Molecular mechanism of Chuanxiong Rhizoma in treating coronary artery diseases

Bang-qiao Yin, Yu-hong Guo, Yuan Liu, Yang-yang Zhao, Shan-mei Huang, Xia-wei Wei, Heng-sheng Wang, Ruo-ya Liu, Ying Liu, Yao-ping Tang

Objective: Most of the studies on the herb Chuanxiong Rhizoma (CR) have focused on the L-arginine-nitric oxide (NO) pathway, but the nitrate-nitrite-NO (NO$_3^-$–NO$_2^-$–NO) pathway was rarely investigated. Therefore, the aim of this study was to evaluate the effects and mechanisms of action of CR in coronary artery disease (CAD).

Methods: The NO$_3^-$, NO$_2^-$ and NO levels were examined in the NO$_3^-$–NO$_2^-$–NO pathway. High-performance ion chromatography was used to quantify NO$_3^-$ and NO$_2^-$ levels. Then, NO was quantified using a multi-functional enzyme marker with a fluorescent probe. The tension of aortic rings was measured using a multi-myograph system.

Results: High content of NO$_3^-$ and low content of NO$_2^-$ was found in CR, and which could potently convert NO$_3^-$ to NO$_2^-$ in the presence of endogenous reductase enzyme. Incubating human coronary artery endothelial cells (HCAECs) with CR-containing serum showed that CR significantly decreased the NO$_3^-$ content and increased the levels of NO$_2^-$ and NO in the cells under hypoxic conditions. In addition, CR significantly relaxed isolated aortic rings when the L-arginine–NO pathway was blocked. The optimal concentration of CR for relaxation was 200 mg/mL.

Conclusion: CR supplements large amounts of NO in cells and vessels to achieve relaxation via the NO$_3^-$–NO$_2^-$–NO pathway, thereby making up for the deficiency caused by the lack of NO after the L-arginine-NO pathway is suppressed. This study also supports the potential use of a traditional Chinese herb for future drug development.

Original Article

1. Introduction

Nitric oxide (NO) is an important signaling molecule that plays an important role in treating coronary artery disease (CAD) via the NO pathway, thereby regulating endothelial cell function (Bian & Murad, 2014). NO also plays significant roles in the regulation of human biological networks such as relaxing blood vessels, regulating blood pressure and maintaining the balance of the intravascular environment (Mas-Capdevila et al., 2019; Oliveira-Paula et al., 2018). Two NO-generating pathways exist in endothelial cells. First, NO can be produced by endothelial NO synthase (eNOS) using L-arginine and oxygen molecules (O$_2$), which is a potent oxidant generated by endothelial cells. Second, NO is also generated via the nitrate-nitrite-NO (NO$_3^-$–NO$_2^-$–NO) pathway. However, previous studies showed that NO synthesis in the L-arginine-nitric oxide (NO) pathway decreases with decreasing O$_2$ concentrations (Carlstrom & Montenegro, 2019; Lundberg et al., 2008). Moreover, there are considerable studies confirming nitrate (NO$_3^-$) reductases exhibit nitrate-reducing capabilities in endothelial cells, which directly synthesizes NO enzymatically from nitrite (NO$_2^-$) (Nie et al., 2019). The NO$_3^-$–NO$_2^-$–NO pathway is gradually activated in low O$_2$ conditions (Sparacio-Watkins et al., 2012). The eNOS activity of vascular endothelial cells of the patients with CAD is inhibited. This leads to the lost eNOS activity in the endothelial cells and inhibition of the L-arginine-NO pathway (Erdogan et al., 2018). Therefore, this study attempts to confirm that when the L-arginine-NO pathway in CAD patients is inadequate to generate sufficient NO; An alternative, the NO$_3^-$–NO$_2^-$–NO pathway, can be used through the ingestion of Chinese herbal medicine to ameliorate this deficiency.

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Herbs like Salvia miltiorrhiza Bge. and Panax notoginseng (Burk.) F. H. Chen (Danshen and Sanqi in Chinese) are mainstream medicines for treating CAD in China because they are rich in both NO3 and NO2 (Tang et al., 2009). The dried rhizome of the umbelliferous plant Ligusticum chuanxiong Hort, called Chuanxiong Rhizoma (CR), has been widely and regularly used for the prevention and treatment of CAD in many Asian countries for many years (Zhang et al., 2019). CR is a commonly used herb that activates blood circulation during CAD treatment and is effective for cerebral ischemic stroke (Chen et al., 2019; Li et al., 2016). CR has been reported to prevent hepatic lipid accumulation and improve circulation in cerebral and diastolic blood vessels (Chan et al., 2007; Jiang et al., 2011). CR has also been developed as a clinical drug formulated mainly as capsules, dropping pills, and injections for the treatment of cardiovascular disease, Alzheimer’s disease, and multiple sclerosis (Chen et al., 2018; Guan et al., 2015). It is also a conventional Chinese medicine for the treatment of acute angina (Zhang et al., 2019). In addition, it has been reported to reduce apoptosis in human umbilical vein endothelial cells (Yang et al., 2016), protect endothelial cells (Mak et al., 2017), repair damaged blood vessels (Savaola & Grassi, 2012), and inhibit platelet activation (Badimon et al., 2016). Previous studies have revealed that S. miltiorrhiza, Trichosanthes kirilowii Maxim., Allium macrostemon Bge., P. notoginseng and other herbs have high reductase activity, and in patients with CAD, generate NO via the NO3–NO2–NO pathway rather than the l-arginine pathway (Tang et al., 2009). However, the mechanism of action of CR in the treatment of patients with CAD is not known.

CAD has become a major public health threat due to changes in human lifestyles (e.g. sedentary, high-fat diets). At present, there are nearly 400 types of traditional Chinese formulations for the treatment of CAD (Ding et al., 2016; Tang et al., 2009; Wang et al., 2019; Zhao et al., 2020). The hearts of patients with coronary atherosclerosis are hypoxic, and under such conditions, eNOS activity is inhibited and the l-arginine-NO pathway is blocked, but NO activity remains. We hypothesized that the therapeutic benefits of CR occur through the NO3–NO2–NO pathway by supplying a secondary source of NO to damaged vascular endothelial cells in various cardiovascular diseases. In this study, we evaluated the NO3/N02/NO bioactivity of CR in the NO3–NO2–NO pathway, which might account for its effects in the herbal extract and on human coronary artery endothelial cells (HCAECs) and aortic rings isolated from mice. Specifically, CR was prepared and subsequently analyzed for NO3 and NO2 content, reductive effects, and NO activity in cells; in addition, its effect on vascular tension was evaluated. This study might uncover a safe candidate for future drug development.

2. Materials and methods

2.1. Chemicals and cells

Vanadyltrichloride (VCl3), N-(1-naphthyl) ethylenediamine dihydrochloride, sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate (MgSO4), potassium dihydrogen phosphate (KH2PO4), sodium bicarbonate (NaHCO3), calcium chloride (CaCl2), glucose, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris-buffered saline plus Tween 20 (TBST), electrophoresis reagents, and the enhanced chemiluminescent substrate were bought from Solarbio Life Sciences and CWBio Co., Ltd., respectively (Beijing, China). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE gel), NO assays, BCA protein assay kits, phenylmethylsulfonyl fluoride (PMSF), and radioimmunoprecipitation assay (RIPA) lysis buffer were all purchased from Beyotime Technology Co., Ltd. (Shanghai, China). The polyvinylidene fluoride (PVDF) membranes were bought from Merck, Millipore Ltd. (Damstadt, Germany). Goat anti-mouse IgG secondary antibody (31430) was bought from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The NG-nitro-l-arginine methyl ester (l-NAME) was bought from Beyotime Technology Co., Ltd. (Shanghai, China). The Dulbecco’s Modified Eagle Medium (DMEM) and HCAECs were bought from the American Type Culture Collection ATCC (Rockville, MD, USA).

HCAECs were incubated in a 37 °C incubator with 5% CO2 according to the ATCC’s guidelines (Ren, Gao, & Liang, 2015). The HCAECs were cultured in DMEM added with 10% fetal bovine serum. The above HCAECs were cultured to the seventh generation and were seeded into 6-well plates at a density of 2 × 104/well. When the cell density reaches 90%, the cells will be intervened.

2.2. Animals

The animal experiments were approved by the Experimental Animal Ethics Committee of Guangxi Medical University (201806009). Male C57BL/6 mice, aged 11 weeks, were used in this study. The CR herb was obtained from Beijing Tongrentang (Beijing, China) and the extract was prepared by boiling at 100 °C for 5 min and then cooling to 35 °C for 20 min. The final concentration of the CR decoction was 100 mg/mL. The amount of CR fed to mice was determined using recommendations from the Food and Drug Administration (Administration, 2005). The daily dose of CR per kilogram of mice (mg/kg) = the daily dose of CR per kilogram of human (mg/kg) × human weight (kg) × 0.0026 (equivalent dose ratio)/mouse weight (kg). On average, a patient weighing 70 kg is given 15 g of CR herb and the average weight of a mouse is 20 g. Therefore, the daily dose of CR of mice equals 15,000 mg/70 kg × 70 kg × 0.0026/0.02 kg × 20 × 10^-3 kg × 40 mg. The CR was then orally administered to C57BL/6 mice at 40 mg/day. An equivalent volume of saline was administered to the control group.

Male C57BL/6 mice, aged 11 weeks, were fed with either 0.4 mL of CR or saline in controls after 7 d of adaptive feeding. Blood from orbits of mice were collected and centrifuged at 5000 × g for 15 min before being subjected to ether overdose for euthanization. CR-containing serum levels were obtained from the blood supernatant.

2.3. Determination of NO3/NO2 content in CR herb

The crude CR material was purchased from a Chinese herbal store (Beijing Tongrentang, Beijing, China), obtained it directly from the natural plant source. The NO3/NO2 levels were measured using high performance ion chromatography (HPIC). The CR suspension analyzed for NO3/NO2 content was prepared in a series of steps. The CR herb was firstly ground into a powder using a mortar and pestle, placed in a mixer, and stirred at high speed for 10 min. Then, 1 g of the powder was dissolved in 5 mL phosphate-buffered saline (PBS, pH 7.4) for 5 min. For the NO3/NO2 analysis, the suspension was mixed with PBS for 15 min to extract NO3/NO2. Methanol was added at 1:1 (volume percentage) ratio, the solution was shaken, and then centrifuged at 12,000 × g for 15 min. The supernatant was passed through a C18 (Carbon 18) column, an Ag (Argentum) column, and a Na (Sodium) column in turn. The amount of NO3/NO2 were tested by using a CIC-100 ion chromatograph (Qingdao Shengyu, Qingdao, China). The levels of NO3/NO2 in CR was determined by comparison with standards of known NO3/NO2 concentration.
2.4. Determination of reducing ability of CR herb

The reducing ability of CR was determined by measuring the NO\textsubscript{3} and NO\textsubscript{2} levels with the addition of 5 \mu g nitrate and nitrite as substrates, respectively. We tested the NO\textsubscript{2} content by adding 5 \mu g NO\textsubscript{3} or NO\textsubscript{2}, which was higher than the NO\textsubscript{3}/NO\textsubscript{2} content in the CR. The CR suspension was treated as previously indicated and was then loaded on to two separate multiple-well plates pre-loaded with 100 \mu M NO\textsubscript{3} and NO\textsubscript{2} solution (final concentration, 20 mg/L). The test CR solution (100 \mu L) was added to each plate as an intervention. The reducing ability of CR at 37 °C and 100 °C was also compared to the NO\textsubscript{2} content after a 40-min incubation using HPIC.

2.5. Determination of NO\textsubscript{3}/NO\textsubscript{2} content in HCAECs

The NO\textsubscript{3} and NO\textsubscript{2} levels in the HCAECs were measured using HPIC. Briefly, cells were grouped into aerobic and anaerobic groups. Anaerobic cells were prepared in anaerobic bags. The cells were subcultured into 24-well plates containing 0.2 mL of 100 \mu M L-NAME, 50 \mu L of L-NAME, and was then loaded on to two separate multiple-well plates pre-loaded with 100 \mu M NO\textsubscript{3} and NO\textsubscript{2} solution (final concentration, 20 mg/L). The test CR suspension (100 \mu L) was added to each plate as an intervention. The reducing ability of CR at 37 °C and 100 °C was also compared to the NO\textsubscript{2} content after a 40-min incubation using HPIC.

2.6. Determination of NO in L-NAME-treated HCAECs

A standard curve for NO was constructed using protocols described in the Supplementary materials (Table S1). A standard curve for NO was shown in Fig. S1. All procedures complied with the protocol of the NO fluorescent probe kit, DAF-FM DA/S0019 (Beyotime Technology Co., Ltd., Shanghai, China) and details of this procedure are provided in the Supplementary materials (Table S1).

2.7. Western blot analysis

To detect eNOS activity in the HCAECs (passage 8), HCAECs were incubated with 100 \mu M L-NAME and CR-containing serum for 24 h. HCAECs were then harvested, and pre-cooled PMSF and RIPA lysis buffer were pre-cooled and added to resuspend the HCAECs after treatment. The cells were then lysed using 37% power ultrasonication on a cycle with a 5-min run time and 10 s intervals. The NO\textsubscript{3} and NO\textsubscript{2} levels were detected after HCAECs were treated with 100 \mu M L-NAME and CR-containing serum for 24 h.

2.8. Arterial tension measurement

Male C57BL/6 mice, aged 11 weeks, were euthanized by anesthesia with an ether overdose, and the aortic rings were isolated as previously described (Tang et al., 2009). The arterial tension of the vessels was measured using a multi myograph system. Samples were incubated in L-NAME in an organ bath for 10 min and then 10 \mu g phenylephrine (PE; Mairuier Chemical Technology Co., Ltd., Shanghai, China) was added, followed by CR (10–200 mg/mL) with continuous mixing. Pre-contracted aortic rings from mice were treated either with water (control), or with 10 mg/mL, 20 mg/mL, 40 mg/mL, 80 mg/mL, 100 mg/mL and 200 mg/mL of CR, and the level of relaxation was determined. The effect of CR on vascular tension is expressed as percent of relaxation. Relaxation was measured and quantified using a multi myograph system (PowerLab) and data acquisition software (Chart version 5.2.2) after stabilization. All data reported are the average values of four vascular rings from three mice.

2.9. Statistical analysis

The statistical package for the social sciences (SPSS) software (version 22.0, IBM Corporation, NY, USA) was used to analyze all experimental data. The Student’s t-test was used to compare two groups, whereas a one-way analysis of variance (ANOVA) and the least-significant difference test were used for more than two groups. A P value < 0.05 based on three or more experiments was considered statistically significant.

3. Results

3.1. Determination of NO\textsubscript{3}/NO\textsubscript{2} levels in CR

The Chinese, Latin, and English names of the herbs according to the Chinese Pharmacopoeia are listed in Table 1. Frankincense and Red peony root are herbs commonly used to treat CAD and were therefore selected for comparison with CR in this study. The CR herb contained large amounts of NO\textsubscript{3} (108 \mu g/g) and a small amount of NO\textsubscript{2} (0.74 \mu g/g) (Table 1), whereas they were not detected in the control sample that without any Chinese herbs. The NO\textsubscript{3} content of CR was higher than that of other Chinese medicines such as Frankincense and Red peony root according to previous studies (Tang et al., 2009). Fig. S2 showed the native forms of CR mentioned in this study.

3.2. Reductive ability of CR

Adding exogenous NO\textsubscript{3} or NO\textsubscript{2} as a substrate will obscure the impact of NO\textsubscript{3} or NO\textsubscript{2} present in CR. Thus, the reductive ability of CR converted NO\textsubscript{3} to NO\textsubscript{2} can be detected to be unaffected by NO\textsubscript{3} or NO\textsubscript{2} in CR. The reductive ability of CR was shown in Fig. 1. When 5 \mu g of nitrate was added as substrate to the CR solution, CR at 37 °C showed higher reducing ability than the Control1 sample (P < 0.01) with NO\textsubscript{3} levels of 1.96 \mu g compared to 0 \mu g in the control, but CR at 100 °C showed no difference with Control1. In the presence of 5 \mu g nitrate as substrate, 2.26 \mu g NO\textsubscript{2} was detected at 37 °C, which was slightly lower than that in the Control2 (P < 0.01). The NO\textsubscript{3} content produced at 100 °C (5.21 \mu g NO\textsubscript{3} detected in CR at 100 °C) was a bit higher than that in the Control2, but the difference was not significant.

3.3. eNOS expression in L-NAME-treated HCAECs

Western blotting was performed to confirm that whether the L-arginine-NO pathway was blocked. This study confirms that CR...
produces NO through the NO$_3$–NO$_2$–NO pathway in CAD patients. Therefore, 100 μmol/L of the NOS inhibitor ʟ-NAME was used for HCAECs to block the ʟ-arginine-NO pathway. The lanes labelled Control 1 and Control 2 in Fig. 2 showed that eNOS was expressed without ʟ-NAME intervention under aerobic conditions, while eNOS activity was lost in anaerobic conditions. However, when ʟ-NAME was added to the HCAECs, none of the lanes (Lane 1 to Lane 4) showed an eNOS single band either in aerobic or anaerobic conditions. Lane 1 and Lane 2 in Fig. 2 showed that under aerobic conditions, the expression of eNOS was significantly inhibited with or without CR-containing serum intervention by ʟ-NAME. Lane 3 and Lane 4 in Fig. 2 also indicated that under anaerobic condition, there was no eNOS activity with or without CR-containing serum intervention. This WB assay was performed to prove that the ʟ-arginine-eNOS-NO pathway was blocked with or without CR treatment. Therefore, the NO produced in HCAECs could occur only passing through the NO$_3$–NO$_2$–NO pathway in these experiments.

3.4. Effects of CR on NO$_3$/NO$_2$ and NO activity in HCAECs

The results of the NO$_3$ measurements in HCAECs were shown in Fig. 3A. Under anaerobic condition, the cellular NO$_3$ content was decreased markedly after treatment with CR-containing serum than Control following treatment with serum lacking CR ($P<0.01$). While the cellular NO$_3$ content was increased slightly after treatment with CR-containing serum under aerobic conditions than Control ($P<0.01$; Fig. 3A). The NO$_2$ content increased to 2.5 mg/L under anaerobic conditions after treatment with CR-containing serum, which was higher than control with serum lacking CR ($P<0.01$; Fig. 3B). The NO$_2$ content did not increase under aerobic conditions after treatment with CR-containing serum, which was the same as that with control. As shown in Fig. 3B and C, the increased amount of NO was proportional to the decreased amount of NO$_2$. When the HCAECs were incubated with CR-containing serum, a significantly larger amount of NO (16.34 μmol/L) was generated compared to cells without CR-containing serum and cells cultured under aerobic conditions ($P<0.001$).

3.5. Effect of CR on vascular tension

To further determine the vasoactive properties of CR, 10–200 mg/mL of CR were added to isolated mouse aortic rings pre-contracted with ʟ-NAME and PE. Fig. 4A showed that in the
presence of L-NAME, which blocked the l-arginine-NO pathway, the relaxation percentages were significantly higher following the treatment with 200 mg/mL CR than with lower concentrations (100 mg/mL), which relaxed the blood vessels by 100% (P < 0.01).

The relaxant effects of different concentrations of CR on the vessels were shown in Fig. 4A with the maximum effect achieved when CR concentration is 200 mg/mL. Control was added an equal volume bath solution. The addition of oxyhemoglobin to the organ bath immediately inhibited the blood vessel relaxant effect of CR, proving that these effects were achieved by the generation of NO (Fig. 4B). Fig. 4B showed that compared to control without CR decoction intervened aortic rings (black line), the force of aortic rings treatment with 200 mg/mL CR (red line) almost decreased to 0 mN.

4. Discussion

To the best of our knowledge, this is the first study to detect the NO3/NO2 content of CR. Many Chinese herbs contain high concentrations of NO3 and NO2 (Salguero, 2007), which together act as an important natural regulator of cardiovascular function (Bryan, 2009; Gladwin et al., 2005). Previous studies have also confirmed that NO2 and NO3 protect against myocardial ischemia–reperfusion injury and play a role in repairing defective endothelial cells in mice (Hernandez-Resendiz et al., 2018). NO3 and NO2 also effectively alter endothelial dysfunction and reduce ischemia or reperfusion injury (Bryan et al., 2007; Stokes et al., 2009; Webb et al., 2004). Therefore, strict compliance with NO3 and NO2 therapy could effectively alleviate pain caused by angina. In the present study, we have determined the NO3/NO2 content of the CR herb. We found that it contained considerable amounts of NO3 and a relatively small amount of NO2. This provides a large source of substrate to generate NO in CAD patients.

In this study, the potential activation of CR by a reductase component has also been explored since the reductase activity of some Chinese herbs surpasses that of tissues by several-fold (Hord et al., 2007). The large amount of NO2 and some NO3. It was speculated NO3/NO2 from CR decoction entered the oral cavity of human beings. Then these high levels of NO3/NO2 were reduced to NO2 by anaerobic bacteria, which were then absorbed in the intestine according to previous study (Duncan et al., 1995; Lundberg et al., 2008). The large amount of NO2 which were absorbed in the intestine enters the blood and tissue under physiologic conditions of hypoxia to accelerate the generation of NO which then exerts its biological effects (Lundberg et al., 2004).

These results suggested that in order to obtain NO2 directly, herbal decoction treatment in the presence of oral bacteria could protect against myocardial ischemia–reperfusion injury (Lundberg et al., 2004), which together act as an important natural regulator of cardiovascular function (Bryan, 2009; Gladwin et al., 2005). Previous studies have also confirmed that NO2 and NO3 protect against myocardial ischemia–reperfusion injury and play a role in repairing defective endothelial cells in mice (Hernandez-Resendiz et al., 2018). NO3 and NO2 also effectively alter endothelial dysfunction and reduce ischemia or reperfusion injury (Bryan et al., 2007; Stokes et al., 2009; Webb et al., 2004). Therefore, strict compliance with NO3 and NO2 therapy could effectively alleviate pain caused by angina. In the present study, we have determined the NO3/NO2 content of the CR herb. We found that it contained considerable amounts of NO3 and a relatively small amount of NO2. This provides a large source of substrate to generate NO in CAD patients.

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be useful for patients. There have been reports of a strong association between intestinal microbial probiotics and atherosclerotic disease and that herbal decoction treatment can improve endothelial function (Gan et al., 2014; Malik et al., 2018). Therefore, herbal medicines can speed up the process of NO2 absorption. Then the infarcted hearts of patients showed NO bioactivity after NO2 absorption.

The reduction experiments also showed some unexpected results. When 5 μg of NO2 was added to the reaction solution as a substrate, the reduction in NO2 is actually equivalent to the amount of NO produced. This result demonstrated that the amount of NO2 contained in CR without boiling (37 °C) is lower than that in the Control2 (Fig. 1). This means that non-boiling CR had the ability to reduce NO2 to NO. Similarly, it is consistent with previous findings that some Chinese Herbs had nitrite reductase activity (Tang et al., 2009). However, the contents of NO2 in the boiled CR decoction (100 °C) did not decrease compared to the Control2, indicating that the boiled CR did not have the ability to reduce NO2 to NO. It leads NO2 accumulation, which become a large source of NO activity.

The contents of NO2/NO2 and NO in HCAECs were determined after treatment with CR. Adding the CR solution directly to HCAECs caused rapid cell death, so our experiments used CR-containing serum from the CR fed mice as a cell intervention. Western blotting confirmed that eNOS activity was inhibited by i-NAME. This confirms that the classical L-arginine-eNOS-NO pathway was blocked by i-NAME (Fig. 2). Under anaerobic conditions, this NO2–NO2–NO pathway is gradually activated (Sparacino-Watkins et al., 2012). Therefore, the NO2–NO2–NO pathway is the main pathway mediating NO production in HCAECs. Cellular-level analyses showed increased levels of NO2 and NO in hypoxic cells treated with CR-containing serum (Fig. 3B and C). The content of NO2 in HCAECs is much greater than that in CR-containing serum (Fig. 3A). Under hypoxic conditions, NO2/NO2 in HCAECs can be used as a physiological reservoir of NO, which has protective effects against cardiovascular diseases such as myocardial ischemia (Bryan & Ivy, 2015). Therefore, when CR-containing serum was added to the cells, NO2/NO2 in the cells and some from CR-containing serum are reduced to NO by certain component(s) in CR which possess reductive ability.

Adding i-NAME to the isolated aortic rings blocks the classical L-arginine-eNOS-NO pathway. This leads to the NO2–NO2–NO pathway taking over as the main pathway mediating NO production in aortic rings. The results showed that different concentrations of CR relaxed the aortic rings, indicating that robust NO bioactivity was induced (Fig. 4A). The blood vessels were relaxed to the highest level (100%) at the concentration of 200 mg/mL of CR (Fig. 4B). How CR produce NO to relax infarcted hear based on this study? Previous reports showed that under normal physiological conditions, approximately 25% of NO2 from the diet was actively absorbed by the salivary glands (Lundberg et al., 2008). Therefore, NO2 from CR must be reduced to NO2 by certain component(s) in CR which possess reductive ability. Otherwise, NO2 from common diets reduced to NO2 by a series of enzymes in the human body, but mammals lack these kinds of enzymes. It has previously been shown that it needs a series of enzymes covert NO2 to NO2 from bacteria. These include nitrate reductases and xanthine oxidoreductase from commensal anaerobic bacteria located at the back of the tongue in the oral cavity (Duncan et al., 1995; Lundberg et al., 2004). While CR decoction already contained generous NO2, which plays a critical role in maintaining NO homeostasis in the body (Qu et al., 2016). Moreover, NO2 is reduced by a series of reductase enzymes, which include hypoxia myoglobin and an electron transport chain enzyme, with the highest reductase activity occurring in the liver and aorta (Feeisch et al., 2008; Lundberg et al., 2009; Omar & Webb, 2014). The released NO from CR reaches the infarcted heart or arterial ring through the blood vessels to achieve a therapeutic effect.

We confirmed that CR protects the ischemic myocardial cells and produced NO bioactivity in the aortic rings through the NO2–NO2–NO pathway. Further studies are needed to identify the active ingredient in CR by determining the mechanisms mediating its NO2–NO2–NO pathway activity at both the cellular and organ level in a cardiovascular disease mouse model. This study provides information on a potential safe candidate for future drug development and contributes important insights into the effects of traditional Chinese herbs.

5. Conclusion

In this study, the mechanisms of action of CR for the clinical treatment and prevention of CAD at the physiological, biochemical, cellular, and tissue levels were investigated. The NO2, NO2 and NO content of CR was evaluated in the CR herbal extract as well as the human endothelial cells, and aortic rings isolated from mice. The NO bioactivity showed an increase. This confirms that the traditional Chinese herb CR can protect cells and isolated arterial rings through the NO2–NO2–NO pathway. It is also interesting to note that there is a reducing substance in CR at 37 °C has the ability to reduce NO2 to NO2 and reduce NO2 to NO. Although the specific reducing substance is uncertain, it was confirmed that is not from the reductase. Therefore, this study supports the potential use of the traditional Chinese herb CR for future drug development.

Author contributions

Y.T. and B.Y. conceived and designed the experiments. S.H., Y.G., Y.Z., Y.L., X.W., H.W. and R.L. performed the experiments. B.Y. and S.H. analyzed and interpreted the data. Y.T., and Y.L. contributed reagents, materials, analysis tools. B.Y. wrote and revised the paper. All authors read and approved the final manuscript.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2021.03.001.

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