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Anti-atherosclerosis and cardio-protective effects of the Angong Niuhuang Pill on a high fat and vitamin D3 induced rodent model of atherosclerosis

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ABSTRACT

Ethnopharmacological relevance: The Angong Niuhuang Pill (ANP) is a well known Chinese traditional therapeutic for the treatment for diseases affecting the Central Nervous System (CNS). Components of the ANP formulation, including *Bovis* Calculus Sativus, Pulvis *Bubali* Comus Concentratus, *Moschus*, Margarita, Cinnabaris, Realgar, *Coptidis* Rhizoma, *Scutellariae* Radix, *Gardeniae* Fructus, *Curcumae* Radix, and Bomeolum Syntheticum, have been used for the treatment of stroke, encephalitis and emergency meningitis across Asia, especially in China for hundreds of years.

Objective: The goal of this study was to investigate the anti-atherosclerosis and cardio-protective effects of ANP administration using a rodent model of atherosclerosis induced by a high fat and vitamin D_3.

Methods: Specific Pathogen-Free (SPF) 78 male SD rats were randomly divided into a control group and 5 atherosclerotic model groups. The atherosclerotic groups were divided to receive either Simvastatin (SVTT, 0.005 g/kg), Low-dose ANP (0.125 g/kg), Medium-dose ANP (0.25 g/kg), and High-dose ANP (0.5 g/kg). Following adaptive feeding for one week, atherosclerosis was induced and the atherosclerosis model was established. Experimental drugs (either simvastatin or ANP) or normal saline were administered intragastrically once daily for 9 weeks starting from the 8th week. A carotid artery ultrasound was performed at the 17th week to determine whether atherosclerosis had been induced. After the atherosclerosis model was successfully established, platelet aggregation rates, serum biochemical indices, apoptosis-related Bcl-2, Bax proteins levels in the heart were assayed. Pathological and histological analysis was completed using artery tissue from different experimental different groups to assess the effects of ANP.

Results: ANP significantly decreased aortic membrane thickness, the maximum platelet aggregation rates, and the ratio of low density lipoprotein cholesterol (LDL) to high density lipoprotein cholesterol (HDL). In addition, ANP significantly reduced serum contents of total cholesterol, low density lipoprotein, malondialdehyde, troponin I, high-sensitivity C-reactive protein, and lactate dehydrogenase. ANP markedly improved abnormal pathological conditions of the aorta and heart, and helped to prevent myocardial apoptosis.

Conclusions: We have demonstrated that ANP has robust ant-atherosclerosis and cardio-protective effects on a high-fat and vitamin D_3 – induced rodent model of atherosclerosis due to its antiplatelet aggregation, lipid regulatory, antioxidant, anti-inflammatory and anti-apoptotic properties.

Abbreviations: ADP, adenosine diphosphate glucose pyrophospheralase; AMI, acute myocardial infarction; ANP, Angong Niuhuang Pill; AS, atherosclerosis; cTnI, cardiac troponin I; CVD, Cardiovascular disease; hs-CRP, C-reactive protein; CK-MB, creatine kinase isoenzyme; ET-1, endothelin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL-C, high density lipoprotein cholesterol; LDH, lactate dehydrogenase; LDL-C, low density lipoprotein cholesterol; MDA, malondialdehyde; NO, nitric oxide; PVDF, polyvinylidene difluoride; SD, Sprague Dawley; SVTT, Simvastatin; SPF, Specific Pathogen Free; SOD, Superoxide dismutase; TXB_2, thromboxane B_2; TC, total cholesterol; TG, triglyceride

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1. Introduction

Cardiovascular disease (CVD) and related chronic diseases are widely considered the leading causes of death globally. More than 80% of all CVD-related deaths occur in low- and middle-income countries, including China (Critchley et al., 2004; Fuster et al., 2011). Atherosclerosis (AS) is well accepted as the primary cause of cardiocerebrovascular disease (such as stroke) and mortality worldwide (Davidson, 2007). Over the past three decades research has primarily been focused on the pathological mechanisms and risk factors for AS. Yet in spite of this research, therapies designed to prevent or, more importantly, reverse the devastating outcomes of AS remain elusive (Major, 2013).

While the roles of hyperlipidemia, oxidative stress, and inflammation in the development and progression of AS have been well documented, the detailed pathogenesis of how those pathogenic factor function in AS is still not completely understood (Mckenney, 2001; Paoletti et al., 2004). Therapeutic measures for treating AS currently include lipid-regulating agents and antihypertensive medicines (Teramoto et al., 2013). However, since the mechanisms of atherogenesis are complex, long-term and high dose applications of single drug therapies like simvastatin, which target single molecules, can increase certain side effects, such as myopathy and liver damage. (Yang et al., 2011). Hence, combination therapy may be more effective to treat AS.

Angong Niuhuang Pill (ANP) is a well-known, traditional Chinese patented medicine in use across Asia and especially in China for hundreds of years to treat stroke, encephalitis and meningitis. ANP has been listed in the *Chinese Pharmacopoeia* for decades. Its main components include *Bovis* Calculus Sativus (*Bovis* Calculus Sativus is prepared with fresh bile of *Bos taurus domesticus* Gmelin as a mother liquor and by adding deoxycholic acid, cholic acid and compound calcium bilirubin, etc), Pulvis *Bubali* Comus Concentratus (Pulvis *Bubali* Comus Concentratus is prepared from the horn of *Bubalus bubalis* Linnaeus.), *Moschus* (*Moschus* is the dried secretion of the musk sac of adult male *Moschus berezovskii* flerov or *Moschus moschiferus* Linnaeus.), Margarita (Margarita is the pearl of *Pteria martensii* (Dunker), *Hyriopsis cumingii* (Lea) or *Cristaria plicata* (Leach).), Cinnabaris (Cinnabaris is a mineral of sulfides of cinnabar group, containing mainly mercuric sulfide (HgS)), Realgar (Realgar is a mineral of sulfides of the realgar group, containing mainly arsenic disulfide (As_2S_2).), *Coptidis* Rhizoma (*Coptidis* Rhizoma is the dried rhizome of *Coptis chinensis* Franch, *Coptis deltoidea* C.Y. Cheng et Hsiao, or *Coptis teeta* Wall.), *Scutellariae* Radix, *Gardeniae* Fructus (*Gardeniae* Fructus is the dried ripe fruit of *Gardenia jasminoides* Eills), *Curcumae* Radix (*Curcumae* Radix is the dried root tuber of *Curcuma wenyujin* Y.H. Chenet C. Ling, *Curcuma Longa* L., *Curcuma kwangsiensis* S.G. Lee et C.F. Liang or *Curcuma phaeocaulis* Val.) and Bomeolum Syntheticum (Bomeolum Syntheticum is a synthetic product consists mainly of borneol) (Editorial Committee of Pharmacopoeia of Ministry of Health PR China, 2010). Studies have shown that *Bovis* Calculus and *Gardeniae* Fructus have anti-inflammatory, anti-oxidative and cardioprotective effects, and may be useful for preventing AS (Liu et al., 2013; Mizuno et al., 2012). *Moschus*, *Coptidis* Rhizoma and *Scutellariae* Radix have been shown to have anti-myocardial ischemia effects (Chan et al., 2011; Kim et al., 2009; Luo et al., 1996). Realgar has been used to alleviate angina pectoris resulting from coronary heart disease (Liu et al., 2002). *Borneolum* has been shown to contain antithrombotic effects as a result of its anticoagulant properties (Li et al., 2008). ANP has been widely used for hundreds of years in the emergency clinical management of cardiocerebrovascular conditions including stroke, encephalitis and meningitis (Guo et al., 2013; Wu et al., 2016).

AS is a disease characterized by the excess buildup of plaque deposits inside arteries. Over time, these deposits harden and narrow the vessels, limiting the flow of blood. In extreme cases, the buildup of plaque can completely seal the vessels and lead to heart attack, stroke, or even death. Since ANP is an effective medicine on treat stroke, and the development of AS is an important pathophysiological predisposing factor for stroke, we attempt to investigate whether ANP also have effects on AS. This following investigating aims to study the effects of ANP on AS and its mechanism of action in a rat model of AS.

2. Materials and methods

2.1. Preparation of ANP

ANP was prepared by Guangzhou Baiyunshan Zhongyi pharmaceutical co., LTD (Guangzhou, Guangdong, China) using the following ingredients: *Bovis* Calculus 100 g, Pulvis *Bubali* Comus Concentratus 200 g, *Moschus* 25 g, Margarita 50 g, Cinnabaris 100 g, Realgar 100 g, *Coptidis* Rhizoma 100 g, *Scutellariae* Radix 100 g, *Gardeniae* Fructus 100 g, *Curcumae* Radix 100 g, Bomeolum Syntheticum 25 g.

Cinnabaris, levigate Margarita and Realgar were ground or pulverized to very fine powders. *Coptidis* Rhizoma, *Scutellariae* Radix, *Gardeniae* Fructus and *Curcumae* Radix were pulverized to a fine powder. *Bovis* Calculus, Pulvis *Bubali* Comus Concentratus, *Moschus* or *Moschus* and Bomeolum Syntheticum were triturated with the above powders, sifted and mixed well. Refined honey was mixed to make 600 big honeyed pills, or alternately coated with a gold film (Editorial Committee of Pharmacopoeia of Ministry of Health PR China, 2010). HLPC was used to verify the formulation to guarantee the quality of the ANP. For details please see the supporting information.

2.2. Reagents

Reagents used in the study were as follows: 1. ANP (Guangzhou Baiyunshan Zhongyi Pharmaceutical Co., Ltd, Lot: S07063M) 2. Simvastatin (Hangzhou Merck pharmaceutical Co., Ltd, Lot:130054) 3. High fat feed composed of 3% cholesterol, 0.5% sodium cholate, 0.2% propylthiouracil, 5% sugar, 10% lard and 81.3% basic feed (Medical Science Experimental Animal Center, Guangdong, China) 3. Vitamin D_3 (Shanghai General Pharmaceutical Co., Ltd, Lot:121123) 4. Kits for superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO), lactate dehydrogenase (LDH) (Jiancheng Bioengineering Institute, Nanjing, China, Lot: 20140724, 20140722, 20140712, 20140725) 5. Kits for triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) (Beijing Beihua Kangtai Clinical Reagent Co., Ltd, Lot: 20130115, 20130116, 20130718, 20130723) 6. Kit for high sensitivity C-reactive protein (hs-CRP) (Wuhan Uscn Co., Ltd, Lot: L140928852) 7. Kits for thromboxane B_2 (TXB_2), endothelin 1 (ET-1), cardiac troponin I (cTnI), creatine kinase isoenzyme (CK-MB) (Wuhan CUSABIO Co., Ltd, Lot: E11015195, E11015194, E11015101, D13015193). 8. Rabbit polyclonal antibodies specific for Bcl-2(Cell Signaling, Beverly, MA, USA, Lot: #2870), Bax (Cell Signaling, Beverly, MA, USA, Lot: #2772). 9. Rabbit polyclonal antibodies specific for GAPDH (Affbiotech, USA, Lot: NO BST09E04A) and 10. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BOSTER, Wuhan, China, Lot: #19U71).

2.3. Animals

Seventy-eight 8-week old Specific Pathogen Free (SPF) male Sprague Dawley (SD) rats (weight: 200 ± 20 g), were provided by the Medical Science Experimental Animal Center of Guangdong Province in China (Certificate no. SCXK (Guangdong) 20130002). Rats were housed in the Jinan University Medical School laboratory animal management center (Certificate no. SCXK (Guangdong) 2012-0117) and were maintained at 24 °C and 65% humidity. Rats were maintained on a 12-h light/dark cycle and were given free access to standard laboratory rat chow and tap water. All animal welfare and experimental procedures were in strict accordance with the Guide for the Care and