Original Article

Molecular mechanism of *Chuanxiong Rhizoma* in treating coronary artery diseases

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ABSTRACT

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.

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 confiming 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 (Sparacino-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 NO_3^- and NO_2^- (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 cardio-cerebrovascular 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 (Savoia & 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 NO_3^-–NO_2^-–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 NO_3^-–NO_2^-–NO pathway by supplying a secondary source of NO to damaged vascular endothelial cells in various cardiovascular diseases. In this study, we evaluated the NO_3^-/NO_2^-/NO bioactivity of CR in the NO_3^-–NO_2^-–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 NO_3^- and NO_2^- 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

Vanadium trichloride (VCl_3), *N*-(1-naphthyl) ethylenediamine dihydrochloride, sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), 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 bougth 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% CO_2 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 2×10^4/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)/mice 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 NO_3^-/NO_2^- 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 NO_3^-/NO_2^- levels were measured using high performance ion chromatography (HPIC). The CR suspension analyzed for NO_3^-/NO_2^- 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 NO_3^-/NO_2^- analysis, the suspension was mixed with PBS for 15 min to extract NO_3^-/NO_2^-. Methanol was added at a 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 C_{18} (Carbon 18) column, an Ag (Argentum) column, and a Na (Sodium) column in turn. The amount of NO_3^-/NO_2^- were tested by using a CIC-100 ion chromatograph (Qingdao Shengyu, Qingdao, China). The levels of NO_3^-/NO_2^- in CR was determined by comparison with standards of known NO_3^-/NO_2^- concentration.

2.4. Determination of reducing ability of CR herb

The reducing ability of CR was determined by measuring the NO_3^- and NO_2^- levels with the addition of 5 μg nitrate and nitrite as substrates, respectively. We tested the NO_2^- content by adding 5 μg NO_3^- or NO_2^-, which was higher than the NO_3^-/NO_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 μL NO_3^- and NO_2^- solution (final concentration, 20 mg/L). The test CR solution (100 μL) 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_2^- content after a 40-min incubation using HPIC.

2.5. Determination of NO_3^-/NO_2^- content in HCAECs

The NO_3^- and NO_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 divided into six groups as follows. Group A1: without 100 μmol/L ʟ-NAME, under aerobic condition, without CR-containing serum; Group A2: without 100 μmol/L ʟ-NAME, under anaerobic condition, without CR-containing serum; Group B1: with 100 μmol/L ʟ-NAME, under aerobic condition, without CR-containing serum; Group B2: with 100 μmol/L ʟ-NAME, under aerobic condition, with CR-containing serum; Group C1: with 100 μmol/L ʟ-NAME, under anaerobic condition, without CR-containing serum; Group C2: with 100 μmol/L ʟ-NAME, under anaerobic condition, with CR-containing serum. In controls, HCAECs were treated with saline containing 100 μmol/L ʟ-NAME (Beyotime Technology Co., Ltd. Shanghai, China) for 24 h. 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_3^- and NO_2^- levels were detected after HCAECs were treated with 100 μmol/L ʟ-NAME and CR-containing serum for 24 h.

2.6. Determination of NO in ʟ-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 μmol/L ʟ-NAME and CR-containing serum for 24 h. HCAECs were then harvested, and pre-cooled PMSF and RIPA lysis buffer added to resuspend the HCAECs. The cells were subsequently lysed using 37% power ultrasonication on a cycle with a 5-min run time and 10-s intervals. After centrifugation at 12,000 × g for 20 min, the supernatant was collected for protein detection with a BCA protein assay kit. A standard curve was generated using bovine serum albumin. Protein samples (10 μg) were separated by 6% SDS-PAGE and transferred to a PVDF membrane. The eNOS and internal control β-actin were detected by incubation with anti-eNOS antibody (1:3000) and anti-β-actin antibody (1:3000). The electrophoresis and film transfer instruments were purchased from Bio-Rad (Hercules, CA, USA).

2.8. Arterial tension measurement

Male C57BL/6 mice, aged 11 weeks, were euthanized by anesthetization 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 ʟ-NAME in an organ bath for 10 min and then 10 μg 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_3^-/NO_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_3^- (108 μg/g) and a small amount of NO_2^- (0.74 μg/g) (Table 1), whereas they were not detected in the control sample that without any Chinese herbs. The NO_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_3^- or NO_2^- as a substrate will obscure the impact of NO_3^- or NO_2^- present in CR. Thus, the reductive ability of CR converted NO_3^- to NO_2^- can be detected to be unaffected by NO_3^- to NO_2^- in CR. The reductive ability of CR was shown in Fig. 1. When 5 μg 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_2^- levels of 1.96 μg compared to 0 μg in the control, but CR at 100 °C showed no difference with Control1. In the presence of 5 μg nitrite as substrate, 2.26 μg NO_2^- was detected at 37 °C, which was slightly lower than that in the Control2 ($P < 0.01$). The NO_2^- content produced at 100 °C (5.21 μg NO_2^- was 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 ʟ-NAME-treated HCAECs

Western blotting was performed to confirm that whether the ʟ-arginine-NO pathway was blocked. This study confirms that CR

Table 1
Nitrate and nitrite in some Chinese herbs.

Abbreviation	Chinese	Latin	English	Nitrate	Nitrite	References
CR	Chuanxiong	*Ligusticum chuanxiong* Hort.	*Chuanxiong Rhizoma*	108.89 ± 7.51	0.74 ± 0.09	This study
RX	Ruxiang	*Boswellia carterii* Birdw.	*Olibanum*	61.0 ± 6.00	0.98 ± 0.59	Tang et al., 2009
CS	Chishao	*Paeonia lactiflora* Pall.	*Paeoniae Raddix Rubra*	37.00 ± 1.50	0.12 ± 0.03	Tang et al., 2009

Data represent the averages of n = 5 ± SEM for nitrite and nitrate (each measurement was of fresh herb on separate days). Control: water; CR: *Chuanxiong Rhizoma*.
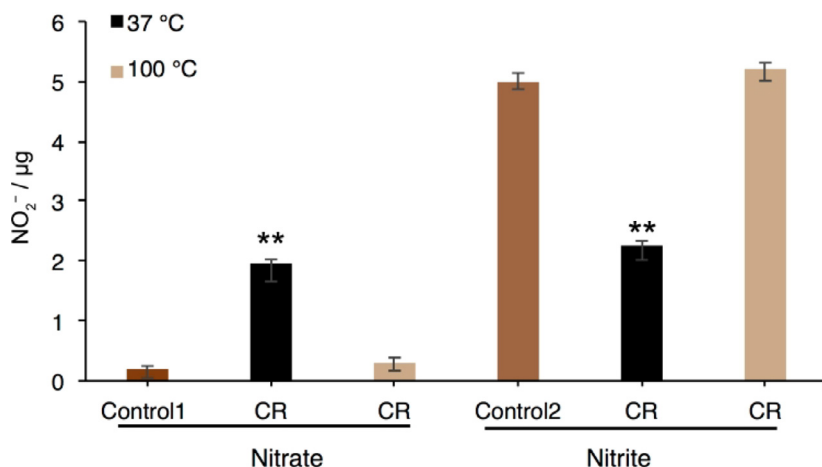

Fig. 1. Determination of NO_3^- and NO_2^- reducing ability of CR (Means ± SEM, n = 5). When 5 μg NO_3^- was added, **$P < 0.01$ CR (unheated at 37 °C) *vs* Control. When 5 μg NO_2^- was added as substrate, **$P < 0.01$ CR (unheated at 37 °C) *vs* Control2. Control1: mixed solution of methanol, PBS buffer and NO_3^-. Control2: mixed solution of methanol, PBS buffer and NO_2^-; CR: *Chuanxiong Rhizoma*; NO_3^-: nitrate, NO_2^-: nitrite.

produces NO through the NO_3^-–NO_2^-–NO pathway in CAD patients. Therefore, 100 μmol/L of the NOS inhibitor L-NAME was used for HCAECs to block the L-arginine-NO pathway. The lanes labelled Control 1 and Control 2 in Fig. 2 showed that eNOS was expressed without L-NAME intervention under aerobic conditions, while eNOS activity was lost in anaerobic conditions. However, when L-NAME was added to the HCAECs, none of the lanes (Lane 1 to Lane 4) showed an eNOS single band either in aerobic or anerobic 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 L-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 L-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 L-NAME and PE. Fig. 4A showed that in the

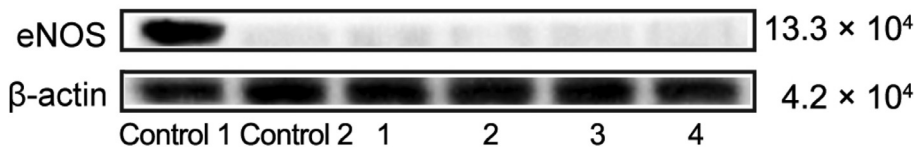**Fig. 2.** eNOS expression in L-NAME-treated HCAECs under aerobic/anaerobic conditions by Western blot. Control 1: HCAECs with no 100 μmol/L L-NAME and no CR-containing serum intervention under aerobic condition; Control 2: HCAECs with no 100 μmol/L L-NAME and no CR-containing serum intervention under anaerobic condition. Lane 1: HCAECs with 100 μmol/L L-NAME intervention under aerobic conditions, without CR-containing serum intervention; Lane 2: HCAECs with 100 μmol/L L-NAME intervention under aerobic conditions, but with CR-containing serum intervention. Lane 3: HCAECs with 100 μmol/L L-NAME intervention under anaerobic conditions, without CR-containing serum intervention; Lane 4: HCAECs with 100 μmol/L L-NAME intervention under anaerobic conditions, but with CR-containing serum intervention. CR: *Chuanxiong Rhizoma*; HCAECs: human coronary artery endothelial cells.
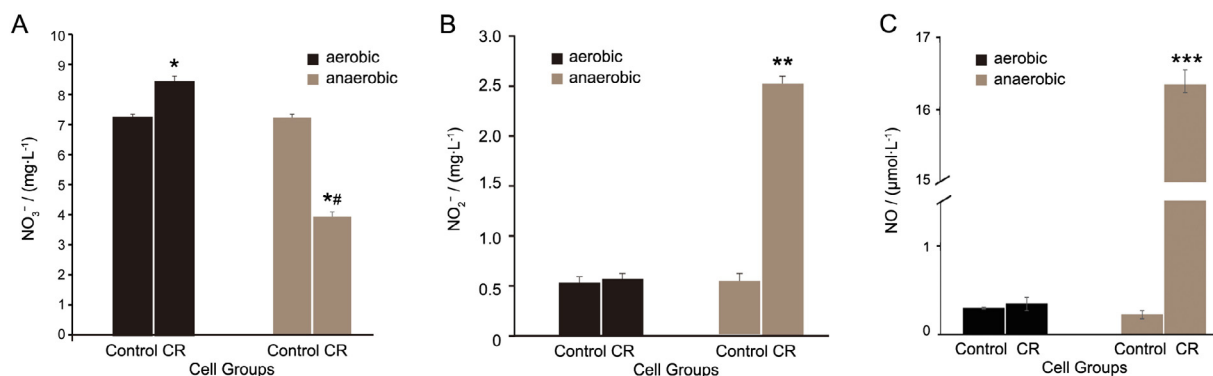
Fig. 3. Effects of CR on NO_3^-/NO_2^- and NO activity in HCAECs (Means ± SEM, $n = 5$). A, NO_3^- reduction after treatment with CR-containing serum in L-NAME (100 μmol/L)-treated HCAECs, *$P < 0.05$ CR (Blank) intervened under aerobic conditions vs Control (Blank) under aerobic conditions; **$P < 0.01$ CR (Brown) intervened under anaerobic condition vs Control (Brown) under anaerobic conditions. B, NO_2^- production after treatment with CR-containing serum in HCAECs. **$P < 0.01$ CR (Brown) intervened under anaerobic conditions vs Control (Brown). C, NO production after treatment with CR-containing serum in HCAECs. ***$P < 0.001$ CR (Brown) intervened under anaerobic conditions vs Control (Brown). Control: HCAECs without CR-containing serum intervention under aerobic/anaerobic conditions; CR: *Chuanxiong Rhizoma*.

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 NO_3^-/NO_2^- content of CR. Many Chinese herbs contain high concentrations of NO_3^- and NO_2^- (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 NO_3^- and NO_2^- protect against myocardial ischemia–reperfusion injury and play a role in repairing defective endothelial cells in mice (Hernandez-Resendiz et al., 2018). NO_3^- and NO_2^- 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 NO_3^- and NO_2^- therapy

could effectively alleviate pain caused by angina. In the present study, we have determined the NO_3^-/NO_2^- content of the CR herb. We found that it contained considerable amounts of NO_3^- and a relatively small amount of NO_2^-. 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., 2009). To determine the reductase activity of CR, body temperature (37 °C) and the boiling temperature (100 °C) were chosen for the reduction test. Interestingly, reduction experiments on NO_3^-/NO_2^- showed that the NO_2^- content of CR at 37 °C was much higher than that of Control1 and 100 °C (Fig. 1). This showed that the reducing ability was from endogenous reductases present in the herb because the enzymes in the decoction lost all reductase activity when boiled to 100 °C. This may be due to the fact that endogenous reductases of CR promote the reduction of NO_3^- to NO_2^-. This is worth exploring in future research. The CR had a large amount of NO_3^- and some NO_2^-. It was speculated NO_2^-/NO_3^- from CR decoction entered the oral cavity of human beings. Then these high levels of NO_3^- were reduced to NO_2^- 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 NO_2^- which were absored 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 NO_2^- directly, herbal decoction treatment in the presence of oral bacteria could
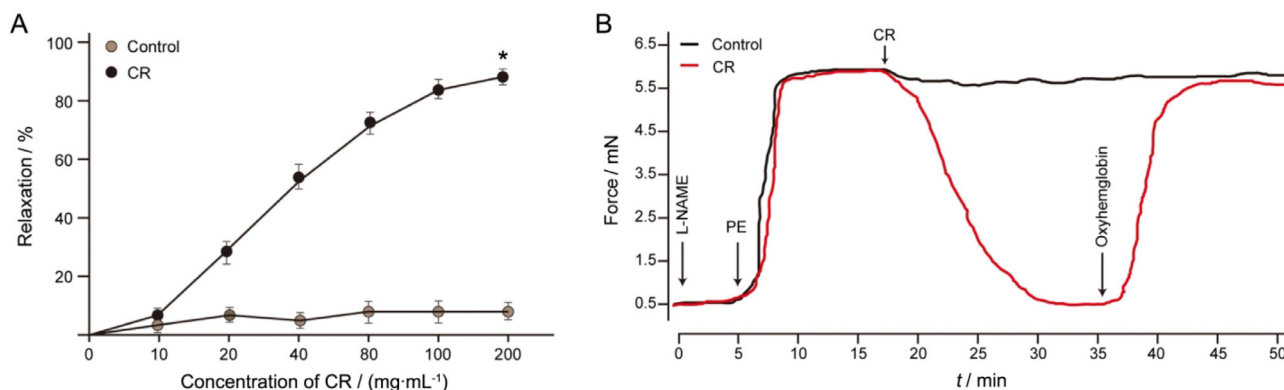

Fig. 4. Relaxation of vascular rings by CR (Means ± SEM, $n = 12$). **A**, Effect of different concentration of CR on vasodilation with L-NAME treatment. The rings were pre-treated with the eNOS-inhibitor L-NAME. The effect of CR on vascular tension is expressed as percent of relaxation. Data are mean ± SEM from the indicated numbers of experiments. *$P < 0.05$ CR with 200 mg/mL vs CR with 100 mg/mL. **B**, Detection of vasodilation by treatment with L-NAME, PE, 200 mg/mL concentration of CR and oxyhemoglobin. Control: water; CR: *Chuanxiong Rhizoma*. eNOS: nitric oxide synthase. The n value indicated independent vascular group experiments.

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 NO_2^- absorption. Then the infarcted hearts of patients showed NO bioactivity after NO_2^- absorption.

The reduction experiments also showed some unexpected results. When 5 μg of NO_2^- was added to the reaction solution as a substrate, the reduction in NO_2^- is actually equivalent to the amount of NO produced. This result demonstrated that the amount of NO_2^- 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 NO_2^- 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 NO_2^- 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 NO_2^- to NO. It leads NO_2^- accumulation, which become a large source of NO activity.

The contents of NO_3^-/NO_2^- 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 *L*-NAME. This confirms that the classical *L*-arginine-eNOS-NO pathway was blocked by *L*-NAME (Fig. 2). Under anaerobic conditions, this NO_3^-–NO_2^-–NO pathway is gradually activated (Sparacino-Watkins et al., 2012). Therefore, the NO_3^-–NO_2^-–NO pathway is the main pathway mediating NO production in HCAECs. Cellular-level analyses showed increased levels of NO_2^- and NO in hypoxic cells treated with CR-containing serum (Fig. 3B and C). The content of NO_3^- in HCAECs is much greater than that in CR-containing serum (Fig. 3A). Under hypoxic conditions, NO_3^-/NO_2^- 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, NO_3^-/NO_2^- in the cells and some from CR-containing serum are reduced to NO by certain component(s) in CR which possess reductive ability.

Adding *L*-NAME to the isolated aortic rings blocks the classical *L*-arginine-eNOS-NO pathway. This leads to the NO_3^-–NO_2^-–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 NO_3^- from the diet was actively absorbed by the salivary glands (Lundberg et al., 2008). Therefore, NO_3^- from CR must be reduced to NO_2^- by certain component(s) in CR which possess reductive ability. Otherwise, NO_3^- from common diets reduced to NO_2^- 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 NO_3^- to NO_2^- 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 NO_3^-, which plays a critical role in maintaining NO homeostasis in the body (Qu et al., 2016). Moreover, NO_2^- 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 (Feelisch 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 NO_3^-–NO_2^-–NO pathway. Further studies are needed to identify the active ingredient in CR by determining the mechanisms mediating its NO_3^-–NO_2^-–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 NO_3^-, NO_2^- 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 NO_3^-–NO_2^-–NO pathway. It is also interesting to note that there is a reducing substance in CR at 37 °C has the ability to reduce NO_3^- to NO_2^- and reduce NO_2^- 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.

Acknowledgments

Appendix A. Supplementary data

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

References

Administration, F.D. (2005). Guidance for industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers.

Badimon, L., Suades, R., Fuentes, E., Palomo, I., & Padro, T. (2016). Role of Platelet-Derived Microvesicles as crosstalk mediators in atherothrombosis and future pharmacology targets: A link between inflammation, atherosclerosis, and thrombosis. *Frontiers in Pharmacology, 7*, 293.

Bian, K. a., & Murad, F. (2014). What is next in nitric oxide research? From cardiovascular system to cancer biology. *Nitric Oxide, 43*, 3–7.

Bryan, N. S. (2009). Cardioprotective actions of nitrite therapy and dietary considerations. *Frontiers in Bioscience-Landmark, 14*, 4793.

Bryan, N. S., Calvert, J. W., Elrod, J. W., Gundewar, S., Ji, S. Y., & Lefer, D. J. (2007). Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America, 104*(48), 19144–19149.

Bryan, N. S., & Ivy, J. L. (2015). Inorganic nitrite and nitrate: Evidence to support consideration as dietary nutrients. *Nutrition Research, 35*(8), 643–654.

Carlstrom, M., & Montenegro, M. F. (2019). Therapeutic value of stimulating the nitrate-nitrite-nitric oxide pathway to attenuate oxidative stress and restore nitric oxide bioavailability in cardiorenal disease. *Journal of Internal Medicine, 285*(1), 2–18.

Chan, S. K., Cheng, T. Y., & Lin, G. e. (2007). Relaxation effects of ligustilide and senkyunolide A, two main constituents of *Ligusticum chuanxiong*, in rat isolated aorta. *Journal of Ethnopharmacology, 111*(3), 677–680.

Chen, R., Wu, P., Cai, Z., Fang, Y., Zhou, H., Lasanajak, Y. i., et al. (2019). *Puerariae Lobatae Radix* with *Chuanxiong Rhizoma* for treatment of cerebral ischemic stroke by remodeling gut microbiota to regulate the brain-gut barriers. *Journal of Nutritional Biochemistry, 65*, 101–114.

Chen, Z., Zhang, C., Gao, F., Fu, Q., Fu, C., He, Y., et al. (2018). A systematic review on the rhizome of *Ligusticum chuanxiong* Hort. (Chuanxiong). *Food and Chemical Toxicology, 119*, 309–325.

Guan, D., Su, Y., Li, Y., Wu, C., Meng, Y. i., Peng, X., et al. (2015). Tetramethylpyrazine inhibits CoCl$_2$-induced neurotoxicity through enhancement of Nrf2/GCLc/GSH and suppression of HIF1α/NOX2/ROS pathways. *Journal of Neurochemistry, 134*(3), 551–565.

Ding, Y. F., Peng, Y. R., Shen, H., Shu, L., & Wei, Y. J. (2016). Gualou Xiebai decoction inhibits cardiac dysfunction and inflammation in cardiac fibrosis rats. *BMC Complementary and Alternative Medicine, 16*, 49.

Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., et al. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nature Medicine, 1*(6), 546–551.

Erdogan, M., Kulaksizoglu, M., Tetik, A., Solmaz, S., Kucukaslan, A. S., & Eroglu, Z. (2018). The relationship of the endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) gene polymorphism in Turkish type 2 diabetic patients with and without diabetic foot ulcers. *The Foot, 37*, 5–10.

Feelisch, M., Fernandez, B. O., Bryan, N. S., Garcia-Saura, M. F., Bauer, S., Whitlock, D. R., et al. (2008). Tissue processing of nitrite in hypoxia: An intricate interplay of nitric oxide-generating and -scavenging systems. *Journal of Biological Chemistry, 283*(49), 33927–33934.

Gan, X. T., Ettinger, G., Huang, C. X., Burton, J. P., Haist, J. V., Rajapurohitam, V., et al. (2014). Probiotic administration attenuates myocardial hypertrophy and heart failure after myocardial infarction in the rat. *Circulation-Heart Failure, 7*(3), 491–499.

Gladwin, M. T., Schechter, A. N., Kim-Shapiro, D. B., Patel, R. P., Hogg, N., Shiva, S., et al. (2005). The emerging biology of the nitrite anion. *Nature Chemical Biology, 1*(6), 308–314.

Hernandez-Resendiz, S., Munoz-Vega, M., Contreras, W. E., Crespo-Avilan, G. E., Rodriguez-Montesinos, J., Arias-Carrion, O., et al. (2018). Responses of endothelial cells towards ischemic conditioning following acute myocardial infarction. *Conditioning Medicine, 1*(5), 247–258.

Hord, N. G., Tang, Y., & Bryan, N. S. (2009). Food sources of nitrates and nitrites: The physiologic context for potential health benefits. *American Journal of Clinical Nutrition, 90*(1), 1–10.

Jiang, F., Qian, J., Chen, S., Zhang, W., & Liu, C. (2011). Ligustrazine improves atherosclerosis in rat via attenuation of oxidative stress. *Pharmaceutical Biology, 49*(8), 856–863.

Li, H., Zhang, C., Fan, R., Sun, H., Xie, H., Luo, J., et al. (2016). The effects of Chuanxiong on the pharmacokinetics of warfarin in rats after biliary drainage. *Journal of Ethnopharmacology, 193*, 117–124.

Lundberg, J. O., Gladwin, M. T., Ahluwalia, A., Benjamin, N., Bryan, N. S., Butler, A., et al. (2009). Nitrate and nitrite in biology, nutrition and therapeutics. *Nature Chemical Biology, 5*(12), 865–869.

Lundberg, J. O., Weitzberg, E., Cole, J. A., & Benjamin, N. (2004). Nitrate, bacteria and human health. *Nature Reviews Microbiology, 2*(7), 593–602.

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery, 7*(2), 156–167.

Mak, S. K., Yu, C. M., Sun, W. T., He, G. W., Liu, X. C., & Yang, Q. (2017). Tetramethylpyrazine suppresses angiotensin II-induced soluble epoxide hydrolase expression in coronary endothelium via anti-ER stress mechanism. *Toxicology and Applied Pharmacology, 336*, 84–93.

Malik, M., Suboc, T. M., Tyagi, S., Salzman, N., Wang, J., Ying, R., et al. (2018). *Lactobacillus plantarum* 299v supplementation improves vascular endothelial function and reduces inflammatory biomarkers in men with stable coronary artery disease. *Circulation Research, 123*(9), 1091–1102.

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Aragonès, G., Aleixandre, A., Bravo, F., et al. (2019). Evidence that nitric oxide is involved in the blood pressure lowering effect of the peptide AVFQHNCQE in spontaneously hypertensive rats. *Nutrients, 11*(2), 225.

Nie, Q. u., Zhu, L., Zhang, L., Leng, B., & Wang, H. (2019). Astragaloside IV protects against hyperglycemia-induced vascular endothelial dysfunction by inhibiting oxidative stress and Calpain-1 activation. *Life Sciences, 232*, 116662.

Oliveira-Paula, G. H., Lacchini, R., Pinheiro, L. C., Ferreira, G. C., Luizon, M. R., Garcia, W. N. P., et al. (2018). Endothelial nitric oxide synthase polymorphisms affect the changes in blood pressure and nitric oxide bioavailability induced by propofol. *Nitric Oxide, 75*, 77–84.

Omar, S. A., & Webb, A. J. (2014). Nitrite reduction and cardiovascular protection. *Journal of Molecular and Cellular Cardiology, 73*, 57–69.

Qu, X. M., Wu, Z. F., Pang, B. X., Jin, L. Y., Qin, L. Z., & Wang, S. L. (2016). From nitrate to nitric oxide: The role of salivary glands and oral bacteria. *Journal of Dental Research, 95*(13), 1452–1456.

Ren, Y., Gao, X. P., & Liang, H. (2015). Atorvastatin prevents angiotensin II-induced high permeability of human arterial endothelial cell monolayers via ROCK signaling pathway. *Biochemical and Biophysical Research Communications, 459*(1), 94–99.

Salguero, C. P. (2007). Medieval Chinese medicine: The Dunhuang medical manuscripts. *Medical History, 51*(1), 126–127.

Savoia, C., & Grassi, G. (2012). Exercise activity and endothelial function: The uprising role of endothelial progenitor cells in vascular protection. *Journal of Hypertension, 30*(11), 2083–2084.

Sparacino-Watkins, C. E., Lai, Y. C., & Gladwin, M. T. (2012). Nitrate-nitrite-nitric oxide pathway in pulmonary arterial hypertension therapeutics. *Circulation, 125*(23), 2824–2826.

Stokes, K. Y., Dugas, T. R., Tang, Y., Garg, H., Guidry, E., & Bryan, N. S. (2009). Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *American Journal of Physiology. Heart and Circulatory Physiology, 296*(5), H1281–H1288.

Tang, Y., Garg, H., Geng, Y. J., & Bryan, N. S. (2009). Nitric oxide bioactivity of traditional Chinese medicines used for cardiovascular indications. *Free Radical Biology and Medicine, 47*(6), 835–840.

Wang, H., Bao, Q., Yi, H., & Xia, Q. (2019). The evaluation of embryotoxicity of *Ligusticum chuanxiong* on mice and embryonic stem cells. *Journal of Ethnopharmacology, 239*, 111895. https://doi.org/10.1016/j.jep.2019.111895.

Webb, A., Bond, R., McLean, P., Uppal, R., Benjamin, N., & Ahluwalia, A. (2004). Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proceedings of the National Academy of Sciences of the United States of America, 101*(37), 13683–13688.

Yang, C., Xu, Y., Zhou, H., Yang, L., Yu, S., Gao, Y., et al. (2016). Tetramethylpyrazine protects CoCl$_2$-induced apoptosis in human umbilical vein endothelial cells by regulating the PHD2/HIF/1alpha-VEGF pathway. *Molecular Medicine Reports, 13*(2), 1287–1296.

Zhang, X., Feng, Z. M., Yang, Y. N., Jiang, J. S., & Zhang, P. C. (2019). Bioactive butylphthalide derivatives from *Ligusticum chuanxiong. Bioorganic Chemistry, 84*, 505–510.

Zhao, Y. L., Hua, F., Li, W., Gong, S., Deng, J. J., Wang, D. J., et al. (2020). Bioassay of anti-gel bioactivity of *Chuanxiong Rhizoma* and related Chinese patent medicines. *Chinese Traditional and Herbal Drugs, 50*(2), 469–473.