometry and analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentage of fibrosis was calculated as the fibrotic area/total myocardial area x 100%.

4.6 | Wheat germ agglutinin (WGA) staining

Heart sections were subjected to WGA (1:200; Sigma, St. Louis, MO, USA) staining to evaluate the size of cardiomyocytes. Images were captured with a fluorescence microscope (Carl Zeiss, Thuringia, Germany). More than 10 fields of view from each section were analysed. The cardiomyocyte size was assessed utilising ImageJ software (National Institutes of Health).

4.7 | Neonatal cardiomyocyte (CM) and cardiac fibroblast (CF) isolation and culture

Neonatal Sprague–Dawley rats were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Heart tissues were gradually minced and digested in a mixed enzyme solution comprising 60% trypsin and 40% collagenase II. After approximately 10 digestions (usually 30 rats in total), the suspended cells were centrifuged and resuspended in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Pasadena, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (PS; Gibco) and then plated in a cell incubator (37°C, 5% CO2 and 95% O2). Nearly 1 h later, the CFs had attached to the culture plates and were ready for subsequent culture and passage. To obtain purer CMs, Percoll gradient centrifugation was used to further purify the unattached cells in the medium. Purified CMs were cultured in appropriate culture plates in DMEM containing 10% horse serum (HS; Gibco, Pasadena, CA, USA), 5% FBS and 1% PS.

4.8 | Cytotoxicity assay

To detect the cytotoxicity of hesperidin (0, 25, 50, 100 μM), nobiletin (0, 25, 50, 100 μM), tangeretin (0, 25, 50, 100 μM), HMF (0, 10, 25, 50 μM) and PMF (0, 5, 10, 20 μM) on CMs, cell counting kit-8 (CCK-8) assay was performed according to the manufacturer's instructions after CMs were respectively administrated with above five bioactive flavonoids for 48 h at different concentrations.

4.9 | Cell treatment

To mimic the lack of blood and oxygen in mice with MI, CMs were maintained in glucose-free Dulbecco's modified Eagle's medium (Gibco) in an anaerobic chamber containing 95% N2 and 5% CO2 at 37°C for 8 h to mimic oxygen–glucose deprivation (OGD). To explore the roles of CRP in OGD-induced cardiomyocyte injury, CMs were treated with CRP 2 days in advance at a dose of 0.25, 0.5 or 1.0 μg/mL. A PPARγ agonist (rosiglitazone, 1 μM) or T0070907 (1 μM) combined with CRP was added to the CM culture medium for 48 h before OGD to investigate whether CRP can protect CMs from OGD-induced injury by upregulating PPARγ expression. To study the anti-apoptotic roles of the five bioactive flavonoids in CRP, CMs were administrated with hesperidin (50 μM), nobiletin (50 μM) and tangeretin (50 μM), HMF (25 μM) and PMF (10 μM) for 48 h before OGD performance.

CFs were used in our study at passage 2. CFs were treated with recombinant human TGF-β (Peprotech, Rocky Hill, NJ, USA) for 24 h to study the roles of CRP in CF proliferation and differentiation into myofibroblasts. CFs were treated with CRP (0.5 μg/mL) for 48 h. To evaluate the mechanisms by which CRP alleviates TGF-β-induced CF activation, rosiglitazone or T0070907 was mixed with CRP and added to the cells 24 h before administration of TGF-β.

4.10 | Western blotting

Tissues or cells were harvested at the experimental endpoint and lysed with RIPA buffer (Beyotime, Nantong, China) with 1 mM PMSF (ST505; Beyotime). A bicinchoninic acid protein assay kit (Thermo Fisher, Waltham, MA, USA) was used to measure the protein
The authors state that there is no conflict of interest.
DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the article.

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