Chinese Herbal Cardiotonic Pill Stabilizes Vulnerable Plaques in Rabbits by Decreasing the Expression of Adhesion Molecules

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Abstract: The cardiotonic pill (CP), consisting of a mixture of Radix Salviae Miltiorrhizae, Radix Notoginseng, and Borneolum Syntheticum, has been widely used in the prevention and treatment of cardiovascular disease. Adhesion molecules, including intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1, are involved in the development of vulnerable plaque. We investigated the effect of the CP in a rabbit model of vulnerable plaque established by local transfection with p53 gene. Compared with the control group, rabbits with vulnerable plaque showed a significantly lower intima-media thickness and plaque burden after CP treatment for 12 weeks. Moreover, the reduction in rate of plaque rupture and vulnerability index was similar. On enzyme-linked immunosorbent assay, real-time polymerase chain reaction, and immunohistochemistry analysis, the expression of intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 was inhibited with CP treatment. CP treatment could postpone atherosclerotic plaque development and stabilize vulnerable plaque by inhibiting the expression of adhesion molecules in treatment of cardiovascular disease.

Key Words: cardiotonic pill, vulnerable plaque, adhesion molecule

INTRODUCTION

Myocardial infarction is a frequent and fatal disease. Vulnerable plaque is an important etiological factor in acute myocardial infarction. The accumulation of inflammatory cells, such as macrophages and T cells, is a characteristic of vulnerable plaque. Adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), are involved in monocyte immigration and inflammation. VCAM-1 and ICAM-1 expression is up-regulated by an atherogenic diet, with oxidized low-density lipoprotein (LDL) and native LDL both playing an important role. Recruitment of monocytes into atherosclerosis-prone areas could be regulated by ICAM-1. In animal models, pre-treatment with antibodies or gene knockout of ICAM-1 reduced the number of macrophages homing into atherosclerotic lesions. In human coronary atherosclerotic plaques, the expression of VCAM-1 and ICAM-1 and the numbers of macrophages and T cells are both increased. These results suggest that VCAM-1 and ICAM-1 may participate in vulnerable plaque. Analysis of correlation between adhesion molecules and maximal intima-media thickness (IMT) suggests that VCAM-1 is a good biohumoral correlate of overt atherosclerosis.

Research into atherosclerosis treatment has achieved a great deal. Many herbs have been found to prevent cardiovascular disease at many steps in the atherosclerotic process. Radix Salviae miltiorrhizae (Chinese herb Danshen), Radix Notoginseng (Sanqi), and Borneolum Syntheticum (Bing Pian) are used by Tianjin Tansy (Tianjin, China) to produce the cardiotonic pill (CP). The CP has been used to treat cardiovascular disease for ~8 years. In ApoE-deficient mice, the CP can downregulate VCAM-1 expression and inhibit atherosclerotic plaque development. In any cells, such as smooth muscle cells, endothelial cells, and fibroblasts, the CP can inhibit cell proliferation and attenuate ICAM-1 and VCAM-1 expression induced by tumor necrosis factor α and in a rat model of myocardial infarction CP was effective. After 12 weeks, administration of CP to men and women with mild-to-moderate elevation in lipid levels is associated with reduced expression of the ICAM-1. The inhibitory effects of Salviae miltiorrhizae on the production of inflammatory mediators are accomplished by inhibiting the nuclear translocation of NF-κB. The CP can interfere in the atherosclerosis progress.

However, whether the CP can stabilize vulnerable plaque in atherosclerosis is unknown. We aimed to explore the effects of the CP on vulnerable plaque in a rabbit model and investigate the possible mechanism.
MATERIALS AND METHODS

Animal Model and Experimental Groups

All animal care and experimental protocols complied with the Animal Care Committee of Shandong University. We used our previously established rabbit model of vulnerable plaque. A total of 45 male New Zealand white rabbits weighing 1.5–2.5 kg were fed an atherogenic diet containing 1% cholesterol (120–140 g/d) for 12 weeks after abdominal aortic balloon injury. At the end of week 12, the rabbits were randomly divided into 3 groups for treatment: low-dose CP (LCP, 5 pills/d), high-dose CP (HCP, 10 pills/d), and control (placebo). At the end of week 12, the groups were switched to an ordinary diet for 12 weeks.

At the end of week 24, all animals were anesthetized by intravenous injection of 30 mg/kg sodium pentobarbital, the abdominal cavity was opened and the abdominal aorta was exposed. To establish the model of vulnerable plaque, under the guidance of intravascular ultrasonography, 0.1 mL of 2.245 × 10⁶ pfu/mL recombinant vector Ad-p53 was injected in the vessel between the right renal and the common iliac arteries showing the largest plaque. The suspension was left in situ for 10 minutes after temporary ligation of the aortic segment. The site of injection was marked, and the abdominal cavity was closed. At the end of week 25, pharmacological triggers induced plaque rupture: 0.15 mg/kg Chinese Russells Viper Venom (Snake Venom Institute of Guangzhou, China) was injected intraperitoneally, then 30 minutes later, 0.02 mg/kg histamine (Sigma, St. Louis, MO) was given intravenously. The animals were killed after 2 triggers of plaque rupture.

Lipid and Inflammatory Factor Study

At the beginning of the experiment and at the end of weeks 12 and 24, blood samples were collected from all rabbits. Serum samples were stored at −80°C for 24 weeks before assay. Serum levels of total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), and LDL cholesterol (LDL-C) were measured by enzymatic methods (TGs), high-density lipoprotein cholesterol (HDL-C), and LDL cholesterol (LDL-C) were measured by enzymatic assays. High sensitivity C-reactive protein (hsCRP), ICAM-1, and vascular cell adhesion molecule 1 (VCAM-1) were assayed by use of high sensitivity enzyme-linked immunosorbent assay (ELISA) (BPB Laboratory).

Abdominal Aortic Ultrasonography and Doppler Measurements

At the beginning of the experiment and at the end of weeks 12 and 25, all rabbits underwent abdominal aortic ultrasonography 3 times with use of an HP Sonos 7500 (HP, Palo Alto, CA) ultrasonic imaging instrument and a 7.5-MHz transducer. Aortic longitudinal and transversal axis views were obtained; IMT (mm) and end-systolic diameter (Ds, mm) were measured. Doppler flow measurement was performed to derive the aortic peak velocity (Vp, cm/s). Ultrasonic integrated backscatters (IBS) from the aortic wall and atherosclerotic plaques were analyzed by the acoustic densitometry technique. The average ultrasonic intensities (AI) of the aortic intima and adventitia in normal segments and atherosclerotic plaques were measured, and the corrected AI was derived by calculating the ratio of AI of the intima to AI of the adventitia.

Intravascular Ultrasonography

Intravascular ultrasonography (IVUS) was performed twice, at the end of week 24 and at the end of week 25, before and after pharmacological triggering, by use of a 3.2-F catheter containing a single rotating-element transducer of 40 MHz connected to an IVUS system (Galaxy, Boston Scientific). After the catheter reached the aortic arch, it was withdrawn by use of a motorized pullback device at a constant speed of 0.5 mm/s. We measured the following parameters in cross-sectional images: external elastic membrane area (EEMA, mm²), lumen area (mm²), plaque area (PA = EEMA − lumen area, mm²), and plaque burden (PB% = PA/EEMA × 100%, %). Plaque rupture was determined by visualizing an echolucent zone within a plaque separated from the lumen by a thin echo-reflecting structure representing the fibrous cap. Intramural thrombus was depicted as intense granular or finely speckled echo reflections that scintillate during real-time imaging and appear mobile with blood flow. The IVUS images were reviewed by 2 independent observers, who were blinded to the study. And the values were averaged for data analysis.

Histopathology and Immunohistochemistry

The abdominal aorta specimens were harvested located between the right renal and the common iliac arteries. Isolated abdominal aortic segments were fixed in 4% formaldehyde for 3–4 hours, with some segments embedded in paraffin and cut into 5-μm-thick sections for staining with hematoxylin and eosin (H&E) or for immunohistochemistry. Cryosections were cut into 8-μm-thick sections for Oil-red O staining. In brief, after deparaffinization and hydration, endogenous peroxidase block was immersed in 3% H₂O₂ for 10 minutes at room temperature. After adding goat serum blocking solution for 30 minutes, mouse anti-α-actin (MAB1522, 1:400; Chemicon) and monoclonal antibodies against rabbit macrophages (RAM11, 1:400; Lab Vision Neomakers) were added and incubated overnight at 4°C. After subsequent washing, biotinylated secondary antibody was added, and color development was carried out following the manufacturer’s instructions. Control slides were treated with mouse IgG instead of primary antibody. Histopathological slides were analyzed by use of Image-Pro Plus 5.0. The fibrous cap thickness was measured at 10 equidistant points around the cap in each slice; 3 slices per section were measured, and the values were averaged. The area of positive staining of lipids, collagen, and macrophages was expressed as a percentage of the staining area divided by the PA in at least 10 high-power fields (×400). The vulnerability index (VI) was calculated as follows: (macrophage staining % + lipid staining %)/(smooth muscle cell % + fiber %). Plaque rupture was defined as thrombosis overlying fissured plaques or fibrous caps buried inside a plaque.

Real-time Polymerase Chain Reaction

Tissue samples were frozen with liquid nitrogen. Total RNA was extracted with use of TRIZol reagent (Invitrogen, Carlsbad, CA), quantified by spectrophotometry, and reverse transcribed with the use of the Moloney Murine Leukaemia
Statistical Analysis

Data are expressed as mean ± SEM and analyzed with use of SPSS v13.0 for Windows (SPSS, Inc, Chicago, IL). The incidence of plaque rupture among groups was compared by \( \chi^2 \) test. The other data were assessed by one-way ANOVA. A \( P < 0.05 \) was considered statistically significant.

RESULTS

Rabbit Parameters

Three rabbits died due to diarrhea because of high cholesterol, so 42 rabbits were randomly divided into 3 groups, and another 3 died during anesthesia at the end of week 24. A total of 39 rabbits completed the experiments: 14 in the LCP group, 13 in the HCP group, and 12 in the control group. At the end of week 12, the weight of all rabbits was increased significantly from baseline, from 1.76 ± 0.14 to 2.98 ± 0.26 kg (\( P < 0.01 \)). Body weight did not change significantly from the end of weeks 12–24 (2.98 ± 0.26 vs. 3.05 ± 0.25 kg, \( P > 0.05 \)).

Lipid Levels

At the end of week 12, levels of TC, TGs, LDL-C, and HDL-C were significantly higher in all rabbits than at baseline (all \( P < 0.01 \), Table 1), with no significant difference between the 3 groups (all \( P > 0.05 \)) at baseline and at the end of week 12. At the end of week 24, levels of TC, TGs, LDL-C, and HDL-C were significantly lower in the 3 groups than at the end of week 12 (all \( P < 0.01 \)). Compared with the control group, the LCP and HCP groups showed lower LDL-C level (\( P < 0.05–0.01 \), Table 2), and the HCP group showed lower TC and TG levels (\( P < 0.01 \), Table 2). The HCP group showed lower TC, TG, and LDL-C levels than the LCP group (\( P < 0.05–0.01 \)).

Inflammatory Factor Levels

At the end of week 12, hsCRP, ICAM-1, and VCAM-1 levels were significantly higher in all rabbits than at baseline (all \( P < 0.01 \), Table 1), with no significant difference between the 3 groups (all \( P > 0.05 \)) at baseline and at the end of week 12. At the end of week 24, hsCRP, ICAM-1, and VCAM-1 levels were significantly lower in all 3 groups than at the end of week 12 (all \( P < 0.01 \)). Compared with the control group, the LCP and HCP groups showed lower hsCRP, ICAM-1, and VCAM-1 levels (all \( P < 0.01 \), Table 2); the HCP group showed lower ICAM-1 and VCAM-1 levels (\( P < 0.01 \)) but not hsCRP level than the LCP group.

Abdominal Aortic Ultrasonography and Doppler Analysis

At week 12, IMT and Ds were significantly higher in all animals than at baseline (both \( P < 0.01 \), Table 1), and Vp and corrected AII were significantly lower than at baseline (both \( P < 0.01 \)), with no significant difference among the 3 groups.

### TABLE 1. Difference in Lipid and Inflammatory Factors and Ultrasonography Results in Rabbits Between Baseline and the End of Week 12

| Parameter          | Control Group (n = 12) | LCP Group (n = 14) | HCP Group (n = 13) |
|--------------------|------------------------|--------------------|--------------------|
| TC (mmol/L)        | 1.55 ± 0.03            | 22.29 ± 0.59*      | 1.58 ± 0.02        |
| TGs (mmol/L)       | 0.91 ± 0.12            | 2.22 ± 0.09*       | 1.03 ± 0.04        |
| LDL-C (mmol/L)     | 0.46 ± 0.02            | 21.74 ± 0.55*      | 0.50 ± 0.01        |
| HDL-C (mmol/L)     | 0.33 ± 0.02            | 1.12 ± 0.07*       | 0.33 ± 0.01        |
| IMT (mm)           | 0.36 ± 0.02            | 1.29 ± 0.08*       | 0.36 ± 0.01        |
| Ds (mm)            | 4.00 ± 0.18            | 6.17 ± 0.25*       | 4.15 ± 0.11        |
| Vp (cm/s)          | 95.14 ± 2.38           | 70.70 ± 1.87*      | 97.81 ± 1.88       |
| AIIc%              | 80.17 ± 1.46           | 63.92 ± 0.94*      | 78.64 ± 0.46       |
| hsCRP (mg/mL)      | 109.99 ± 2.99          | 166.43 ± 6.28*     | 110.15 ± 1.67      |
| ICAM-1 (pmol/mL)   | 242.28 ± 3.82          | 331.78 ± 4.18*     | 241.13 ± 4.24      |
| VCAM-1 (mmol/L)    | 5.73 ± 0.21            | 11.63 ± 0.35*      | 5.63 ± 0.15        |

Data are expressed as mean ± SEM.

*Compared with baseline, \( P < 0.01 \).

AIIc%, corrected AII.
Compared with the control group, the LCP and HCP groups showed lower IMT (both $P$, 0.05–0.01, Table 2). The HCP group showed significantly faster Vp than the LCP and control groups (both $P < 0.05$–0.01) and lower IMT and Ds than the LCP group (both $P < 0.01$).

**FIGURE 1.** Intravascular ultrasonography results after treatment. A, Control group; (B) LDCP group; (C) HDCP group; (D) plaque rupture. White arrow: fractured intima.

**TABLE 2.** Difference in Lipid and Inflammatory Factors and Ultrasonography Results in Rabbits at the End of Week 24

| Parameter     | Control Group (n = 12) | LCP Group (n = 14) | HCP Group (n = 13) |
|---------------|------------------------|--------------------|--------------------|
| TC (mmol/L)   | 8.68 ± 0.66            | 8.00 ± 0.55        | 3.48 ± 0.24*†      |
| TG (mmol/L)   | 1.14 ± 0.11            | 0.96 ± 0.04        | 0.67 ± 0.06*†      |
| LDL-C (mmol/L)| 6.48 ± 0.51            | 5.43 ± 0.13†       | 3.00 ± 0.20*†      |
| HDL-C (mmol/L)| 0.55 ± 0.04            | 0.51 ± 0.02        | 0.55 ± 0.02        |
| IMT (mm)      | 0.84 ± 0.03            | 0.64 ± 0.02*       | 0.48 ± 0.02*†      |
| Ds (mm)       | 5.76 ± 0.14            | 5.79 ± 0.09        | 4.99 ± 0.12*†      |
| Vp (cm/s)     | 92.03 ± 2.77           | 87.05 ± 2.19       | 99.72 ± 1.88††     |
| AIIc%         | 66.42 ± 0.81           | 68.36 ± 0.94       | 71.85 ± 1.01§      |
| hsCRP (ng/mL) | 154.55 ± 3.69          | 138.73 ± 2.13*     | 137.78 ± 4.91††    |
| ICAM-1 (pmol/L)| 269.79 ± 3.30         | 247.47 ± 1.98*     | 223.95 ± 2.76††    |
| VCAM-1 (nmol/L)| 7.77 ± 0.20           | 6.87 ± 0.08*       | 5.65 ± 0.14*†      |

Data are expressed as mean ± SEM.

* $P < 0.01$, compared with the control group.
† $P < 0.05$, compared with the control group.
‡ $P < 0.05$, compared with the LCP group.
§ $P < 0.05$, compared with the LCP group.

AIIc%, corrected AII.
Intravascular Ultrasonography

Figure 1 shows results of IVUS imaging of the 3 groups, a direct sign of plaque rupture being fractured intima and an indirect sign being low echoic thrombus in lumen of vessels (Fig. 1D). Compared with the control group, the LCP and HCP groups showed lower EEMA, PA, and PB% (all \( P < 0.05 \)–\( 0.01 \), Fig. 2A); the HCP group showed lower PA than the LCP group (\( P < 0.01 \), Fig. 2A).

Histopathology and Immunohistochemistry

Plaque rupture could be found with thrombosis overlying fissured plaques (Fig. 2B) or fibrous caps buried inside a plaque. The LCP and HCP groups showed less lipid core, less macrophage cells in plaque, more smooth muscle cells, and thicker caps than the control group (Figs. 3A–D), as well as lower VI (both \( P < 0.01 \), Fig. 2C). The total plaque rupture rate after pharmacological triggering at the end of week 25 was 43.59% [control group, 9/12 (75%); LCP group, 5/14 (35.71%); and HCP group, 3/13 (23.08%)]. The rate differed among the 3 groups (\( P < 0.05 \)) but not between the LCP and HCP groups.

The LCP and HCP groups showed significantly less positive staining for ICAM-1 and VCAM-1 than the controls (Fig. 4).

Real-time Polymerase Chain Reaction Analysis of Cytokines

The LCP and HCP groups showed lower expression of ICAM-1 and VCAM-1 than the control group (all \( P < 0.01 \), Table 2). In addition, the HCP group showed significantly lower ICAM-1 and VCAM-1 levels than the LCP group (both \( P < 0.01 \)).

DISCUSSION

We studied whether treatment with the CP, used for cardiovascular treatment, has an effect on vulnerable plaque and found that the CP could stabilize vulnerable plaque by decreasing ICAM-1 and VCAM-1 expression and modulating lipid levels in rabbits with vulnerable plaque. Treating the rabbits with CP could downregulate IMT, PA, and PB% because CP can inhibit the development of atherosclerotic plaque. In addition, the VI and the rate of plaque rupture were significantly lower with CP treatment than with control treatment, which indicates that the CP could stabilize vulnerable plaque. From these results, we conclude that the CP can inhibit and stabilize vulnerable plaque by decreasing ICAM-1 and VCAM-1 expression.

Atherosclerosis is an inflammatory disease, and the recruitment of inflammatory cells is involved in early
FIGURE 3. H&E staining, Oil-red O staining, and immunohistochemical staining of macrophages and α-actin in atherosclerotic lesions of the abdominal aorta, vulnerability index analysis and anatomy specimen of rupture plaque. A, H&E staining of the control group (A1), LCP group (A2), HCP group (A3); (B) Oil-red O staining of control group (B1), LCP group (B2), HCP group (B3); (C) macrophage immunohistochemical staining of the control group (C1), LCP group (C2), HCP group (C3); (D) α-actin immunohistochemical staining of the control group (D1), LCP group (D2), HCP group (D3). Bar = 100 μm.
atherosclerotic development, predominantly mediated by cellular adhesion molecules such as ICAM-1 and VCAM-1. Endothelial cells, smooth muscle cells, and leukocytes can upregulate the adhesion molecule gene expression. ICAM-1 and VCAM-1 mediate the attachment of leukocytes, such as monocytes and T cells, to endothelium. ICAM-1 deficiency protects against the development of atherosclerosis. Partial deficiency of VCAM-1, whose total absence is lethal to the embryo, is also protective against atherosclerosis. Vulnerable plaques typically have a thin fibrous cap, large lipid-rich core, and abundant macrophages. Inflammatory cell accumulation is a characteristic of vulnerable plaque. ICAM-1 can induce the regulation of monocyte recruitment into atherosclerosis-prone areas. Observations of human coronary atherosclerotic plaques suggest that VCAM-1 and ICAM-1 may participate in the development of vulnerable plaque. In addition, analysis of the association of adhesion molecule expression and IMT suggests that VCAM-1 is a good biohumoral correlate of overt atherosclerosis.

The CP is a mixture of Radix Salviae miltiorrhizae, Radix Notoginseng, and Borneolum Syntheticum. Radix Salviae miltiorrhizae is one of the most popular herbs for treating cardiovascular disease. It can reduce LDL and TG levels and inhibit the expression of adhesion molecules. Radix Notoginseng is said to adjust the supply and demand of oxygen, which increases coronary blood flow and reduces myocardium consumption. As we know, the CP contains multiple active chemical constituents. However, evidence is lacking of the effect of the CP on vulnerable plaque. The CP can inhibit smooth muscle cell proliferation and attenuate ICAM-1 and VCAM-1 expression in vitro. It can also downregulate VCAM-1 expression and inhibit atherosclerotic plaque development in vivo. All this evidence suggests that the CP might have an effect on vulnerable plaque, and adhesion molecules might be involved in this pathophysiological process. In our study, serum lipid levels were lower with CP treatment than with control treatment. In addition, IMT, PA, PB%, and rate of plaque rupture showed a similar reduction with CP treatment, which suggests a positive effect of the CP on vulnerable plaque. With CP treatment for 12 weeks, the expression of ICAM-1 and VCAM-1 was low, and the development of atherosclerotic plaque was postponed, even reversed. So decreasing ICAM-1 and VCAM-1 expression with CP treatment could be a novel target for inhibiting atherosclerosis development.

FIGURE 4. Immunohistochemical staining of ICAM-1 and VCAM-1 in atherosclerotic lesions of abdominal aorta. A, ICAM-1 staining of control group (A1), LCP group (A2), HCP group (A3); (B) VCAM-1 staining of control group (B1), LCP group (B2), HCP group (B3). Bar = 100 μm.
In our study, CP could reduce the circulating levels of ICAM-1 and VCAM-1, and protein and mRNA expression of ICAM-1 and VCAM-1 in atherosclerotic plaque was down-regulated, with high-dose CP showing better effects. The effects of CP on reducing LDL and TG levels have been shown.23 In this study, we also showed the CP as reducing the circulating LDL-C level. Native LDL-C and oxidized LDL both upregulated VCAM-1 and ICAM-1 expression.5,26 In animal models, the CP can inhibit atheroscleroplaque development with VCAM-1 expression downregulation, but the mechanism was not clear.9 The CP can attenuate ICAM-1 and VCAM-1 expression induced by tumor necrosis factor α.12–15 These suggest that CP may be associated with inflammation. In our study, we could not show whether CP inhibits the expression of ICAM-1 and VCAM-1 directly or through lowering serum lipid levels, but we did show CP with effects on vulnerable plaque. Further experiments are needed to address this problem. And the reason could be found through many studies which focus on the bioactive components in CP and the elucidation of their targets.16

In conclusion, our study revealed that the CP could postpone and stabilize vulnerable plaque by decreasing the expression of ICAM-1 and VCAM-1 as well as lowering serum lipid levels, with high-dose CP possibly having a better effect than low-dose CP.

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