The study on therapy effect of Danqi pill to renin-angiotensin-aldosterone system (RAAS) and lipid metabolism disorder in coronary heart disease

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Accepted 3 September, 2012

The objective of this work is to inquire whether the myocardial ischemia (MI) could lead to plasma lipid metabolism disorders and Danqi Pill (DQP)’s pharmacological effects on coronary heart disease (CHD). Ameroid ring was placed on left anterior descending coronary artery (LAD) to prepare CHD model of chronic MI on Chinese mini-swine. Echocardiography was employed to measure cardiac function. Enzyme-linked immunosorbent assay (ELISA) and Western blot was used to detect key molecules such as nitric oxide (NO), angiotensin II (Ang II), aldosterone (Ald), oxidized-low density lipoprotein (ox-LDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) and NADPH oxidase (NOX4). Radioimmunoassay (RIA) was applied to evaluate the level of reactive oxygen species (ROS). Lipid metabolism disorder was mediated by MI, represented by the up-regulation of ox-LDL, LDL and VLDL, caused by RAAS activation. Plasma Ang II and Ald in model group were increased while NO decreased by 25.20%. NOX4 and ROS were also up-regulated significantly. Compared with model group, plasma Ang II and Ald in DQP group were decreased, while ox-LDL and LDL decreased, respectively and NO decreased by 47.60%. NOX4 and malondialdehyde (MDA) almost returned to normal level. Echocardiograph showed that ejection fraction (EF) improved significantly, with the improvement of left ventricular end-diastolic diameter (LVEDd). MI can lead to plasma lipid metabolism disorders and RAAS activation. DQP can down-regulate plasma Ang II and Ald, thus to reduce the ox-LDL and LDL level mediated by NOX4-ROS pathway; it also can increase NO levels, eventually promote heart function.

Key words: Danqi pill, myocardial ischemia, lipid disorder, renin-angiotensin-aldosterone system (RAAS).

INTRODUCTION

Coronary heart disease (CHD) remains the major cause of death for adults worldwide (He et al., 2005). Lipid metabolism disorder is an independent risk factor for CHD (Jaswal et al., 2011); lipid peroxidation induced by lipid infiltration is considered to be the main pathological mechanism of myocardial ischemia (MI) caused by coronary stenosis and atherosclerosis (Stephen et al., 2002). Even some critical proteins in lipid metabolism are used as new drug targets to treat or reduce the risk of CHD (Frayn et al., 2005).

That is to say, lipid disorder can eventually lead to MI. But whether the MI can induce the plasma lipid metabolism disorder in turn is poorly studied. Meanwhile, renin-angiotensin-aldosterone system (RAAS) activation is one of the most important cause contributing to ventricular hypertrophy and cardiac remodeling (Madamanchi et al., 2005), some researchers showed that they can induce vascular endothelial dysfunction thus to mediate lipid metabolism disorders (Papaharalambus and Griendling, 2007), these changes indicate that RAAS play...
the central roles in CHD. Traditional Chinese medicine (TCM) has effective remedies against CHD and its related diseases for more than 1000 years. It provides a complementary and alternative treatment, and has definite clinical effects. The TCM patent prescription Danqi pill (DQP), a basic formula contains 2 Chinese herbs (Salvia miltiorrhiza Bunge and Panax notoginseng), is widely produced in China and strictly fulfill the China Pharmacopoeia standard of quality control (Ministry of Health of the People’s Republic of China Pharmacopoeia Committee, 2005), and is commonly used in routine treatment of CHD of clinical practice in China. It contains large-scale epidemiological survey in the randomized, controlled clinical trials with a definite effect on improving heart function (Gong and Zhang, 2009), while a lot of studies are carried out to investigate in active monomers among them, and make great progress, for example, tanshinone IIA (monomer of S. miltiorrhiza Bunge) is found in cardioprotective effects and attenuating myocardial hypertrophy (Tu et al., 2009), however, monomer pharmacological effects cannot present overall efficacy of the whole formula, studies involving all the compounds are rarely carried out.

Therefore, the study on relationship between RAAS and lipid disorder can eliminate the potential drug targets for the formula, and understand the pathological mechanisms of CHD, thus, to provide prevention and treatment for CHD.

In this experiment, the model of MI was induced by constriction of left anterior descending coronary artery (LAD) in miniature swine. Angiotensin (Ang II), aldosterone (Ald), NADPH oxidase (NOX4), reactive oxygen species (ROS), oxidized low density lipoprotein (ox-LDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), nitric oxide (NO) and heart function in each group were estimated, the efficacy of DQP was evaluated, thus, to reveal the relationship between lipid disorder and the RAAS, and to find pharmacological targets of DQP.

MATERIALS AND METHODS

Eighteen (18) healthy Chinese miniature swines (25 ± 4 kg), which were obtained from China Agricultural University (SYXK: 2008-0007), were divided randomly into sham-operated, model and DQP group with 6 swine in each group. All animals were maintained and treated in accordance with the Principles of Laboratory Animal Care, the medicine were formulated by the National Society for Medical Research, and the experiments were guided by the Care and Use of Laboratory Animals, published by the National Institutes of Health and local laws about laboratory animal care. The local ethics committee of Beijing University of Chinese Medicine specifically approved this study (Wang et al., 2012).

Animal model of CHD was induced as follows (Guo et al., 2007): Diazepam and ketamine were administered intravenously to induce anesthesia. After the animal was intubated, isoflurane (0.5 to 3.0%) in oxygen (40 to 100%) was administered to maintain general anesthesia. A 5 to 6 cm left thoracotomy was then performed in the fifth intercostal space.

The pericardium was opened along the atrioventricular groove, and the LAD was dissected free at its bifurcation from the left main coronary artery. An ameroid constrictor was placed on the LAD artery by a cardiovascular surgeon blinded to the angiographic measurements, and the chest was closed in the standard fashion. The swines were transferred to the intensive care unit and monitored for signs of distress or pain.

Analogic agents were administered as needed. All the treatments previously described were also performed in control group animals but for placement of ameroid constrictor. At the end of the experiment, the heart was harvested and rapidly frozen in liquid nitrogen; blood samples were harvested quickly and stored in -80°C for subsequent detection.

Echocardiography detection

Echocardiography was used to detect left ventricular end-systolic diameter (LVEDs), left ventricular end-diastolic diameter (LVEDd), ejection fraction (EF), fractional shortening (FS) of operated-animals in 4, 8 and 12 weeks. A PST 65A sector scanner (8-MHz probe, HP, America) was used, which generated two-dimensional images at a frame rate ranging from 300 to 500 frames/s. Left ventricular dimension (LVD) was measured by M-model, and FS% was calculated by using the equation as follows:

\[ FS\% = \frac{(LVEDd - LVEDs)}{LVEDd} \times 100 \]

Preparation and dose consideration of DQP

The DQP used in the present study were manufactured by Tongren Tang (Beijing, China, Z11020471) using 2 Chinese herbs at a composition of 1:1(150 g S. miltiorrhiza Bunge and 150 g P. notoginseng).

Briefly, after extraction with 95% ethanol, the residue of S. miltiorrhiza Bunge and P. notoginseng were mixed, extracted by hot water twice/2 h, and water extract was concentrated till paste, then ethanol were added into paste. After 24 h, the filtration was collected to form the final product. Based on the recommendation of daily human dosage (20 g/d) and the equivalent conversion between animal and people by body surface area, dosage of 25.68 g/kg was chosen in the present study. It was given to animals from 4 to 8 weeks after surgery.

Determination of plasma malondialdehyde (MDA) by radioimmunoassay (RIA)

The plasma was homogenized in saline containing enzyme inhibitor [0.3 M ethylene diamine tetraacetic acid (EDTA)-Na 10 μl, 0.34 M 8-hydroxyquinoline 10 μl, 0.32 M dimercaproproanol 5 μl] (1 ml blood) on ice. The homogenate was centrifuged at 8000 × g for 10 min.

The supernatant was used for determination of MDA which can reflect the level of ROS using a RIA kit (Beijing Kangyuan Ruide Biotechnology Co. Ltd., Beijing, China) following the instructions of the company.

Measurement of plasma indicators by enzyme-linked immunosorbent assay (ELISA)

Levels of indicators were quantified in duplicate using commercial ELISA kits (Crystal Chem Inc., Downer’s Grove, USA). Each assay was performed following the related instructions. Standards at a series of concentrations were run in parallel with the samples. The concentrations in the samples were calculated in reference to the corresponding standard curves and expressed as ng/ml.
Measurement of indicators by western blot

The cardiac tissue was homogenized in RIPA buffer [50 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS)] and total protein was extracted from this homogenate. The protein concentration in each sample extract was measured using a protein assay kit (Pierce Company, USA, lot number: MB155207A) and then was adjusted to the same value in all samples with 2X 4% SDS sample buffer. The samples were boiled for 5 min followed by loading on a 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel (50 mg protein/10 μl per well) for electrophoresis using a Bio-Rad mini gel apparatus at 100 V for 2 h. The fractionated protein on the gel was transferred onto NC membrane (Beijing Pu Lilai Gene Technology Co., Ltd, Beijing, China) and electrophoresed at 300 mA for 90 min. The membrane was first probed with NOX4 primary antibody (Abcam, 1:500; Rabbit polyclonal to NOX4 antibody, ab19134, Abcam, 1:500) and secondary antibody (donkey polyclonal secondary antibody to rabbit IgG-HRP, ab97064, Abcam, 1:5000), and then treated with ECL plus western blotting detection reagent (ECL; GE Healthcare, America) for 1 min at room temperature. The bands in the membrane were visualized and analyzed using UVP Biolimage Systems. After obtaining the NOX4 blot density, the membrane was then treated using Restore Western Blot Stripping Buffer (Thermo Scientific, America) to remove the NOX4 signal, followed by probing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies (GAPDH mouse monoclonal IgG, ab8245, Abcam, 1:2000). The final reported data were the normalized NOX4 band densities by GAPDH.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical analysis was carried out on three or more groups using one-way analysis of variance (ANOVA) and Dunnett’s test. The values of P < 0.05 were considered statistically significant.

RESULTS

Pathology

Hematoxylin-eosin (HE) staining of sham group showed neat and clear myocardial cells while CHD model control group swines showed large area of disordered, swelling, degeneration and necrosis of myocardial cells in myocardial infarction area. Meanwhile, disorder, swelling, degeneration and necrosis cells in DQP group were less than the model group after treatment with DQP for 4 weeks (Figure 1).

Echocardiographic studies

Eight (8) weeks after surgery, echocardiography showed that, compared with the sham-operated animals, model group exhibited the expansion of LVEDd and LVEDs dimensions and volume. The front wall of the LVEDd and LVEDs thickness decreased, especially the change of front wall of the LVEDd thickness was more significant. In contrast, sham group were all normal. EF and FS values in the model group were significantly different (P< 0.05) when compared with sham group. EF value of model group dropped down to 29.41% compared with sham group, suggesting a steady CHD model is established. After treated by DQP for 4 weeks, the EF value recovered by 18.75% compared with model group. What’s more, DQP seemed to improve the LVED d in some distant (P < 0.05), the others indicators did not show any statistic significance (Figure 2 and Table 1).

Plasma concentration of Ang II, Ald and NO

ELISA results showed that the levels of Ang II and Ald in CHD model control group up-regulated by 63.41 and 56.25%, respectively when compared with control (P < 0.05). However, the NO concentration in plasma which reflected endothelial functions decreased by 25.20% (P < 0.01). After treatment with DQP for 4 weeks, 35.49 and 28.00% reduction of Ang II and Ald were detected in DQP

Figure 1. The change of HE staining in different group (×200). A, Sham group; B, model group; C, Danqi pill group.
The change of echocardiograph of 8 weeks swines in different groups. A, Sham group; B, model group; C, DQP group.

Table 1. Echocardiographic changes observed in different groups (\(\bar{x} \pm s\)).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>LVEDd (cm)</th>
<th>LVEDs (cm)</th>
<th>EDV (ml)</th>
<th>ESV (ml)</th>
<th>EF</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6</td>
<td>3.21 ± 0.25*</td>
<td>2.08 ± 0.09*</td>
<td>9.11 ± 2.49*</td>
<td>24.67 ± 8.02*</td>
<td>0.68 ± 0.06*</td>
<td>37.39 ± 5.19*</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>4.16 ± 0.21▲</td>
<td>2.97 ± 0.56▲</td>
<td>24.3 ± 18.27▲</td>
<td>44.5 ± 14.74▲</td>
<td>0.48 ± 0.03▲</td>
<td>30.68 ± 4.55▲</td>
</tr>
<tr>
<td>Danqi pill</td>
<td>6</td>
<td>3.81 ± 0.32*</td>
<td>2.72 ± 0.50</td>
<td>24.62 ± 10.17</td>
<td>46.43 ± 17.96</td>
<td>0.57 ± 0.03*</td>
<td>33.84 ± 6.90</td>
</tr>
</tbody>
</table>

ps, ▲ P < 0.05, versus sham-operated group; * P < 0.05, versus model group.

Table 2. The change of Ang II, Ald and NO in different groups (\(\bar{x} \pm s\)).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Ang II (pg/ml)</th>
<th>Ald (ng/ml)</th>
<th>NO (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>6</td>
<td>231.38 ± 56.99*</td>
<td>0.32 ± 0.18*</td>
<td>66.03 ± 4.32*</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>378.10 ± 97.82▲</td>
<td>0.50 ± 0.05▲</td>
<td>49.39 ± 7.03▲</td>
</tr>
<tr>
<td>Danqi pill</td>
<td>6</td>
<td>243.92 ± 47.15*</td>
<td>0.36 ± 0.03*</td>
<td>72.90 ± 4.05*</td>
</tr>
</tbody>
</table>

ps, ▲ P < 0.05, versus sham-operated group; * P < 0.05, versus model group.

The change of NOX4 and MDA

The western blot of NOX4 showed that at the end of the study, the NOX4 in model group increased by 62% (\(P < 0.05\)) compared with sham, while when treated with DQP for 4 weeks, the level of NOX4 showed 35.80% reduction compared with model group (\(P < 0.05\)), which had no statistical significance compared to sham (Figure 3). The result of MDA showed that in model group, its level increased by 27.72% (\(P < 0.05\)) compared with sham, after treatment with DQP, the level of MDA showed 35.46% reduction compared with model group (\(P < 0.05\)) (Table 4 and Figure 3).

DISCUSSION

Based on lipid infiltration theory, lipid metabolism disorder
is the dependent risk to cause the CHD with MI. However, there is no experimental research to identify whether MI can cause lipid metabolism derangement; conversely, if we can confirm this pathological mechanism by experiments, we will get further acknowledgement of the occurrence and development of CHD with MI. Thus, to predict the prognosis and provide prevention and treatment for CHD, the present study was undertaken; what's more, it also can give us new idea for treatment of other ischemic disease including cerebral ischemia.

In our previous study, we used MI model referred in this paper to investigate the biomarkers for CHD. The model was induced by amiodarone constrictor which was implanted in swines around the LAD as previously mentioned and no lipid intervention such as high fat diet were given during the experiment. Proteomics and metabolomics were applied to analyze the change of plasma. The results showed that although no lipid interventions, abnormal up-regulation of apolipoprotein and lipoprotein levels were detected in plasma. The proteomics research based on two-dimensional gel electrophoresis (2-DE) showed significant up-regulation of apolipoprotein A-I (ApoA-I) and apolipoprotein A-IV (ApoA-IV) (Guo et al., 2010).

Meanwhile, the metabolomics research based on gas chromatography coupled with mass spectrometry (GC-MS) showed pathologic changes of metabolites related to lipoprotein such as beta-hydroxybutyric acid (Yong et al., 2011). Based on previous studies, we carried out further research on possible pathological mechanism of plasma lipid disorders caused by MI in this paper. Studies also had shown that activation of RAAS system including the up-regulation of Ang II and Ald activated NOX4 through AT1 receptor pathway (Imanishi et al., 2005), and induced ROS by the electron transformation (Quinn and Gauss, 2004). ROS can modify LDL to be OX-LDL (Truman et al., 2012; Galle et al., 2003) and impaired endothelial function especially the NO content. What's more, ox-LDL is the critical risk factors of lipid metabolism disorder that can contribute to CHD (Koenig et al., 2011). In this study, we found that plasma Ang II and Ald of CHD model control group increased by 63.41 and 56.25%, the level of LDL which reflects plasma lipid metabolism disorders increased by 29.46%, the level of ox-LDL increased more significantly, and the plasma NO decreased by 25.20%. What's more, the levels of NOX4 and the MDA were up-regulated, which was in accordance with reference (Yong et al., 2011) after 4 weeks' treatment by DQP. Compared with model group, plasma Ang II and Ald of DQP group decreased by 35.49 and 28.00%, while plasma NO increased and plasma ox-LDL decreased to the level close to the values of sham-operated group, accomplished by the down-regulation of NOX4 and MDA, which suggested that DQP can inhibit the activation of RAAS system, improve vascular endothelial function, so as to improve the disorder of lipid metabolism through the NOX4-ROS pathway. In addition, echocardiographic study of CHD model control group showed significant difference in left ventricular cavity structure, left ventricular anterior wall structure, contractile capacity, left ventricular systolic and diastolic function compared with sham-operated swines, which suggested the appearance of ventricular hypertrophy. After 4 weeks' treatment with DQP, LVEDd, ESV showed no significant difference compared with CHD model control group; however, EF value which reflected heart function increased and LVEDs decreased. These may be related with cardiac decompensation, reduction of cardiac after load by decreasing Ang II, and reduction of the peripheral resistance by Ald (Karl et al., 2010).

In conclusion, we applied a CHD model with chronic MI of swines to validate whether ischemia could lead to

### Table 3. the change of ox-LDL, LDL and VLDL in different groups (x±s).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>ox-LDL (ng/ml)</th>
<th>LDL (ng/ml)</th>
<th>VLDL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>6</td>
<td>10.30 ± 1.77*</td>
<td>88.97 ± 16.08</td>
<td>5.41 ± 0.35</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>34.66 ± 8.59▲</td>
<td>115.18 ± 16.83▲</td>
<td>5.91 ± 0.69</td>
</tr>
<tr>
<td>Danqi pill</td>
<td>6</td>
<td>18.63 ± 6.94▲</td>
<td>96.59 ± 11.14*</td>
<td>4.41 ± 0.43*</td>
</tr>
</tbody>
</table>

*p, ▲P < 0.05, ▲▲P < 0.01 versus sham-operated group; *P < 0.05, versus model group.

### Table 4. Concentration of NOX4 and MDA after 8 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>NOX4 (mg/ml)</th>
<th>MDA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>6</td>
<td>1.00**</td>
<td>5.41 ± 0.356*</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>1.62 ± 0.208▲</td>
<td>6.91 ± 0.697▲</td>
</tr>
<tr>
<td>Danqi pill</td>
<td>6</td>
<td>1.04 ± 0.142**</td>
<td>4.46 ± 0.435*</td>
</tr>
</tbody>
</table>

*p, ▲P < 0.05, ▲▲P < 0.01 versus sham-operated group; *P < 0.05, versus model group.

Figure 3. The western blot results of NOX4 in different groups. DQP, Danqi pill.
plasma lipid disorder, and to ascertain how the DQP acts on CHD. The results showed that ischemia caused plasma lipid disorder probably mediated by Ang II-NOX4-ROS-ox-LDL pathway. DQP can inhibit the RAAS system activation, improve endothelial cell function and promote heart function, thereby providing an experimental basis for therapy effect to CHD for clinical practice.

ACKNOWLEDGMENTS

We thank Yue Tang in Fuwai cardiovascular hospital for the guidance on Echocardiographic Assessment. The work was supported by grants from the Creation for Significant New Drugs Project of China (No. 2012ZX09103-201-011). The Project of the Beijing University of Traditional Chinese Medicine (No.2011-JYBZZ-JS055), the National Natural Science Foundation of China(no.81202788) and National Science & Technology Pillar Program (no. 2012BAI29B07).

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