Investigations of the Anti-hypertensive and Anti-atherosclerotic Properties of Danshen-Gegen Formula

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Investigations of the Anti-hypertensive and Anti-atherosclerotic Properties of Danshen-Gegen Formula

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Abstract

Hypertension and atherosclerosis are two main risk factors for cardiovascular diseases (CVDs) in the modern world. CVDs are the top killers in the world. In addition, the management of CVD patients is costly, and places a heavy economical burden to the health care system. In order to search for an economical but effective solution, we seek from the old Chinese wisdom, the traditional Chinese medicines. As recorded in "Zhong yao fang ji xian dai yan jiu da dian" (中藥方劑現代研究大典), Danshen-Gegen-Yanhusuo forms the simplest formula for treating cardiovascular disease, and Yanhusuo is mainly for analgesic purpose. Therefore, Danshen-Gegen formula becomes our target of study. In the present study, the anti-hypertensive and anti-atherosclerosis effects of Danshen-Gegen (DG) 7:3 water extract on aorta were being investigated. The action mechanisms of DG extract on vasodilation were also elucidated.

To evaluate the anti-hypertensive effects of DG extract, *in vivo* model of spontaneous hypertensive rats (SHR) of 6-weeks old (for preventive study) and 14-weeks old (for therapeutic study) were used. In study involving SHR rats, Wistar-Kyoto (WKY) rats of same age was used as a normotensive control, in which no treatments were given. For both preventive and therapeutic studies, daily oral

gavage of DG extract was given to the SHR rats for 12 weeks. The systolic blood pressure (SBP) was measured biweekly by tail-cuff method. Our results showed that the positive control raloxifene (6.2mg/kg), DG extract of 90.2mg/kg (new clinical relevant dose) and 300 mg/kg (old clinical relevant dose) could significantly reduce the plateau of SBP in the prevention group, though not capable of reverting the SBP back to normal level. Besides, all three treatments could significantly lower the blood pressure of the therapeutic group. According to the blood lipid profile test, 90.2mg/kg of DG extract could significantly lower the cholesterol level, as compared with SHR control.

Mechanistic studies of vasodilation can be divided into 2 parts: *ex vivo* mechanism elucidation and *in vivo* confirmation. The first part was performed using *ex vivo* isolated aorta ring organ bath. Aorta was pretreated with inhibitors or blockers for the elucidation of vasodilation pathways involved in DG extract-induced relaxation. Our results showed that 1 and 2mg/ml DG extract could induce full relaxation, and 0.5mg/ml of DG extract could induce about 50% relaxation of the aorta ring after U46619-induced contraction. The addition of inhibitors such as L-NAME and indomethacin, as well as denudation of aorta ring, could also give similar relaxation profiles to intact aorta ring. Hence, our results indicated that the vasodilation observed is not related to nitric oxide (NO) or prostacyclin, and is in fact endothelium-independent. Besides, TEA, a non-selective blocker of potassium channels, also inhibited the relaxation of 1mg/ml DG extract to 50%. Furthermore, blockers of Kir and K_v channels also showed similar patterns of DG extract-induced relaxation to TEA, while blocker of K_{ATP} channel significantly inhibited the DG extract-induced relaxation at all doses. On the other hand, the addition of BK_{ca} channel blocker did not inhibit the DG extract-induced relaxation at all. These results

suggest the involvement of potassium channels, especially Kir, K_v and K_{ATP} channels but not BK_{ca}, in DG extract-induced relaxation.

In the second part of the mechanistic studies, in order to confirm the results with *in vivo* model, SHR rats were fed with positive control raloxifene (6.2mg/kg), or DG extract (90.2mg/kg or 300 mg/kg) for 1 month, and aorta rings were isolated for study. In SHR rats, intake of raloxifene or DG extract did not significantly potentiate the increasing dose of acetylcholine-induced relaxation, in both the presence and absence of inhibitors or blockers. Aorta rings from SHR rats presented a significantly lower relaxation than those from WKY rats, induced by single and high dose of acetylcholine. Besides, intake of raloxifene or DG extract could slightly potentiate the relaxation in this condition. Moreover, it was found that the contractile force of the aorta was reduced by raloxifene or DG extract intake, which gave another explanation to the anti-hypertensive effect of DG extract.

To investigate the effect of DG extract on anti-atherosclerosis, rabbits were initially fed with 1% cholesterol diet for 4 weeks. Then, 45.1mg/kg, 150mg/kg or 450mg/kg of DG extract, or 300mg/kg vitamin E (serve as positive control) were orally fed daily for another 8 weeks with 1% cholesterol diet *ad libitum*. The intima-media thickness (IMT) of aorta, which reflects the degree of atherosclerosis, was significantly and dose-dependently suppressed by DG extract. Our results showed that DG extract had a slight hypolipidemic effect on cholesterol-fed rabbits, which partially explains its anti-atherosclerosis effect. However, both vitamin E and various dosages of DG extract did not have significant effect on plasma glucose level and lipid peroxidation.

In conclusion, our results confirmed the efficacy of DG 7:3 water extract on anti-hypertension and anti-atherosclerosis, possibly due to the reduction in contractile force, vasodilative and hypolipidemic effect of DG extract. The promising effects of DG extract on hypertension and atherosclerosis give scientific evidence towards our current clinical trial, and shear light to the future research of DG extract on anti-hypertension and anti-atherosclerosis.

論文摘要:

心血管病是世界的頭號殺手，其護理與治療成本高昂，對世界各國造成大量的人命及經濟損失。而高血壓和動脈粥樣硬化是心血管病的主要風險因素，亦是防止心血管病的主要戰線，爲了找尋經濟而有效的治療方法，我們從中藥中追尋答案。在《中藥方劑現代研究大典》內，丹參葛根元胡片乃治療心血管痛之最簡單複方，而元胡則因其主要功效爲止痛而被剔除。是次研究的丹參葛根的比例爲7:3，而是次研究將集中丹參葛根複方的抗高血壓和抗動脈粥樣硬化能力，以及其引起血管舒張的基理。

在抗高血壓研究中，丹參葛根複方對 Wistar-Kyoto (WKY)大鼠以及自發性高血壓大鼠(SHR)模型的收縮壓的影響進行研究。丹參葛根複方及陽性對照品 Raloxifene 在防止 6 週大自發性高血壓大鼠的高血壓形成及治療 14 週大自發性高血壓大鼠的高血壓均有顯著成效。在兩次實驗中，正常自發性高血壓大鼠收縮壓爲 200mmHg 左右，而每天服食丹參葛根複方或 Raloxifene 的自發性高血壓大鼠收縮壓均被控制於約 180mmHg。另外，90.2mg/kg 的丹參葛根複方明顯地降低了血液中膽固醇含量。

血管舒張基理的研究分爲兩部分 - 體外基理分析及體內確認結果。在體外基理分析中，我們使用了由 Sprague-Dawley (SD)大鼠分離的大動脈模型，以其拉力作爲測定因素，測定丹參葛根複方會否引致血管舒張，並加入各種抑制劑以測定不同的基理是否參與在血管舒張的過程之中。結果顯示，在使用 U46619 引起血管收縮後，1 至 2mg/ml 的丹參葛根複方能令血管完全舒張，而 0.5mg/ml 丹參葛根複方則能引起血管 50%的舒張。抑制劑 L-NAME 及 indomethacin，以及去除血管內皮均不能降低丹參葛根複方引起的舒張，由此證明了丹參葛根複方引起的舒張跟一氧化氮及前列環素，還有內皮的存在與否無關。鉀離子通道抑制劑四乙基銨(TEA)能降低 1mg/ml 丹參葛根複方引起的血管舒張至 50%，而 Kir 及

K_v 通道抑制劑均有跟四乙基銨同樣的效果，K_{ATP} 通道抑制劑則明顯地抑制了各劑量丹參葛根複方引起的血管舒張，反之，BK_{ca} 通道抑制劑不能降低丹參葛根複方引起的舒張。由此可見，鉀離子通道，特別是 Kir、K_v 跟 K_{ATP} 通道，跟丹參葛根複方引起的血管舒張有密切關係。

體內確認結果的方法則是使用自發性高血壓大鼠(SHR)模型，每天餵食 Raloxifene 或丹參葛根複方，一個月之後，把大動脈分離，以乙醯膽鹼(ACh)作血管舒張測定。實驗結果證明，服食 Raloxifene 或丹參葛根複方均不能令逐漸增加的乙醯膽鹼引起更大的血管舒張程度，但可以令單一、高劑量的乙醯膽鹼引起稍大的血管舒張，此外，Raloxifene 或丹參葛根複方能減低血管的收縮力度。以上兩點能部份解釋丹參葛根複方抗高血壓的原因。

而在抗動脈粥樣硬化方面，我們使用高膽固醇糧食在雄性新西蘭兔身上引起動脈粥樣硬化，4 週之後，我們每天餵食 45.1mg/kg、150mg/kg、450mg/kg 丹參葛根複方或 300mg/kg 維他命 E(陽性對照品)。我們測定了動脈粥樣硬化的指標 - 內膜中層厚度(IMT)，發現丹參葛根複方及維他命 E 均能降低內膜中層厚度，而對丹參葛根複方的反應呈劑量依賴性。丹參葛根複方能輕微降低血液中膽固醇含量，但對血糖及脂質過氧化過程沒明顯效果。

總結而言，實驗結果認證了丹參葛根複方的抗高血壓和抗動脈粥樣硬化能力，原因可能在於其減低血管的收縮力度、血管舒張及降血液中膽固醇含量的效果。丹參葛根複方在抗高血壓和抗動脈粥樣硬化的顯著成效，對臨床研究提供了科學理據，亦爲將來對丹參葛根複方的研究點亮了明燈。

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Abbreviations

4-AP	4-aminopyridine
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ADI	Average daily intake
$BaCl_2$	Barium chloride
BK_{Ca} channels	Large-conductance Ca^{2+}-activated K^+ Channels
Ca^{2+}	Calcium ion
CABG	Coronary artery bypass grafting
CAM	Complementary and alternative medicine
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DG extract	Danshen-Gegen 7:3 extract
eNOS	Endothelial Nitric oxide synthase
GC	Guanylate cyclase
H&E	Haematoxylin and eosin
HDL	High density lipoprotein
HED	Human equivalent dose
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IbTX	Iberiotoxin
ICAM-1	Intercellular adhesion molecule-1
ICM	Institute of Chinese Medicine
IMT	Intima-media thickening/thickness
iNOS	Inducible Nitric oxide synthase
K^+	Potassium ion
K_{ATP} channels	ATP-sensitive K^+ Channels
Kir channels	Inward Rectifier K^+ Channels
Kv channels	Voltage-dependent K^+ Channels
LDL	Low density lipoprotein
L-NAME	NG-Nitro-L-arginine methyl ester
MCP-1	Monocyte chemoattractant protein 1
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
NCCAM	National Center for Complementary and Alternative Medicine
nNOS	Neuronal Nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
oxLDL	Oxidized low density lipoprotein
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin/ Prostaglandin I_2
Phe	(R)-(-)-Phenylephrine
PKA	Protein kinase A
PKG	Protein kinase G
Ral	Raloxifene
ROS	Reactive oxygen species
SD rat	Sprague-Dawley rat
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SHR	Spontaneously hypertensive rat
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TCM	Traditional Chinese Medicine
TEA	Tetraethylammonium
TGF-β	Transforming growth factor-β
TLC	Thin-layer chromatography
TMOP	1,1,3,3-Tetramethoxypropane
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein

VPR Volume Pressure Recording

WKY rat Wistar-Kyoto rat

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Chapter 1

Introduction

1.1 Introduction to Cardiovascular Disease, Hypertension and Atherosclerosis

1.1.1 Cardiovascular Disease

Cardiovascular diseases (CVD) are classified as coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism, etc. CVDs are the major cause of death and caused more than 17.1 millions death each year globally, in which a 7.2 million was due to coronary heart disease and 5.7 million due to stroke. CVDs accounted for about 30% of deaths in 2004 (World Health Organization, 2009). Low- and middle-income countries account for 82% of the deaths, due to higher exposure to risk factors, lower exposure to preventive measures and proper education, and less effective treatment and health care. Although the mortality in developed-countries is lower, the continuous health cares for CVD patients become a heavy economical burden in developed-countries. This trend is spreading to the developing countries, due to adverse lifestyle such as lack of exercise, western style high fat/high caloric diet and increased tobacco use (Smith, *et al.*, 2004). Therefore, CVD cause an economical loss and burden in the whole world, with an increasing trend in prevalence.

In Hong Kong, heart disease is the second leading cause of death in 2008, which accounts for 15% and 18% of mortality of male and female, respectively; while the cerebrovascular disease is the fourth leading cause of death, which accounts for 8% and 10% of mortality of male and female, respectively. Overall, CVD is the second and the first leading cause of death in Hong Kong for male and female, which account for 23% and 28% of mortality, respectively. The percentage is very close to first and

the second leading cause of death - malignant neoplasm, which cause 32.7% and 26.7% of mortality for male and female, respectively (Department of Health, 2010).

Hypertension is the major cause of hemorrhagic stroke (Rashid, *et al.*, 2003, Smith, *et al.*, 2004). It also increases the risk of recurrent myocardial infarction in patients with diagnosed coronary heart disease. Atherosclerosis is the major cause of atherosclerotic vascular disease – stroke, heart attack and peripheral arterial disease. As mentioned, stroke and heart attack were leading causes of death in Hong Kong in 2008, and have a high chance of recurrence (Rodgers, *et al.*, 1996). In rare case, atherosclerosis and hypertension would cause aortic aneurysm or dissection, which has extremely high mortality (Smith, *et al.*, 2004). Therefore, management of hypertension and risk factors of atherosclerosis can broadly reduce the risk of a large variety of CVD events.

1.1.2 Hypertension

1.1.2.1 Background

Hypertension refers to the elevated arterial pressure. The World Health Organization, International Society of Hypertension, European Society of Hypertension and the European Society of Cardiology definition of different stages of hypertension is listed in Table 1.1. It is a very common public health problem in developed countries. Although it is common, easy to diagnose and manage, the patients would dependent on the anti-hypertensive drugs and the complications of hypertension are usually lethal. In most case, the etiology is unknown. There are also difficulties in preventing (Chobanian, *et al.*, 2003) or curing (Hagemeister, *et al.*, 2001). So, the patients would depend on the anti-hypertensive drugs, which is a large economical burden to the society and individuals.

Table 1.1 Classification of blood pressure for human adults

Category	*Systolic Pressure (mmHg)*	*Diastolic Pressure (mmHg)*
Optimal	<120	<80
Normal	<130	<85
High normal	130–139	85–89
Hypertension		
Stage 1 (mild)	140–159	90–99
Stage 2 (moderate)	160–179	100–109
Stage 3 (severe)	≥180	≥110

1.1.2.2 Causes of Hypertension

There are two types of hypertension – primary (essential or *idiopathic)* and secondary. Hypertension which caused by generalized or functional abnormalities are named as primary hypertension, while hypertension caused by disease of other structural organs or gene defects are named as secondary hypertension. However, regulation of blood pressure involves interactions among different systems - peripheral and/or central adrenergic, renal, hormonal, and vascular system. This makes the mechanism elucidation becomes very difficult.

A) Primary Hypertension

Primary hypertension is defined as high BP in which secondary causes such as renovascular disease, renal failure, aldosteronism, or other causes of secondary hypertension are not present (Carretero and Oparil, 2000). There are some common abnormalities in hypertensive patient, which claimed to be the primary causes of hypertension as described below.

1) Environment

There are several environmental factors that increase the risk of hypertension, including salt intake, obesity, occupation, alcohol intake, family size, and crowding. These factors thought to be an explanation of increasing blood pressure with age in wealthy countries (Burt, *et al.*, 1995, Kasper and Harrison, 2005).

2) Salt Sensitivity

In about 60% of hypertensive patients, the blood pressure is especially responsive to the amount of salt intake (Kasper and Harrison, 2005). Blood pressure of Asians and Blacks are more sensitive to salt intake than Caucasians (Jurgens and Graudal, 2004). The pathophysiology of this sensitivity is unknown. But the possible causes are modification of renin activity, chloride intake, calcium intake, insulin resistance, and "nonmodulation".

3) Modification of Renin Activity

Renin is an enzyme secreted by juxtaglomerular cells of the kidneys. It is linked with aldosterone in a negative feedback loop. However, it is essential for the production of angiotensin II. Angiotensin II causes vasoconstriction, induces the release of aldosterone to retain sodium and lose potassium (adrenal response), and acts on proximal tubules in kidneys to increase sodium reabsorption (renal response). In normotensive individuals, with sodium restriction, adrenal responses are enhanced and the renal vascular responses reduced. Sodium loading has the opposite effect.

About 20% of hypertensive patients have their renin activity down-regulated (Kasper and Harrison, 2005). Thus, plasma aldosterone levels are not fully suppressed by dietary sodium salt loading, resulting an excessive amount of aldosterone that

retain sodium. They would have an increased extracellular fluid volume and can be managed by diuretics (Bhattacharya, *et al.*, 2009, Brunner, *et al.*, 1972). About 15% of hypertensive patients have their renin activity up-regulated. Their hypertension can be managed by antagonist of angiotensin II, suggesting the involvement of renin-angiotensin system (Kasper and Harrison, 2005).

4) Chloride and Calcium Intake

It is assumed that sodium plays a key role in hypertension. However, chloride and calcium ions may also play a role. Some studies showed that sodium salt without chloride actually fails to induce blood pressure elevation (Kotchen, *et al.*, 1983, Kurtz, *et al.*, 1987). Interestingly, a low calcium intake seems to be related to an increased blood pressure (Lal and Dakshinamurti, 1995). Nevertheless, calcium channel blockers are potent anti-hypertensive drugs. All of these indicate chloride and calcium ion play a role in hypertension.

5) Insulin resistance

Insulin resistance or hyperinsulinemia can lead to hypertension. An assumption is that the tissues involved in glucose homeostasis are not responsive, while the tissue involved in hypertensive process still work fine. Hypertension is caused by one or more of these three mechanisms: (1) Hyperinsulinemia induces sodium retention in kidneys and stimulate sympathetic activity, which can lead to an increase in blood pressure (Natali, *et al.*, 1993, Quinones-Galvan and Ferrannini, 1997); (2) Vascular muscle hypertrophy due to the mitogenic effect of insulin (Rizzoni and Agabiti Rosei, 2006); (3) Insulin may modify ion transport across the cell membrane, causing cytosolic calcium accumulation in insulin-sensitive vascular smooth muscles (Lebeche, *et al.*, 2008).

6) Nonmodulation

Some hypertensive patients have impaired modulation of adrenal and renal response to sodium restriction and loading. These patients would have difficulties in excretion of sodium through kidneys. The accumulation of sodium in the body would cause hypertension, and this type of hypertension seems to be salt-sensitive (Hollenberg and Williams, 2006).

B) Secondary Hypertension

Secondary hypertension is the complications of other diseases, like renal diseases or endocrine diseases. Renal diseases affect blood pressure by (1) changing the secretion of vasoactive molecules, like renin (Kalra, 2007), or (2) damaging the excretion function of kidneys leading to accumulation of salt and fluid (Campese and Nosrati, 1999). Endocrine diseases, mainly hormone-secreting tumour, induce hypertension mainly through the over-secretion of glucocorticoids or aldosterone, which cause retention of sodium (Sica, 2008). In rare case, it can be caused by tumour secreting epinephrine and norepinephrine, which act on adrenergic receptor and cause vasoconstriction (Amodeo, *et al.*, 1989). To treat the hypertension, curing the primary abnormality is the main goal.

1.1.2.3 Current Western Management and Medication

Current treatments to hypertension mainly include lifestyle modification and drug treatments.

A) Lifestyle Modification

Several changes to lifestyle are needed for all hypertensive patients: (1) Prevent stressful activities; (2) Low sodium, low cholesterol and fat, low alcoholic, low

caloric (for obesity patients) diet; (3) Regular aerobic exercise; (4) Quit smoking; (5) Weight reduction (for obesity patients); and (6) Control the risk factors contributing to atherosclerosis (Kasper and Harrison, 2005).

B) Drug Therapy

1) Diuretics

Thiazides are most frequently used in this family. The acute function is diuresis (increase excretion of water through urine) by impairing sodium reabsorption and reduces the blood volume (Wilson and Freis, 1959). In long term intake, reduction in peripheral vascular resistance was found in some studies (Freis, 1983). However, its possible side effects include potassium depletion, hyperglycemia, hyperuricemia, hypercholesterolemia, dermatitis, purpura, depression, and hypercalcemia.

2) Angiotensin-Converting Enzymes (ACE) Inhibitors

ACE inhibitors act on multiple ways: (1) Inhibit the production of angiotensin II, a potent vasoconstrictor; (2) Inhibit the degradation of bradykinin, a potent vasodilator; (3) Modify prostaglandin production; and (4) Modify the activity of the adrenergic nervous system (Bravo and Tarazi, 1979). It is effective for most hypertensive patients, even the malignant ones. There are also fewer side effects, include cough, hyperkalemia in patients with renal disease and angioedema.

3) Angiotensin Receptor Antagonists

The effect of these antagonists is similar to ACE inhibitors, but with a higher selectiveness. They compete with angiotensin II for the binding to angiotensin II AT_1 receptor subtype (Tofovic, et al., 1991). Therefore, they have fewer side effects than ACE inhibitors, including excessive cough or angioedema.

4) Calcium Channel Blockers

They act on α_1 subunit of the L-type voltage-dependent calcium channels (Luft and Haller, 1993). It acts to reduce calcium entry to the cell, lower the cytosolic calcium and causing vasodilation. The side effects include tachycardia, flushing, gastrointestinal disturbances, hyperkalemia, edema, and headache.

5) β-adrenergic Receptor Blockers

The actions of β-adrenergic hormone include the elevated heart rate and strengthen the contractile force of the heart. The hormone also induces renin secretion in juxtaglomerular cells in kidneys. Therefore, the blockers of its receptor can reduce the cardiac output and renin secretion (Distler, *et al.*, 1978). β-adrenergic receptor blockers are commonly use with vasodilator, which causes increased heart rate, or use with diuretics, which causes increased renin activity (Kasper and Harrison, 2005). Side effects includes dizziness, depression, bronchospasm, nausea, vomiting, diarrhea, constipation, heart failure, fatigue, Raynaud's phenomenon, hallucinations, hypertriglyceridemia, hypercholesterolemia, and psoriasis

6) Vasodilator

Vasodilator causes relaxation in vascular smooth muscle cells. However, the effect usually compensate by increased heart rate by homeostasis of the body (Kasper and Harrison, 2005). Therefore, they are mainly used for hypertensive emergencies by intravenous injection. Side effects include tachycardia, nausea, vomiting, and fluid retention.

The above medications can be used in single drug or in combination, depending on the degree and malignance of the hypertension. Patients with other diseases,

especially renal diseases, have to pay attention to the use of drugs, as many of them would have side effects that possibly overload or damage specific organs.

1.1.3 Atherosclerosis

1.1.3.1 Background

Atherosclerosis is the thickening of artery walls due to the build up of foam cells and smooth muscles. It progresses slowly in human, usually takes years to decades to build up. Atherosclerotic lesion initiates at branching points of arteries and regions with disturbed blood flow, and in a discontinuous spots manner. The condition remains asymptomatic for a long time, until the blood flow is seriously occluded. Chronic symptoms include reproducible angina pectoris after exercise and intermittent claudication. Acute clinical expressions differ according to the location of atherosclerosis. Atherosclerosis of coronary arteries causes myocardial infarction. Atherosclerosis of cerebroarteries causes stroke and mini stroke. Peripheral atherosclerosis causes gangrene. The ruptured plaque has a chance to become embolus and cause embolism, which cause ischemia in other organs. Most of the acute clinical expressions have a high mortality rate, and strongly affect the quality of life of patients after recovery.

Structure of Arteries

Arteries are known to have three distinct layers – *tunica adventitia*, *tunica media* and *tunica intima*. Intima is the innermost layer in arteries, which consists of a single layer of endothelial cells. Intima is in direct contact with blood. Media is the middle layer in arteries, which consists of smooth muscle cells and elastic fibers. Media is responsible for contraction and relaxation of arteries. Adventitia is the outermost layer of arteries, which consists of collagen and some elastic fibers. Adventitia anchors the

arteries to nearby tissues and keeps them in position.

1.1.3.2 Pathogenesis of Atherosclerosis

Initiation of Atherosclerosis

Atherosclerosis is initialized by the fatty steak accumulation. The early lesion starts at the infiltration of low density lipoprotein (LDL) to the intima. LDL binds with the proteoglycan molecules of the arterial extracellular matrix, and retain in the intima area (Williams and Tabas, 2005). The matrix separates LDL from the plasma antioxidants, and favors the oxidation. Oxidation of trapped LDL by reactive oxygen species (ROS) and free radicals occurs, and oxidized LDL (oxLDL) is the final product (Chisolm and Steinberg, 2000).

Leukocyte Recruitment

After the fatty steak accumulation, recruitment of leukocytes occurs. OxLDL can induce cytokine release and trigger the local inflammatory response. By expression of adhesion molecules (Vascular cell adhesion molecule-1 (VCAM-1)) or receptors, monocytes and lymphocytes attach to the adhesion molecules (Iiyama, *et al.*, 1999). OxLDL also elicits the production of chemoattractant cytokines such as monocyte chemoattractant protein 1 (MCP-1). MCP-1 directs monocytes and lymphocytes to penetrate through the endothelium and stay in the intima layer (Libby, 2002).

Foam cell Formation

After migration, monocytes differentiate into macrophages. The macrophage expresses the scavenger receptors to endocytose oxLDL (van Berkel, *et al.*, 2005). Some of them can leave the intima and clear the LDL from the arteries. However, if the entry of LDL is faster than the clearance of LDL, the macrophages take up excess

LDL and transform into foam cells (Kasper and Harrison, 2005), named after its foamy appearance under microscope. Foam cells secrete pro-inflammatory cytokines that worsen the local responses to inflammation and ROS. Foam cells also secrete matrix metalloproteinases (MMPs), which degrade the extracellular matrix (Rajavashisth, *et al.*, 1999). The strength of the plaque's fibrous cap is reduced, and rupture. As a consequence, foam cells-originated pro-coagulant protein tissue factor contacts with the blood (Williams and Tabas, 2005). Foam cells are trapped in the blood clot, and undergo apoptosis. The dead foam cells mediate more local inflammatory response and form lipid-rich "necrotic core" (Williams and Tabas, 2005) in more advanced atherosclerotic lesions. The whole process is illustrated in Fig 1.1.

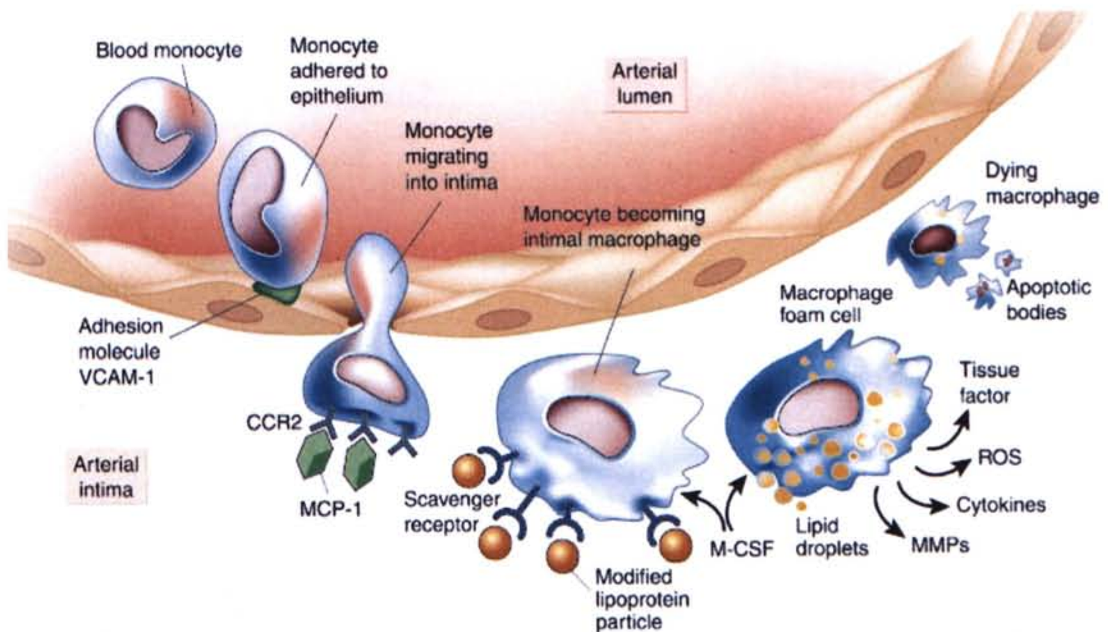


Fig 1.1 Process of leukocyte recruitment and foam cells formation (Extracted from Libby, 2002)

Smooth Muscle Migration

After rupture of plaque, blood coagulation is induced by thrombin and other coagulants. On the other side, thrombin activates wound healing response stimulate

smooth muscle cells proliferation. At the same time, activated platelets secrete platelet-derived growth factor (PDGF), contributing to the smooth muscle cells migration (Badimon, *et al.*, 1993), and transforming growth factor-β (TGF-β), stimulating collagen production (Border and Noble, 1994). With increased smooth muscle cell proliferation, migration and extracellular matrix reconstruction, intima further thicken towards an inward direction. Finally, a narrower lumen is formed (Fig 1.2). The blood flow is restricted, which may lead to observable symptoms like angina pectoris during physically demanding activities. In serious cases, ischemia of the destination organ is resulted.

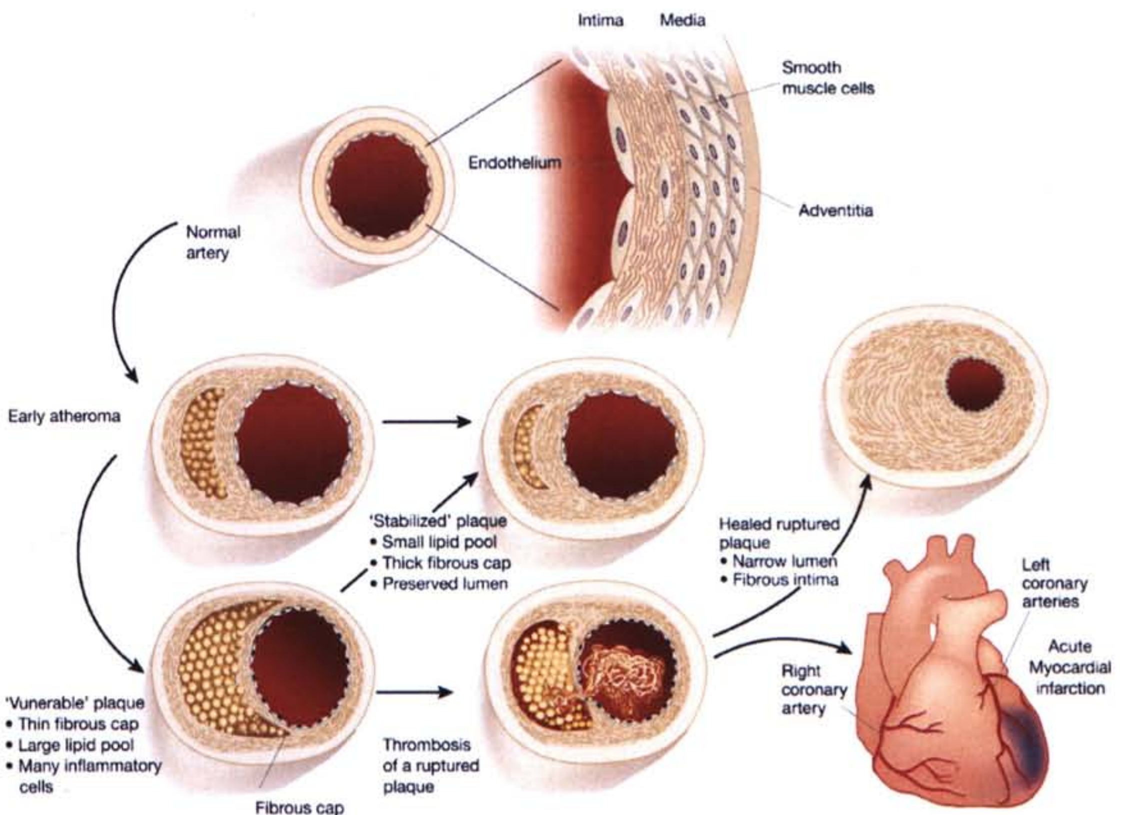


Fig 1.2 Schematic of the life history of an atherosclerotic plaque (Extracted from Libby, 2002)

1.1.3.3 Current Western Treatment and Medication

There is not yet a treatment protocol for atherosclerosis. However, risk factors that contribute to atherosclerosis have been identified. By correcting some of the

modifiable risk factors, the progress of atherosclerosis can be slowed down. These risk factors include hypercholesterolemia, hypertension, disregulation of coagulation or fibrinolysis, hyperhomocystinemia, insulin resistance and inflammation.

Lipid-lowering Therapies

Most well-known lipid-lowering drug is "statin". Statin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor. HMG-CoA reductase is a major enzyme participating in cholesterol synthesis in liver. By inhibiting it, the endogenous production of cholesterol halt, and the level of liver cholesterol would decrease (Ginter and Simko, 2009). The liver senses the reduction in liver cholesterol, and draws LDL and very low density lipoprotein (VLDL) from the bloodstream as a feed-back. LDL and VLDL would be consumed by liver to produce bile salt and excreted.

Statin also found to be increasing endothelium-dependent vasomotion and reduce thrombosis. These functions are suspected to be related to improved endothelial function. The final outcome is the stabilization of the atherosclerotic plaque (de Lorenzo, *et al.*, 2006).

Anti-hypertensive Therapy

The therapy is as stated in 1.1.2.3. From other studies, there is a relationship between hypertension and atherosclerotic risk. And drug treatment of hypertension can also reduce the risk of stroke and heart attack.

Anti-thrombosis

In complication of atherosclerosis, like stroke or myocardial infarction, the

triggering factor is thrombosis. Anti-thrombotic drug, like aspirin, is a solution to this. Aspirin is a cyclooxygenase-1 inhibitor and cyclooxygenase-2 action modifier. Low-dose aspirin treatment (81 mg to 325 mg daily) can inhibit most of the production of thromboxane A_2, a prothrombotic molecule, in platelets (de Gaetano, *et al.*, 1986). It is also used to control stable ischemic heart disease, and in treatment of acute heart failure and stroke (Calverley and Roth, 1998). However, it has side effects of increased chance of hemorrhagic stroke, gastrointestinal bleeding, and prolong bleeding after operation.

Advance Stage of atherosclerosis

If atherosclerosis developed into ischemic heart disease, drug therapies are used to treat the angina pectoris. Drugs involved include vasodilator (Nitrites), β-adrenergic blocker, and calcium channel blockers mentioned in 1.1.2.3. Surgery is also a choice for coronary revascularization (Kasper and Harrison, 2005).

Percutaneous Coronary Intervention (PCI)

PCI is also known as angioplasty. It was first developed by Andreas Gruentzig in Zurich, Switzerland in 1977. The surgery involves the use of a balloon catheter. The catheter penetrates through the skin and goes along the lumen of the artery with the guiding by X-ray. After reaching the destination, the balloon is inflated to open up the artery (Gruntzig, *et al.*, 1979). A stent is placed at the inflated area to keep it expanded (Newsome, *et al.*, 2008). It is mainly for stenosis of one or two coronary vessels. The complication risk is about 2.1% to 12.7%. The possible, major side effects include arrhythmias, vascular complications, contrast reaction, renal failure and haemodynamic complications. The minor side effects include contrast reaction, nausea, and groin complications. Restenosis is possible in revascularized vessels

(Department of Medicine, 2007).

Coronary Artery Bypass Grafting (CABG)

CABG is the anastomosis of internal mammary arteries or radial arteries to the coronary arteries distal to obstruction site. If no arteries available, saphenous vein from leg would also do the job. The new vessels serve as a bypass for transportation of blood to the heart (Fig 1.3). The newly grafted vessels have a lower chance of obstruction compare with PCI. However, it is more expensive and has higher mortality than PCI due to the fact that CABG is a major surgery. This surgery is mainly for stenosis of two or more coronary arteries, especially ones with diabetes. The mortality is about 3% to 4% (Queen Mary Hospital, 2008).

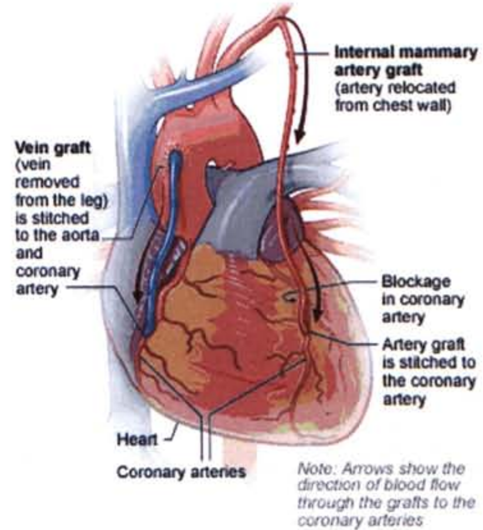


Fig 1.3 Ways of vein and artery bypass grafts are attached to the heart. (Extracted from National Heart Lung and Blood Institute, 2010)

1.2 Selection and Introduction of Current Chinese Medicine Formula

1.2.1 Cardiac Syndrome in Traditional Chinese Medicine

Heart-related diseases were recorded in ancient Chinese medicine records, mainly as heart pain, extending to the back and shoulder, and difficulties in breathe. It can be named in short as “胸痹心痛”. Below were the abstracts from the ancient records:

1) 《素問・臟氣法時論篇》： “心病者，胸中痛，脇支滿，脇下痛，膺背肩胛間痛，兩臂內痛。”
2) 《素問・舉痛論篇》： “經脈流行不止，環周不休，寒氣入經而稽遲，泣而不行，客於脈外則血少，客於脈中則氣不通，故卒然而痛。”
3) 《靈樞・厥病篇》：“真心痛，手足青至節，心痛甚，旦發夕死，夕發旦死。”
4) 《難經・六十難》： “其五臟氣相干，名厥心痛。其痛甚，但在心，手足青者，即名真心痛。其真心痛者，旦發夕死，夕發旦死。”
5) 《金匱要略》： “胸痹不得卧，心痛徹背者，栝蔞薤白半夏湯主之。”
6) 《聖濟總錄》： “胸痛者, 胸痹痛之類也……，胸膺兩乳間刺痛，甚則引背胛，或徹背膂。”
7) 《類証活人書》：“包絡之痛，亦有失血之後，瘀血留滯，胸中隱隱痞痛……，亦有痰涎停伏，窒礙不通而痛，更有本經血滯氣郁，久從火化而虛……皆經所謂心痛也。”

These symptoms are highly similar to the clinical manifestation of angina pectoris, including (1) chest discomfort presented as a pressure, heaviness, tightness, squeezing, burning, or choking sensation; and (2) anginal pains in heart, spreading to upper central abdominal region, back, neck area, jaw, or shoulders. Therefore, “胸痹心痛” is believed to be Chinese medicine version of angina pectoris, which mainly due to ischemic heart diseases.

The main treatments in Chinese medicine involve the use of circulation promoting herbs, such as Radix Salviae miltiorrhizae (丹參), Rhizoma Corydalis (延胡索), Flos Carthami (紅花), Rhizoma Chuanxiong (川芎), and Radix Curcumae (鬱

金). There is also a famous formula that mentioned above "gua lou xie bai ban xia tang" (瓜蔞薤白半夏湯), which was used for a very long time and widely investigated in China (張建敏, *et al.*, 2004, 連樂燊, *et al.*, 2006, 劉貴京, *et al.*, 2004). In the commercial market, the heart tonic drug Kyushin (救心) is also made from traditional Chinese medicines. The above evidences show the effectiveness of traditional Chinese medicine on treatment of heart diseases.

1.2.2 Traditional Chinese Medicine as an Complementary or Alternative Medicine

Complementary and alternative medicine (Campbell and Falck) is a group of diverse medical and health care systems, practices, and products that are not generally considered to be part of conventional medicine. Complementary medicine is used together with conventional medicine, while alternative medicine is used to replace conventional medicine. Traditional Chinese Medicine (TCM) falls into the type of whole medical systems, which defined as systems that are built upon complete systems of theory and practice (Bethesda, 2007). Western medicine is simple and effective in clearly located and well defined disease. However, when the pathology is more complicated, Western medicine becomes ineffective and defective. The disappointment leads to the use of CAM (Leung, *et al.*, 2003).

In United States, an estimated 3.1 million US adults had used acupuncture in the previous year. In the same study, natural product, like herbs, is the top alternative therapy used, with 17 percent of US adult using it (Bethesda, 2009). In China, Chinese medicine hospitals also provide diagnostic and treatment services in Western medicine (Leung, *et al.*, 2003). Some hospital of integrated therapy use TCM as treatment in the problem areas of Western medicine, such as vascular diseases,

dermatitis and rheumatology. Despite of the widespread use of TCM, NCCAM stated that TCM is "Although TCM is used by the American public, scientific evidence of its effectiveness is, for the most part, limited." (Bethesda, 2009). Therefore, scientific researches were widely done to identify the efficacy and mechanisms of action of TCMs.

Scientific researches show TCMs are effective on cardiovascular disease (Li, *et al.*, 2009, Zhao, *et al.*, 2010, Zhou, *et al.*, 2003), rheumatic diseases (Lu, *et al.*, 2009, Zhang, *et al.*, 2010), various cancers (Li, *et al.*, 2010, Luk, *et al.*, 2007, Meng, *et al.*, 2009, Wang, *et al.*, 2010, Woo, *et al.*, 2007) and dermatitis (Makino, *et al.*, 2008, Zhang, *et al.*, 2005). These results further support the use of TCM as CAM.

1.2.3 Selection of TCM Formula from Pharmacopoeia

As recorded in "Zhong yao fang ji xian dai yan jiu da dian" (中藥方劑現代研究大典) (Huang and Shi, 1996), the cardiovascular disease treatment formula were identified. Among them, the simplest formula consisted of three herbs, Radix Salviae miltiorrhizae (Danshen), Radix Puerariae (Gegen) and Rhizoma Corydalis (Yanhusuo) in a weight ratio of 6:3:1. The formula is first used in the clinical trial for treatment of coronary heart disease in 1986. The formula was found to be effective in controlling angina pectoris, lowering triglyceride and improving electrocardiogram. This formula was used as the basic formula for our study.

1.2.3.1 Compound Formula

Traditionally, Chinese herbal medicine was prescribed in compound formula. Comparing with the single herb, compound formula is more potent and have less side effects (Lei, *et al.*, 1995). Through the synergistic effect or formation or new

compounds, the formula can have higher effectiveness. A single Chinese herbal medicine contains a pool of active compounds, which contributes to the multi-functional Chinese herb. When only one or one group of function is needed, the other functions become side effects. Through the antagonistic effect, the formula can reduce or remove unrelated effect or side effects. Thus, compound formula of herbs is a special and advantageous tradition in Chinese medicine. In our research, we keep this tradition to attain a higher efficacy in cardiovascular disease treatments.

1.2.4 Introduction to Constitutional Herbal Medicine

Three herbal medicines would be introduced in this part. Danshen and Gegen were included in our currently investigated formula in a weight ratio of 7:3. The exclusion of Yanhusuo (which originally present in the formula) in the current formula would also be explained.

1.2.4.1 Danshen (Radix Salviae miltiorrhizae)

Salvia miltiorrhiza, which is called Danshen (丹參) in Chinese, is widely used for heart-related diseases in China for long time. The root and rhizome are used. In the perspective of traditional Chinese medicine, Danshen is bitter, a bit cold and belong to heart and liver circulation. It has the function of promoting blood circulation, clearing heat from blood, healing carbuncle, and tranquilizing the mind. It was first recorded in 《神農本草經》 as "主心腹邪氣……破癥除瘕，止煩滿。". In traditional use, it is mainly for treatment of amenorrhea, dismenorrhea or abdominal pain after labour. It can be used for treatment of chest and heart pain, or anxiety and insomnia due to "heat" (Lei, *et al.*, 1995).

In scientific studies, Danshen also have multiple pharmacological functions. The

cardiovascular related ones include: (1) anti-thrombosis (Shen and Li, 1997); (2) cardioprotection (Cao, *et al.*, 2003); (3) anti-hypertension (Kang, *et al.*, 2002); (4) anti-atherosclerosis (Wu, *et al.*, 1998); and (5) improve microcirculation in brain and heart (Yu, 1988). Other functions include promotion of repair and regrowth of the ischemia-damaged cells, anti-oxidation (Niu, *et al.*, 2000) and heptoprotection (Wasser, *et al.*, 1998).

1.2.4.2 Gegen (Radix Puerariae lobatae)

Pueraria lobata, which is called Yegegen (野葛根) in Chinese, has a long history being drug and food in China. Its root was used in this experiment. In the perspective of traditional Chinese medicine, Gegen is bitter, spicy, and cold and belong to pancreas and stomach circulation. It has the function of purging heat, increase saliva secretion, improving the "yang qi", curing diarrhea, and detoxification. It was first recorded in 《神農本草經》 as "主消渴，身大熱，嘔吐，諸痹，起陽氣，解諸毒。". In traditional use, it is mainly used to treat diseases caused by weather change, disease with symptoms of thirsty, measles and diarrhea (Lei, *et al.*, 1995).

In scientific studies, Gegen also have multiple pharmacological functions. The cardiovascular related ones include: (1) anti-hypertension (Sun, *et al.*, 2007); (2) lowering plasma cholesterol level (Yan, *et al.*, 2006); (3) reduce heart rate; and (4) anti-thrombosis (Choo, *et al.*, 2002). Other functions include anti-diabetic (Xu, *et al.*, 2005) and anti-oxidation (Han, *et al.*, 2005).

In the latest edition of Chinese Pharmacopoeia (2010), another species of Gegen, *Pueraria thomsonii*, was mentioned. The contents of the components daidzein, daidzin and puerarin in *Pueraria lobata* are three to five times higher than that of

Pueraria thomsonii. Due to this, *Pueraria lobata* has more potent (about five times stronger) anti-oxidant activity than *Pueraria thomsonii* (Jiang, *et al.*, 2005). Besides, *Pueraria lobata* was also shown to exhibit higher efficacy on vasodilation (Chan, 2006) and hence it was chosen to be used in our study.

1.2.4.3 Yanhusuo (Rhizoma Corydalis)

Corydalis yanhusuo, which is called Yanhusuo (延胡索) in Chinese, is also widely used in China for long time. The rhizome is cooked in boiling water and dried for use. In the perspective of traditional Chinese medicine, Yanhusuo is spicy, bitter, and warm and belong to liver, pancreas and heart circulation. Its main functions are promoting blood and "qi" circulation, and pain killing. It was first recorded in 《雷公炮炙論》 as "治心痛欲死。". In traditional use, it is mainly used to cure blood circulation problem or heart related pain. Sometimes it is used for other analgesic purposes (Lei, *et al.*, 1995).

1.2.4.4 Composition of the Final Formula Used in the Present Study

The main function of Yanhusuo is pain killing, although it also has mild cardiovascular effects. The inclusion of this herb may hide the pain in early stage of cardiovascular disease, like stroke, or heart attack. This may lead to a delay in treatment, which would adversely affect the survival of the patients. For the safety of the patient and considerably small contribution to the cardiovascular function, Yanhusuo is excluded from the formula. So the final formula consists of Danshen and Gegen. With 1 part of Yanhusuo replaced by 1 part of Danshen, the Danshen-Gegen ratio becomes 7:3.

The animal doses used in this study were calculated according to the new and old

clinical doses. For example, the new clinical dose includes a treatment of 2 capsules per day. Each 450 mg capsule (Danshen: Gegen 7:3) contains 436.5 mg herbal extract and 13.5 mg excipient.

- 2 capsules per day, 436.5 mg x 2 = 873 mg
- For a 60kg human (average weight of adult human),

  HED = 873 mg/ 60kg

  = 14.55 mg/kg

- Rat dose = 14.55 mg/kg x 6.2

  = 90.2 mg/kg

- Rabbit dose = 14.55 mg/kg x 3.1

  = 45.1 mg/kg

Conversion factors of 6.2 and 3.1 are for the conversion of human equivalent doses to rat dose and rabbit dose, respectively, based on body surface area. The conversion is recommended by U.S. Department of Health and Human Services, Food and Drug Administration (U.S. Department of Health and Human Services, 2005).

Similarly, for the old clinical dose, the equivalent animal doses calculated were 300 mg/kg for rat and 150 mg/kg for rabbit.

1.2.5 Previous work on Danshen-Gegen Formula and its limitations

In previous studies conducted within our research team, Danshen-Gegen compound formula was shown to have anti-oxidative (Chan, 2006, Koon, 2006),

cardioprotective, vasodilative (Chan, 2006), anti-hypertensive (Tam, 2004) and anti-atherosclerotic (Koon, 2006). In *in vitro* and *in vivo* systems, the formula can scavenge free radicals and increase the expression of antioxidant enzymes (Koon, 2006, Leung, 2003). It also induced vasodilation in *ex vivo* rat aorta (Chan, 2006). The *in vivo* anti-atherosclerotic effect was shown in terms of area of coverage of plaque in rabbits' aorta (Koon, 2006).

However, there are limitations in these experiments. Some of the experiments were done using Radix Puerariae thomsonii instead of Radix Puerariae lobatae. Radix Puerariae lobatae is having much higher bioactivity than Radix Puerariae thomsonii, and was used in our clinical trial and will be used in present study. Some of the experiments were done using a different weight ratio of Danshen and Gegen. The mechanism elucidation did not include all types of potassium channels, but indeed potassium channels involvement was found. The mechanism elucidation included a single dosage of Danshen-Gegen only. For anti-atherosclerotic study, only the amount of plaque, instead of the actual thickness of the plaque, was measured. Therefore, the findings were incomplete, and there may be inconsistence between different data.

To improve these, Danshen-Gegen of weight ratio 7:3 would be used. Same batch of water extract is used throughout all experiments to prevent inconsistence in this study.

In anti-hypertension part, the mechanism elucidation included all pathways of vasodilation, with investigation of the involvement of all four types of potassium channels. Different dosages of DG extract were used including dosage that range from full to no vasodilation. The incubation time after addition of DG extract was extended

to 2 hours, in order to ensure no vasodilation effect was missing.

In anti-atherosclerosis part, intima-media thickness measurements were used to replace the plaque amount for quantification of extent of atherosclerosis, which reflects the situation more accurately.

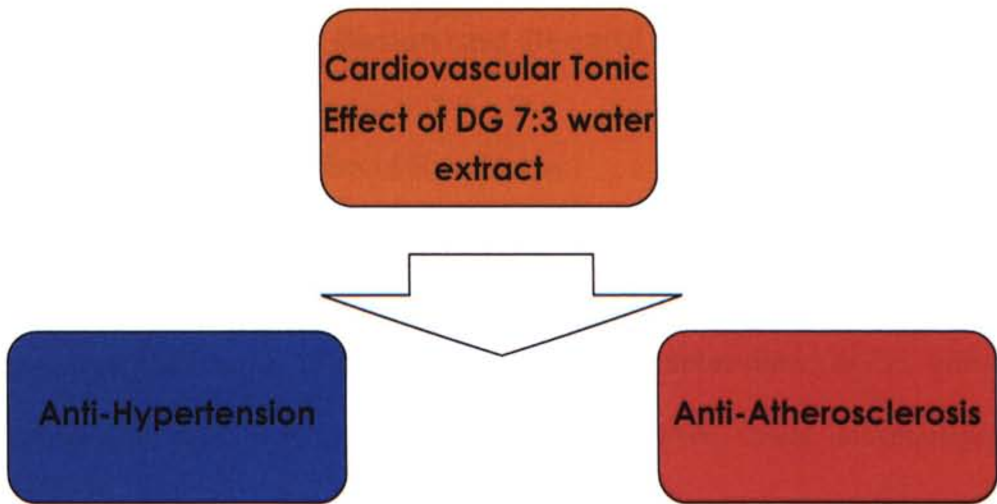
1.3 Objectives of the Present Study

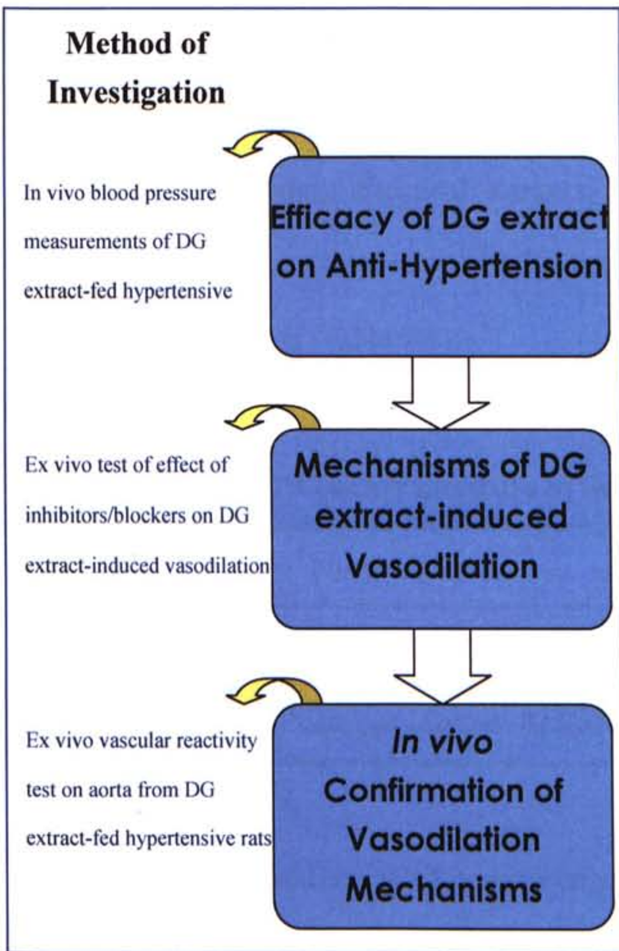
The objective of the present study is to further investigate the vasodilative and anti-atherosclerotic effects of DG 7:3 water extract, in order to give a full picture to cardiovascular tonic effect of DG formula.

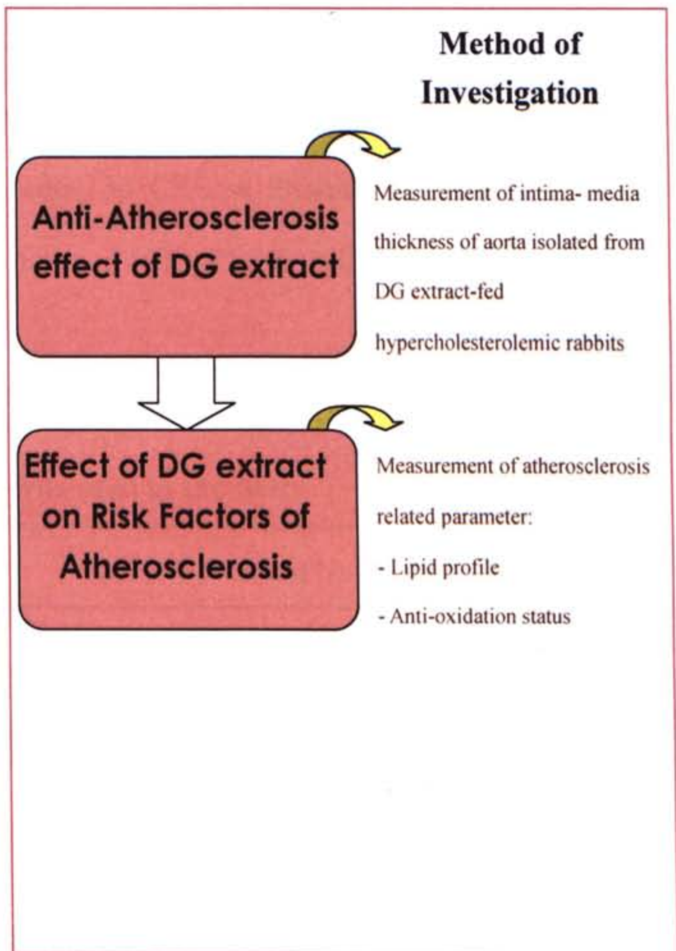
The main objectives include:

1) Verification of previous results and investigation of the new aspects using a single batch of DG extract (produced according to standardized extraction protocol) with *Pueraria lobata* as Gegen, which enabled comparison of results with previous studies, in which a variety of DG ratio, extraction solvents were used and the closely related species of *Pueraria*, *Pueraria thomsonii* was used as Gegen.
2) Verification of DG extract's vasodilative effect in hypertensive rats.
3) Mechanism elucidation of the vasodilative effect of DG extract (in a concentration range) on isolated rat aorta rings, especially the potassium channels which are not investigated before.
4) Study the anti-atherosclerotic effect of DG extract in terms of intima-media thickness in cholesterol-fed rabbits. Although the coverage of plaque in aorta was investigated in previous studies, the main factor causing the blockage of aorta is in fact intima-media thickening.
5) Determination of whether DG extract has anti-oxidative and hypolipidemic effects on cholesterol-fed rabbits, as these effects hinder the progression of intima-media thickening.

1.3.1 Research Plan







Chapter 2

Experimental Design and General Methodology

2.1 Source and Authentication of Raw Herbs

Dried roots of *Salvia miltiorrhiza* (Danshen abbreviated as D), grown in Sichuan province, and dried roots of *Pueraria lobata* (Gegen abbreviated as G), grown in Guangdong province were purchased. The herbs were firstly morphologically authenticated by a botanist, Dr. Cao Hui (National Engineering Research Center for Modernization of TCM, Zhuhai, Guangdong, China). Thin-layer chromatography (TLC) was then utilized to identify the herbs by checking against reference herbs and their corresponding chemical markers, according to Chinese Pharmacopoeia 2005. Voucher specimens were deposited at the museum of ICM, CUHK, with the following voucher numbers (Table 2.1):

Table 2.1 Voucher numbers of the herbs used in the study

Name of Herbs	Voucher Specimen Numbers
Radix Salviae miltiorrhizae (丹參)	2008-3166b
Radix Puerariae lobatae (野葛根)	2008-3167b

With the authenticated herbs, extraction was performed. The raw herbs Danshen and Gegen were used in the weight ratio of 7:3. Extraction was carried out as below:

- ❖ Raw herbs were washed gently with tap water to remove dust and other contaminants
- ❖ The herbs were cut and weighed in the ratio of 7:3

- ❖ The herbs were extracted with water in 1:10 (w/v) under reflux for 1 hour, then the water extract was collected and the process was repeated for another hour
- ❖ The extracts were pooled and centrifuged at 7700 x g for 20 minutes and supernatant was filtered and collected
- ❖ The extract was concentrated using rotary evaporator under reduced pressure
- ❖ The concentrated extract was dried by lyophilization
- ❖ The dried powdered extract was stored in dry location

The yield of extraction was around 35-40%. The same batch of herbs and water extracts were used throughout the present study.

2.2 Materials

Krebs solution stock (10X) was prepared by dissolving 68.96g of NaCl, 3.5g of KCl, 3.675g of $CaCl_2 \cdot 6H_2O$, 2.033g of $MgCl_2 \cdot 6H_2O$ and 1.63g of KH_2PO_4 in 1 liter of distilled water. The stock was diluted to 1X using distilled water with the addition of 2.433g glucose and 2.1g $NaHCO_3$ per liter. Krebs solution (1X) contained 118mM of NaCl, 4.7mM of KCl, 2.5mM of $CaCl_2 \cdot 6H_2O$, 1mM of $MgCl_2 \cdot 6H_2O$, 1.2mM of KH_2PO_4, 13.5mM of glucose and 25mM of $NaHCO_3$. The pH was adjusted to 7.4 and stored at -4℃.

Glucose assay kit was purchased from BioSystems S.A. (Barcelona, Spain).

Trichloroacetic acid (TCA) was purchased from Merck (Darmstadt, Germany).

Phosphate-buffered saline (PBS) (10X), pH 7.4 was obtained from Invitrogen (CA, USA). It was diluted using distilled water.

Ketamine 10% and **Xylazine 2%** were purchased from Alfasan (Woerden, Holland) through Laboratory Animal Services Centre.

Spontaneously hypertensive rats (SHR), Wistar-Kyoto (WKY) rats and Sprague-Dawley (SD) rats were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. All animals were kept at room temperature with a regular 12-hour light/dark cycle, and maintained on a standard rodent chow and water.

Rodent chow (Labdiet 5P14) were purchased from Purina Mills LLC (Minnesota,

USA). The diet was kept unopened in dry condition and protected from light until use.

New Zealand White Rabbits were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. All animals were kept at room temperature with a regular 12-hour light/dark cycle, and maintained on standard/special rabbit chow and water.

Standard rabbit chow (Guinea Pig and Rabbit Pellets) and rabbit chow with 1% cholesterol (SF00-221) were purchased from Specialty Feeds Pty Ltd. (Glen Forest, Australia). The diet was kept unopened in dry condition and protected from light until use.

All triglyceride and cholesterol LiquiColor Tests were purchased from Stanbio Laboratory (Texas, USA). **All H&E staining related reagents** were supplied by division of anatomy. **All other reagents** were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.3 Ethical Approval

All animal studies were conducted under the license from Department of Health, Hong Kong SAR, People's Republic of China, and the procedures were approved by the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (Ref: 09/016/MIS, 09/064/MIS and 09/065/MIS).

2.4. General Methods

2.4.1 Blood Pressure Measurement

Rats were put into the rodent holders so that their noses protruded through the front of nose cones while the tails exit through the rear hatch opening of the holder, to give a comfortable condition for the rats (Fig 2.1). Then, the occlusion cuff and the volume pressure recording cuff were mounted on the rat's tail. After 10 minutes of acclimation, the pressure measurement was obtained using CODA system (Kents scientific Co., USA) with CODA6 Software, installed in a laptop computer with Windows XP system. Six animals were handled under warming at the same time. Minimum of five data of similar systolic blood pressure were obtained for each animal. Average systolic blood pressure of each rat was then calculated.

Fig. 2.1 Setup of CODA system

2.4.2 Blood Profile Measurement

Blood Sample Storage and Preparation

Blood collected from the animals were injected into vacuum tubes with sodium heparin as the anti-coagulant. The tubes were stored in ice during transport. By centrifugation of whole blood at 1500 x g for 10 minutes at 4℃, plasma was collected as the supernatant. Plasma was then stored in 1.5ml eppendorf tube and kept frozen at -80℃ in deep freezer. All assays were performed within 1 month after sampling.

Blood Profile Assay

For all lipid and glucose profile assays, the standards were 2-fold, 4-fold, 8-fold and 16-fold diluted. For total, HDL- and LDL-cholesterol assays, the samples of 1% cholesterol treated rabbits were 10-fold diluted. The procedures of all assays were prepared according to technical manuals of the manufactures

In total cholesterol, triglyceride and glucose assays, 10μl of sample was added into the 96-well plate in duplicate. The reaction reagent (300μl) for each assay was added to each well and incubated at 37°C for 5 minutes. After incubation, absorbance was measured at 500nm.

In HDL- and LDL-cholesterol assays, 10μl of sample was added into the 96-well plate in duplicate. The reaction reagent A (210μl) was added to each well and incubated at 37°C for 5 minutes. After incubation, 70μl of reaction reagent B was added to each well and incubated at 37°C for another 5 minutes. Finally, absorbance was measured at 600nm and 700nm. Differences in absorbance at 600nm and 700nm were used to determine the cholesterol concentration.

$$\textbf{Chol. Conc. of Sample} = \frac{\textbf{Sample } OD_{600nm} - OD_{700nm}}{\textbf{STD } OD_{600nm} - OD_{700nm}} \times \textbf{Chol. Conc. of STD}$$

Anti-oxidation assay

In thiobarbituric acid reactive substances (TBARS) assay, plasma concentrations of TBARS were determined. Thiobarbituric acid (TBA, 67mg) was dissolved in 1ml DMSO with addition of 9ml H_2O. Trichloroacetic acid (TCA, 10%w/v) was prepared using distilled water. The standard 1,1,3,3-tetramethoxypropane (TMOP, 4.167µL of 500µM) was dissolved in 1ml ethanol and 49ml of water was added. The mixing protocol for TMOP standards is shown in Table 2.2.

Table 2.2 The mixing protocol for TMOP standard

Concentrations of TMOP (µM)	H_2O (µl)	Volume of TMOP used (µl)
0	500	------------
0.625	500	500 from 1.25µM standard
1.25	500	500 from 2.5µM standard
2.5	500	500 from 5µM standard
5.	500	500 from 10µM standard
10	800	200 from 50µM standard
50	500	500 from 100µM standard
100	800	200 of 500µM stock

Plasma samples (200µl) were placed into a labeled 1.5mL micro-centrifuge tubes.

Ice cold TCA (10% w/v) (400μl) was added in each tube and incubated in ice for 15 minutes to precipitate protein. After that, the samples were centrifuged at 2200 x g for 15 min at 4℃. Supernatant/standard (250μl) in each tube was transferred to a new 1.5ml eppendorf tube. After that, 250μl of 0.67% (w/v) TBA was added to each tube and incubated in 100℃ for 60 min. The samples/standards were cooled down in ice. 150μl of each sample/standard was loaded into a 96-well plate in duplicate. Absorbance at 532nm was recorded.

The absorbance and concentration of the standards were used to plot the standard curve. The slope was determined. The amount of TBARS was calculated using the formula below:

$$\textbf{TBARS Conc. of Sample} = \frac{\textbf{Sample } \mathbf{OD_{532nm}} \textbf{ - Blank}}{\textbf{Slope of Standard Curve}}$$

2.4.3 Vascular Reactivity Studies

Aorta Isolation and Mounting

Tested rats were sacrificed by cervical dislocation. Blood was drained from abdominal vein using 10ml syringe with 22G needle to prevent excessive blood clot bin aorta. Thoracic aorta was isolated and kept in gassed ice cold-Krebs solution. Fat and connective tissues were trimmed away and the aorta was divided into 8-10 rings of length 2-3mm. The aorta ring was mounted between 2 hooks and kept in the organ bath (Fig 2.2). The organ bath was filled with Krebs solution, kept at 37℃ and gassed with 95% O_2/5% CO_2. The aorta ring was equilibrated for 5 minutes. The tension of the aorta ring was adjusted by 3 times to 1.5g as baseline tension, and Krebs solution was replaced before each adjustment, with 8ml of Krebs solution was added to before last adjustment (Chan, 2006, Woodman and Boujaoude, 2004).

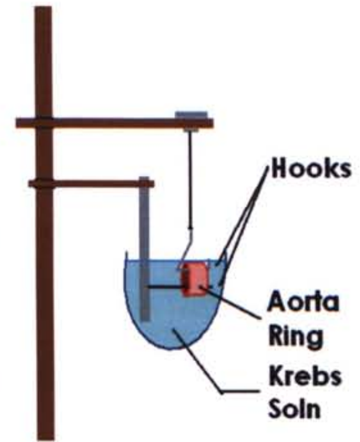


Fig. 2.2 Setup of aorta ring organ bath

Integrity and Function of Aorta Ring

To confirm the integrity of the aorta ring, the contraction and relaxation of it was tested. To induce contraction, 2.4μl of 1mM (R)-(-)-phenylephrine hydrochloride (Phe) (final conc. = 0.3μM) was added, and incubated for 30 minutes to induce plateau vasoconstriction. After incubation, 2.4μl of 1mM acetylcholine chloride (ACh) (final conc. = 0.3μM) was added, and incubated for 5 minutes to induce endothelial-dependent vasodilation. Those aorta rings, with 80% or more relaxation compared to the baseline tension and plateau contraction tension, were regarded as possessing intact endothelium and were accepted for further drug test (Iizuka, *et al.*, 2006, Woodman and Boujaoude, 2004). Aorta rings were then washed with Krebs

solution for 3 times and equilibrated for 20 minutes. After that, the aorta rings were adjusted to baseline tension.

DG Extract-induced Vasodilation

Firstly, 1.2μl of 100μM U46619 (final conc. = 15nM) was added, and incubated for 30 minutes to induce plateau vasoconstriction. DG extract was dissolved in Krebs solution. After incubation, 2, 1, 0.5, and 0.25mg/ml of DG extracts were added into the organ baths, and the tension was recorded by MacLab data acquisition system through Grass force-displacement transducers FT-03E (ADInstruments, Sydney, Australia) for 2 hours. The tension data were analyzed and exported by LabChart 7 Reader. Function of the DG extract was represented by percentage of relaxation after the addition of DG extract, which was calculated using the following formula:

$$\textbf{\% of relaxation} = \frac{\textbf{Plateau tension - Minimum tension after relaxation}}{\textbf{Plateau tension – Baseline tension}}$$

Relaxation curve was plotted by % of relaxation over concentration of DG extract added.

2.5 Statistical Analysis

The verifications of inhibitory effect of the inhibitors were performed by Student's t-test or Mann-Whitney test, depending on the distribution of data. Multiple groups of parameters, such as systolic blood pressure, plasma lipid profile, plasma glucose concentration, TBARS concentration, intima-media ratio and vasodilation effect of DG extract, were compared with no treatment group by ANOVA (followed by post-hoc Dunnett test) or Kruskal-Wallis test (followed by post-hoc Dunns test), depending on the distribution of data.

All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., California, USA). The data were expressed as mean ± standard error of the mean (SEM). A value of $p < 0.05$ was considered statistically significant.

Chapter 3

Anti-hypertensive Studies of Danshen-Gegen Formula on Rat

3.1 Introduction

3.1.1 *In vivo* Anti-Hypertensive Studies

From the previous study by colleagues in our laboratory, Danshen and Gegen (Radix Puerariae thomsonii) compound formula was found to have vasodilative effect (Chan, 2006). Based on that finding, present study aims to investigate the underlying mechanisms of vasodilation. However, Radix Puerariae thomsonii was replaced by Radix Puerariae lobatae in the formula in the present study due to the higher content of components such as daidzein, daidzin and puerarin which have been previously shown to possess vasodilative activities (Chan, 2006). Therefore, before investigation of vasodilation mechanisms, the vasodilative effect of new DG extract, with the use of Radix Puerariae lobatae as Gegen, on rats must be confirmed. Two dosages of DG extract (90.2mg/kg and 300mg/kg per day) were tested, which are rat equivalent dose of the new and old clinical trial, respectively (see section 1.2.4.4). Blood pressure would be used as the parameter to determine the effectiveness of DG extract.

Raloxifene was chosen as the positive control. The dose of 6.2mg/kg was shown to have blood pressure lowering effect on SHR rats (Chan *et al.*, 2007). It is a selective estrogen receptor modulators (SERMs) that inhibits vasoconstriction (Pavo *et al.*, 2000) and potentiates vasodilation, which functions by (1) increasing basal NO release; (2) reducing vascular smooth muscle tone; and (3) improving the effect of NO on vascular smooth muscle in SHR (Chan *et al.*, 2007; Chan *et al.*, 2010). Raloxifene was also found to have cholesterol lowering effect in other studies (Lundeen, *et al.*, 1997). The dose of raloxifene was converted from daily dose of 60mg to the human equivalent dose of 1mg/kg using the same formula (see section

1.2.4.4). Therefore, daily dose of 6.2mg/kg of raloxifene was given to the SHR rats in our study.

3.1.1.1 Spontaneously Hypertensive Rat (SHR)

Spontaneously hypertensive rat (SHR) is a strain of inbred rat that would develop hypertension from 5-6 weeks old. The blood pressure would increase from 120 to 150mmHg at 5-6 weeks old to between 180 to 200 mmHg in about 14 weeks old adult (Okamoto, *et al.*, 1966). The strain was generated by mating a male Wistar rat with spontaneous hypertension with a female Wistar rat with a blood pressure slightly over the average. The spontaneous hypertensive offspring was selected to mate and repeat for six generations. The offspring would then have nearly 100% chance to have spontaneous hypertension. However, SHR was still genetically matched with WKY rat (Okamoto and Aoki, 1963). These factors made SHR to be a very reliable subject to study with an easily obtainable normotensive control.

At the older age of 40 to 50 weeks, the SHR would develop cardiovascular disease character, such hypertrophy of heart and blood vessels. Some were even prone to stroke (Kundu and Rao, 2008).

SHR was chosen instead of other hypertensive models (e.g. angiotensin-induced hypertensive rat or Dahl salt-sensitive hypertensive rat; more details to be found in section 5.2) since its hypertension occurs naturally and no extra manipulation is required. The fact that if manipulation is introduced, such as the use of injection or oral gavage of other drugs to induce hypertension, this will produce stress to the animals (i.e. may affect the blood pressure), and the potential of drug interactions with DG extract is also unknown.

3.1.1.2 Tail-cuff Blood Pressure Measurement

In the experiment, tail-cuff blood pressure measurement was used. A tail-cuff was used to occlude the blood flow. Another non-invasive blood pressure sensor cuff was placed distal to the occlusion-cuff. There are mainly three types of sensor-cuff: photoplethysmography, piezoplethysmography and Volume Pressure Recording (VPR). Photoplethysmography illuminates a small spot on the tail and record the first appearance of the pulse during deflation of the occlusion cuff or the disappearance of pulses during inflation of the occlusion cuff. Piezoplethysmography uses the same theory, but piezoelectric ceramic crystals are used instead of light. Both methods are very susceptible to the tail movement. Last type is VPR. The VPR method transfers the pressure from the cuff to the sensor, which reflects the tail blood volume change. Appearance and disappearance of pulse would cause the tail blood volume varies. Thus, the blood pressure is deduced (Malkoff, 2005).

Direct blood pressure measurement provides most accurate results. However, it involves surgical procedures, which cause a relatively high morbidity and is very undesirable for our long term animal studies.

Therefore, in our present study, non-invasive tail-cuff blood pressure measurement using VPR method was selected, mainly due to the advantages of: (1) non-invasive but comparable accuracy to direct blood pressure measurement; (2) more accurate, less stress and less easily disturbed than photoplethysmography and piezoplethysmography; and (3) 6 Channels in one test making measurement on large number of rats become possible (Malkoff, 2005).

3.1.2 Detailed Underlying Mechanistic Studies

Danshen-Gegen (7:3) formula was demonstrated to have anti-hypertensive effect on SHR rats. However, the underlying mechanism is unknown. Vasodilation may be one of the underlying causes for the lowered blood pressure. Therefore, aorta rings were used to investigate the effect of DG extract on vasodilation. Aorta, like other blood vessels, consist of a thick layer of smooth muscle cells (SMC) and a single layer of endothelial cells. In normal vasodilation, vasodilators would stimulate endothelium to produce signals, in form of nitric oxide (NO) (Ignarro, *et al.*, 1987) or prostacyclin (PGI_2), etc. These signals would act in enzyme cascades on SMC, causing the dephosphorylation of myosin and detachment of myosin from actin. Thus, the smooth muscle would relax and cause vasodilation.

Exogenous chemicals may stimulate endothelium to modify the production of NO or PGI_2. They may also act on the potassium channels directly, causing the hyperpolarization of SMC and cause vasodilation directly.

3.1.2.1 Nitric Oxide-mediated Vasodilation

L-arginine is converted to nitric oxide (NO) by nitric oxide synthase (NOS) (Bredt and Snyder, 1990). NO serves many different functions, like inhibiting platelet aggregation (Mellion, *et al.*, 1981) and leukocyte adhesion. However, NO is also released by phagocytes as weapons (Hibbs, *et al.*, 1987). During ischemic reperfusion process, NO would react with superoxides to form peroxynitrite, which is responsible for oxidative damage of the tissue (Szabo, 1996). NO is mainly produced by three isoforms of NOS: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS).

eNOS is the predominant NOS within endothelial cell. Upon stimulation, L-arginine is converted to nitric oxide by eNOS (Rees, *et al.*, 1989). NO released from the endothelium would diffuse to the SMC and activates guanylate cyclase (GC) to produce cyclic GMP (cGMP) (Gruetter, *et al.*, 1979). The increased cGMP level would activate more protein kinase G (PKG) (Fig 3.1). PKG phosphorylates and activates myosin light chain phosphatase (MLCP) (Murphy and Walker, 1998). MLCP catalyzes the dephosphorylation of myosin light chain, which cause myosin detaches from actin. The SMC relaxes and causes vasodilation.

3.1.2.2 Prostacyclin-mediated Vasodilation

In endothelial cell, cyclooxygenase converts arachidonic acid to prostaglandin H_2 (PGH_2) (MacIntyre, *et al.*, 1978). PGH_2 is converted to prostacyclin (PGI_2), which is released and binds to the G-protein coupled receptor on SMC. This causes the activation of adenylate cyclase and the increased production of cyclic AMP (cAMP) (Tanaka, *et al.*, 2004). The increased cAMP would activate protein kinase A (PKA) (Fig 3.1) (Haynes, *et al.*, 1992). PKA phosphorylates and inactivates myosin light chain kinase (MLCK) (de Lanerolle, *et al.*, 1984, Somlyo and Somlyo, 2003). The myosin light chain kinase is responsible for phosphorylation of myosin to bind with actin, which is the cause of vasoconstriction. Reduced amount of active myosin light chain kinase can reduce vasoconstriction and make the vessels to dilate.

3.1.2.3 Hyperpolarization-mediated Vasodilation

Hyperpolarization happens to change the membrane potential to be more negative. In vascular SMC, potassium (K^+) channels are mainly responsible for that. The opening of K^+ channels causes an efflux of potassium ions. This event is followed by the closure of voltage-dependent calcium channels. The entry of calcium ions is

reduced and the intracellular calcium is reduced. Vasodilation is the final result. Tetraethylammonium is the commonly used non-selective blocker for potassium channels. Four different types of potassium channels involved would be discussed below:

Inward Rectifier K^+ Channels (Kir channels)

Kir channels have mainly 2 possible functions. The first one is the maintenance of resting potential of the membrane (Park, *et al.*, 2007). The second one is the activation in response to the moderate increase in extracellular K^+ concentration, to cause vasodilation (Chrissobolis, *et al.*, 2000). Kir channels allow a large inward current to pass the charge into the cell, but only allow a small outward current. Barium salts are specific blockers to this type of channel (Fig 3.1) (Nelson and Quayle, 1995).

Voltage-dependent K^+ Channels (Kv channels)

Kv channels open in response to membrane depolarization, allowing the efflux of K^+ (Nelson and Quayle, 1995). This causes the repolarization of membrane to return to the resting potential. 4-Aminopyridine is the blocker that targets this channel (Nelson and Quayle, 1995), with little inhibition on K_{ATP} channels (Fig 3.1).

Large-conductance Ca^{2+}-activated K^+ Channels (BK_{Ca} channels)

BK_{Ca} channels can be activated by both membrane depolarization and change in intracellular Ca^{2+} to maintain membrane potential. Its function includes counteracting the pressure- or chemical-induced depolarization and vasoconstriction (Brayden and Nelson, 1992, Tanaka, *et al.*, 2004). Iberiotoxin and charybdotoxin are used as blockers of this type of channels (Brayden and Nelson, 1992). However, iberiotoxin is

more specific, while charybdotoxin may also block Kv channels and intermediate conductance Ca^{2+}-activated K^+ channels (Fig 3.1).

ATP-sensitive K^+ Channels (K_{ATP} channels)

K_{ATP} channels are found in cardiac muscle and vascular smooth muscle (Fujita and Kurachi, 2000). Blockage of K_{ATP} channels cause depolarization and vasoconstriction. K_{ATP} channels activation occur mainly during disease state, which leads to vasodilation (Ko, *et al.*, 2008). Glibenclamide is one of the most commonly used blocker of K_{ATP} channels (Fig 3.1) (Nelson and Quayle, 1995).

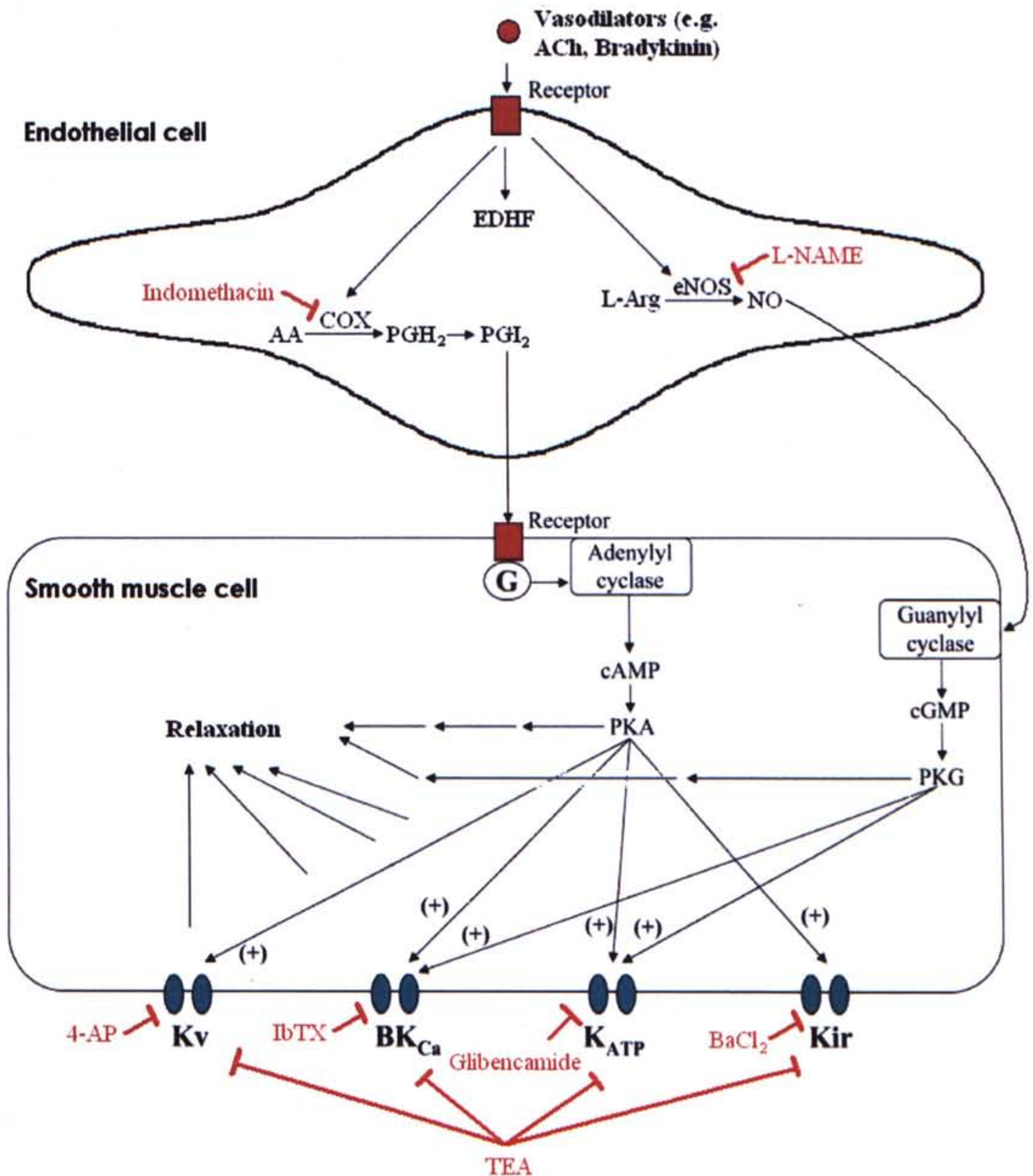


Fig 3.1 Vasodilation mechanisms and involvements of inhibitors or blockers. Black arrows are the main pathways and red blunt lines are the action sites of inhibitors or blockers (Modified from Ko *et al.*, 2008)

3.1.2.4 Endothelium-dependent/-independent Vasodilation

Apart from the involvements in different pathways, the vasodilator can act with/without endothelium. Intrinsically, vasodilation undergoes the whole NO-mediated/prostacyclin-mediated vasodilation pathway and then causes the hyperpolization and relaxation. However, the extrinsic vasodilator may directly act on

the smooth muscle cells. Sodium nitroprusside (SNP), a NO-donor, can directly activate guanylate cyclase in SMC and cause vasodilation (Hayashi and Hester, 2010, Taylor, *et al.*, 1988). A number of vasodilative Chinese herbs acting on potassium channels are also endothelium-independent (Pan, *et al.*, 2008, Xia, *et al.*, 2008). Therefore, determination of endothelium dependence of DG extract can help identifying its mode of action.

3.1.3 Long Term Underlying Mechanistic Studies

The effect of long term oral intake of DG extract on aorta rings, which mimics the real situation in human, is still unknown. As a follow-up of previous parts, Danshen-Gegen 7:3 formula is able to induce vasodilation on isolated aorta rings. The SHR rats model would be applied again. To coherent with the methods in section 3.1.1, the SHR rats used are 14 weeks old, which already have hypertension. DG extract treatment of 1 month is already able to lower the blood pressure. Aorta rings are isolated and tested as in section 3.1.2. In order to test the effects of DG extract that were orally taken, a serial dose of acetylcholine replaces the direct addition of DG extract to induce vasodilation. By comparing the acetylcholine-induced relaxation curve between different groups, the long term effect of DG extract on aorta ring can be shown. The relaxation curve reflects DG extract ability to potentiate vasodilation, while the contractile force reflects the vasoconstriction and stiffness of the aorta.

3.2 Methods

3.2.1 *In vivo* Anti-Hypertensive Studies

Animals, Diet and Treatment

Male SHR rats of 6 weeks-old and 14 weeks-old were used for the preventive and therapeutic studies of the effect of DG extract, respectively. The rats were divided into 4 groups, which were intragastrically-fed (using metal intragastric feeding tube) daily with either distilled water (negative control), 6.2mg/kg raloxifene (positive control), 90.2mg/kg or 300mg/kg of DG extract dissolved in distilled water. WKY rats of same age were used as normal control and were fed with distilled water in same manner (Table 3.1). The rats were given *at libitum* assess to food and water.

Table 3.1 Treatment and sample size of each group in the preventive and therapeutic studies of DG extract on hypertension

Rats	Treatments	Sample size in Preventive study	Sample size in Therapeutic study
WKY	Water (4ml/kg), as negative control	6	6
SHR	Water (4ml/kg), as negative control	6	6
SHR	90.2mg/kg DG extract in water (4ml/kg)	6	6
SHR	300mg/kg DG extract in water (4ml/kg)	6	6
SHR	6.2mg/kg Raloxifene in water (4ml/kg), as positive control	6	6

Blood Pressure Measurement

Systolic blood pressure measurement was carried out bi-weekly from 6 (preventive study) or 14 weeks (therapeutic study) for 12 weeks, according to protocol in 2.4.1. The average systolic blood pressure of each group was plotted against time.

Sampling of Animal Tissue

After 12 weeks of treatment, the rats were sacrificed by cervical dislocation. All rats were fasted for 12 hours before blood collection. Blood sample was collected from abdominal vein and plasma was prepared for total cholesterol, triglyceride and glucose measurements as stated in protocol 2.4.2.

3.2.2 Detailed Underlying Mechanistic Studies

Animals and Sampling of Aorta

Male SD rats of 295 – 305g were sacrificed by cervical dislocation. Sampling was done according to "Aorta Isolation and Mounting" of section 2.4.3.

Mechanistic Study

Preparation and confirmation of function of aorta was done according to "Aorta Isolation and Mounting" and "Integrity and Function of Aorta Ring" of section 2.4.3.

The experimental protocol was similar to "DG extract-induced vasodilation" of section 2.4.3, except that different concentrations of various blockers (Table 3.2) were added and incubated for additional 30 minutes (Park, *et al.*, 2003) before addition of U46619. The doses of blockers, vasoconstrictors (Phe or U46619) and vasodilator (ACh) used in the present study were based on previous literatures (Park, *et al.*, 2003; Ko, *et al.*, 2008). U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) is a prostaglandin H_2-analogue and has similar biological activity as thromboxane A_2, which can cause vasoconstriction (Coleman, *et al.*, 1981). ACh is the neurotransmitter in both CNS and PNS. In vascular endothelial cells, it acts on acetylcholine muscarinic M3 receptor, which leads to increased the synthesis of NO and PGI_2 and causes vasodilation (Spence, *et al.*, 2004). On the other hand, Phe is an alpha-1 (α1) adrenergic receptor agonist. Alpha-1 adrenergic receptor is responsible for the fight-or-flight response, which cause vasoconstriction in arteries when stimulated (Varma and Deng, 2000).

Relaxation curves of DG extract treatments with different blockers were compared to the relaxation curve of DG extract treatment alone.

For endothelium-independent vasodilation, denudation was performed by gentle rubbing of aorta ring against a wooden toothpick. The denudation was confirmed by the inability of ACh to induce vasodilation, as ACh-induced vasodilation is endothelium-dependent.

Table 3.2 The various blockers used in the mechanistic studies (*ex vivo*)

Blockers	Full Name	Properties	Concentrations used	Vasoconstrictors used
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride	Non-specific nitric oxide synthase inhibitor	100μM	U46619
Indomethacin		Non-specific cyclooxygenase inhibitor	10μM	U46619
TEA	Tetraethylammon-ium chloride	Non-selective K channels blocker	10mM	U46619
$BaCl_2$	Barium chloride	Kir channels	100μM	U46619
4-AP	4-aminopyridine	K_v channels blocker	1mM	U46619
IbTX	Iberiotoxin	BK_{Ca} channels blocker	100nM	U46619
Glibenclamide	---	K_{ATP} channels blocker	10μM	Phenylephrine

3.2.3 Long Term Underlying Mechanistic Studies

Animals, Diet and Treatment

Male SHR rats of 14 weeks-old were purchased and kept in the University Centralized Science Laboratory Building. Diet and treatment of the SHR rats were the same as that described in section 3.1.2 (Table 3.3).

Table 3.3 Treatment and sample size of each group in the mechanistic study of DG extract on hypertension

Rats	Treatments	Sample size
WKY	Water (4ml/kg), as negative control	6
SHR	Water (4ml/kg), as negative control	6
SHR	90.2mg/kg DG extract in water (4ml/kg)	6
SHR	300mg/kg DG extract in water (4ml/kg)	6
SHR	6.2mg/kg Raloxifene in water (4ml/kg), as positive control	6

Blood Pressure Measurement

Blood pressure measurement was carried out at 14th week (Before treatments) and 18th week (after treatments), according to protocol in section 2.4.1.

Sampling of Aorta

After 4 weeks of treatment, the rats were sacrificed by cervical dislocation. Sampling was done according to "Aorta Isolation and Mounting" of section 2.4.3.

Mechanistic Study

Preparation and confirmation of function of aorta was done according to "Aorta Isolation and Mounting" and "Integrity and Function of Aorta Ring" of section 2.4.3.

Different concentration of various blockers (Table 3.4) were added and incubated for 30 minutes.

Table 3.4 The various blockers used in the mechanistic studies (*in vivo* confirmation)

Blockers	Full Name	Properties	Concentrations used	Vasocontrictors used
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride	Non-specific nitric oxide synthase inhibitor	100μM	Phenylephrine
Indomethacin		Non-specific cyclooxgenase inhibitor	10μM	Phenylephrine
TEA	Tetraethylammon -ium chloride	Non-selective K channel blocker	10mM	Phenylephrine

2.4μl of 1mM Phe (final conc. = 0.3μM) was added, and incubated for additional 30 minutes to induce plateau vasoconstriction. After incubation, 6 concentrations of ACh ranging from 10^{-9} to 10^{-4}M were added into the organ baths in 5 minute-intervals, and the tension was recorded by MacLab data acquisition system through Grass force-displacement transducers FT-03E (ADInstruments, Sydney, Australia). Percentages of relaxation after each addition of ACh were calculated using following formula:

$$\textbf{\% of relaxation} = \frac{\textbf{Plateau tension - Minimum tension before next ACh addition}}{\textbf{Plateau tension – Baseline tension}}$$

The contractile force after addition of Phe was calculated using following formula:

Contractile force = Plateau tension - Minimum tension before Phe addition

The relaxation curves with/without blocker were compared. The maximum contractile force was recorded as the difference between plateau tension and baseline tension. The differences in relaxation curves among different groups were also compared.

3.2.4 Statistical analysis

***In vivo* Anti-Hypertensive Studies**

In systolic blood pressure and weight measurement, ANOVA was used for analysis. The data for WKY group were compared with that of untreated SHR group, while the data for the treatment groups were compared with that of untreated SHR.

In blood profile assays, student's t-test was used for comparison between WKY group and untreated SHR group. ANOVA was used to compare the data for the treatment groups with that of untreated SHR.

Detailed Underlying Mechanistic Studies

In the study of the effect of Danshen-Gegen 7:3 water extract on dilation of intact rat aorta rings, all treatment groups were compared with the Kreb's group using ANOVA. In assays using denuded aorta, or with inhibitors/blockers treated, the data of denuded or inhibitors/blockers treated groups were compared with the intact, untreated aorta with same dose of DG extract added, using student's t-test.

Long Term Underlying Mechanistic Studies

In systolic blood pressure measurement and vasodilation assay using increasing dose of ACh, ANOVA was used for analysis. The data for WKY group were compared with that of untreated SHR group, while the data for the treatment groups were compared with that of untreated SHR.

In contractile force assay and vasodilation assay using single dose of ACh, student's t-test was used for comparison between WKY group and untreated SHR group. ANOVA was used to compare the data for the treatment groups with that of

untreated SHR.

3.3 Results

3.3.1 *In vivo* Anti-Hypertensive Studies

3.3.1.1 Preventive Effect in Hypertension

Danshen-Gegen 7:3 water extract was tested for the preventive effect in hypertension in SHR rats of 6 weeks old, in which hypertension have not yet been developed. In Fig 3.2, the SHR rats had an increasing trend of systolic blood pressure from 6 weeks onwards. SHR rats reached a significantly higher systolic blood pressure (~200mmHg) starting from week 10, comparing with WKY rats. Treatments of 6.2mg/kg of raloxifene and 90.2mg/kg and 300mg/kg of DG extract could significantly lower the plateau systolic blood pressure to about 180mmHg. The anti-hypertensive effect of 90.2mg/kg and 300mg/kg of DG extract had no significant difference.

Regarding other plasma parameters, SHR rats had significantly lower plasma triglyceride (Fig 3.3 b). Comparing with SHR rats without treatments, 6.2mg/kg of raloxifene could significantly lower the plasma total cholesterol (Fig 3.3 a). There was no significant difference among groups in plasma glucose level (Fig 3.4).The weights of the rats have no significant difference among groups (Fig 3.5).

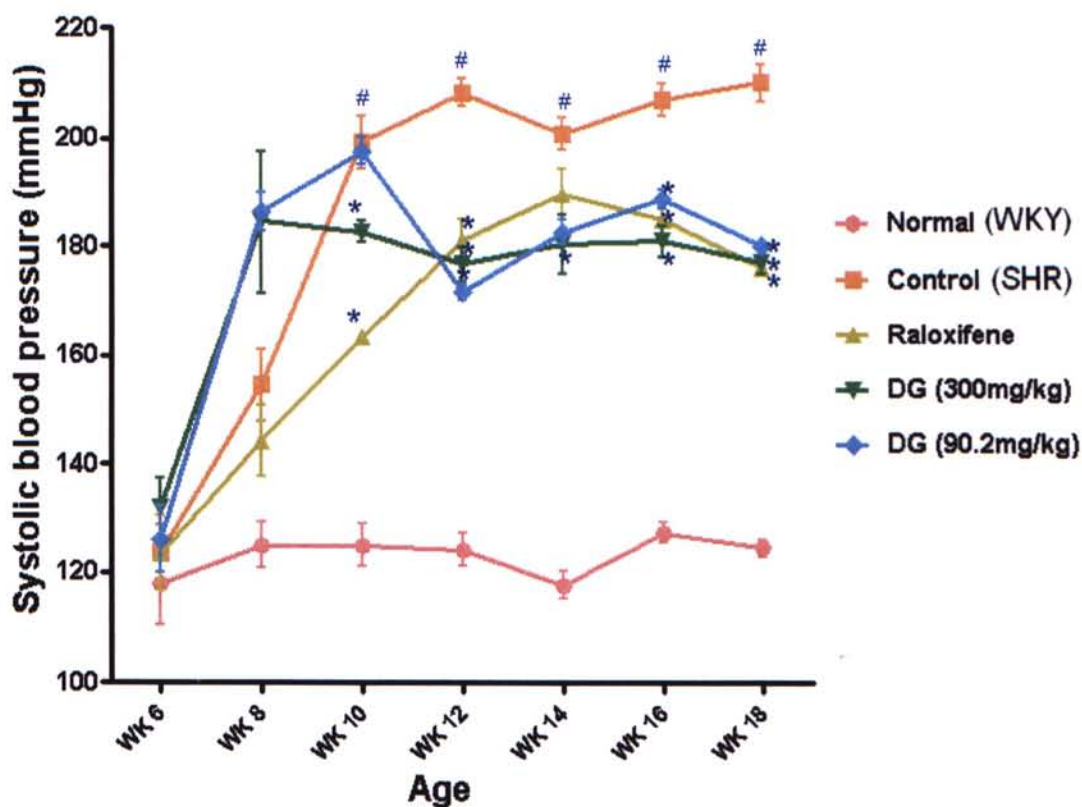


Fig 3.2 Preventive Effect of Danshen-Gegen 7:3 water extract on hypertension in SHR rats. All extracts/drug/water were administered daily by oral gavage. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. WKY vs SHR, # $p < 0.05$; SHR vs treatment groups, * $p < 0.05$.

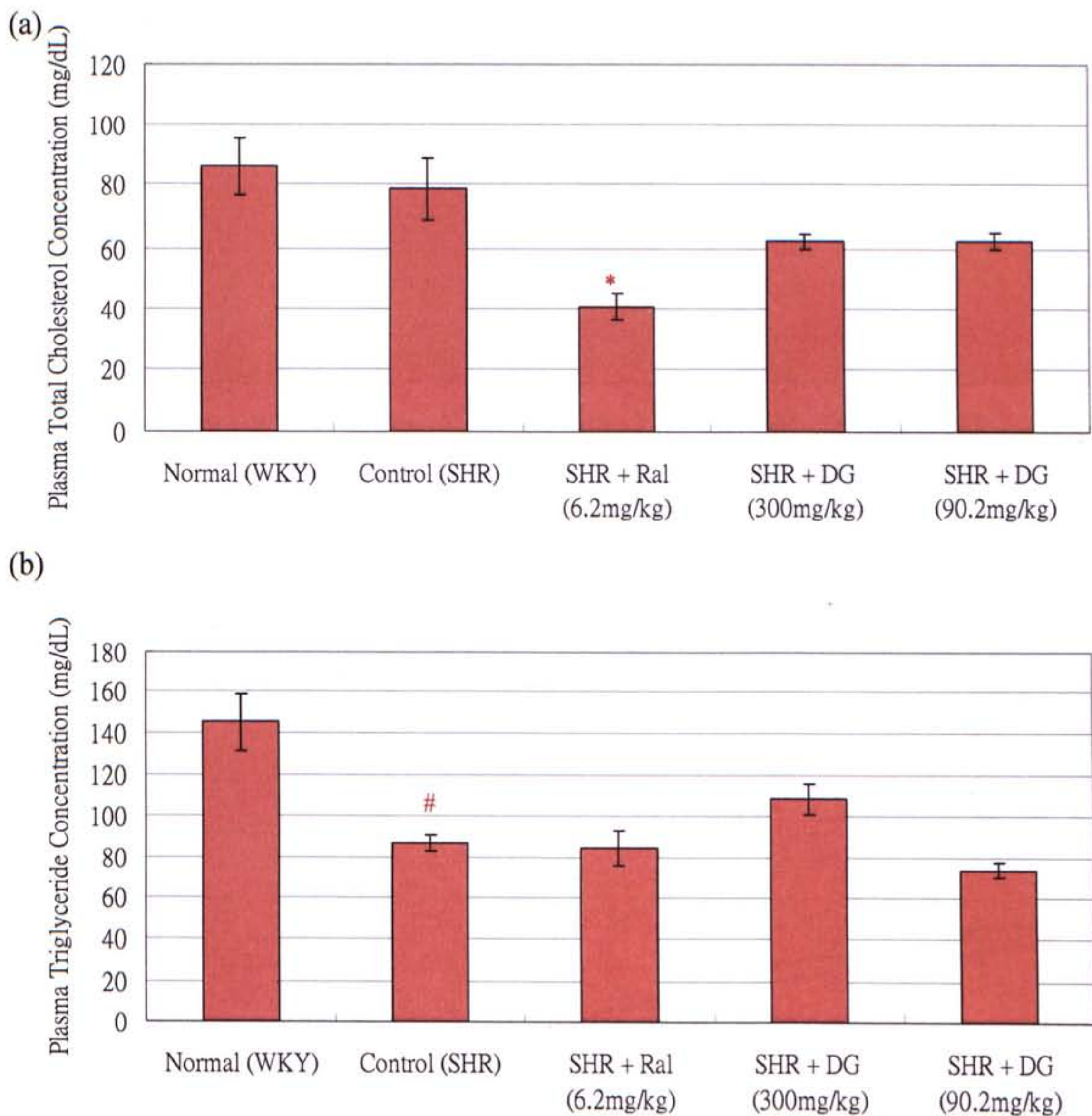


Fig 3.3 Effect of Danshen-Gegen 7:3 water extract on plasma lipid level in SHR rats (Preventive Study). (a)Plasma total cholesterol level; (b) Plasma triglyceride level. SHR group is compared with WKY groups. All treatment groups are compared with SHR group. All data are presented in mean ± SEM (n=6). WKY vs SHR using student's t-test, # $p < 0.05$; SHR vs treatment groups using ANOVA, * $p < 0.05$.

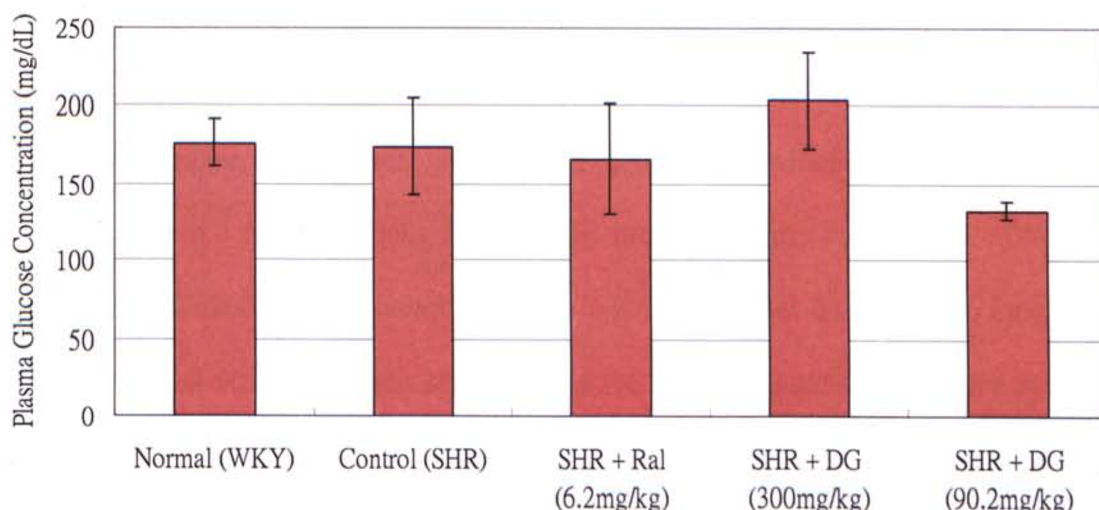


Fig 3.4 Effect of Danshen-Gegen 7:3 water extract on plasma glucose level in SHR rats (Preventive Study). All data do not show a significant difference. All data are presented in mean ± SEM (n=6). WKY vs SHR using student's t-test, # $p < 0.05$; SHR vs treatment groups using ANOVA, * $p < 0.05$.

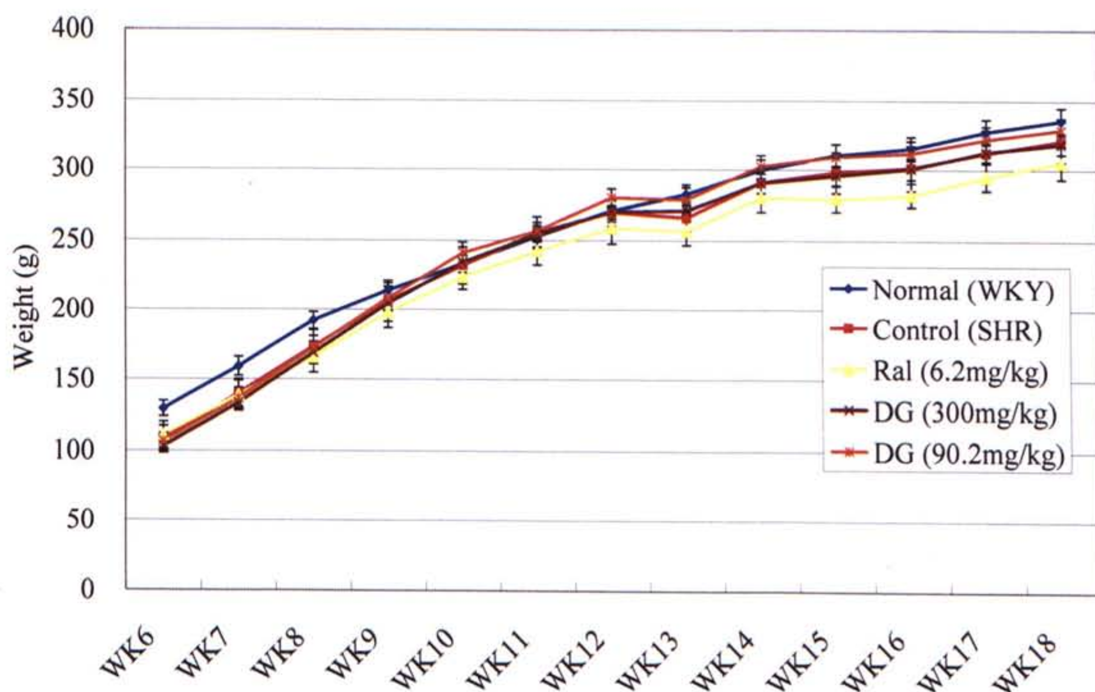


Fig 3.5 Effect of Danshen-Gegen 7:3 water extract on the weight of SHR rats (Preventive Study). All data do not show a significant difference. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. * $p < 0.05$.

3.3.1.2 Therapeutic Effect in Hypertension

Danshen-Gegen 7:3 water extract was tested for the therapeutic effect against hypertension in SHR rats of 14 weeks old, during which hypertension was developed. In Fig 3.6, from weeks 14, the SHR rats had significantly higher systolic blood pressure (~200mmHg), compared with WKY rats. After treatments, 6.2mg/kg of raloxifene and 90.2mg/kg and 300mg/kg of DG extract could significantly lower the plateau systolic blood pressure to about 180mmHg. Again, no dose dependent response was observed between 90.2mg/kg and 300mg/kg of DG extract treatments.

Regarding other plasma parameters, SHR rats had significantly lower cholesterol and triglyceride (Fig 3.7). Comparing with SHR rats without treatments, 6.2mg/kg of raloxifene and 90.2mg/kg of DG extract could significantly lower the plasma total cholesterol (Fig 3.7 a). There was no significant difference among groups in plasma glucose level (Fig 3.8). The weights of the rats have no significant difference among groups (Fig 3.9).

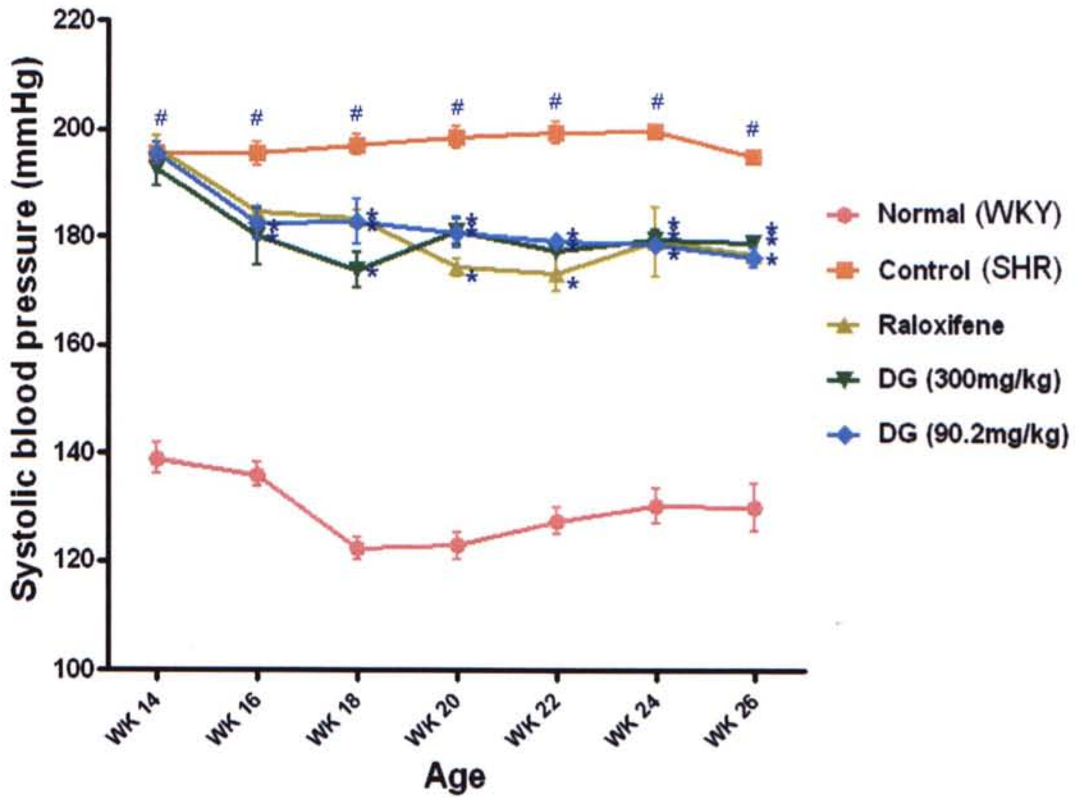


Fig 3.6 Therapeutic effect of Danshen-Gegen 7:3 water extract on hypertension in SHR rats. All extracts/drug/water were administered daily by oral gavage. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. WKY vs SHR, # $p < 0.05$; SHR vs treatment groups, * $p < 0.05$.

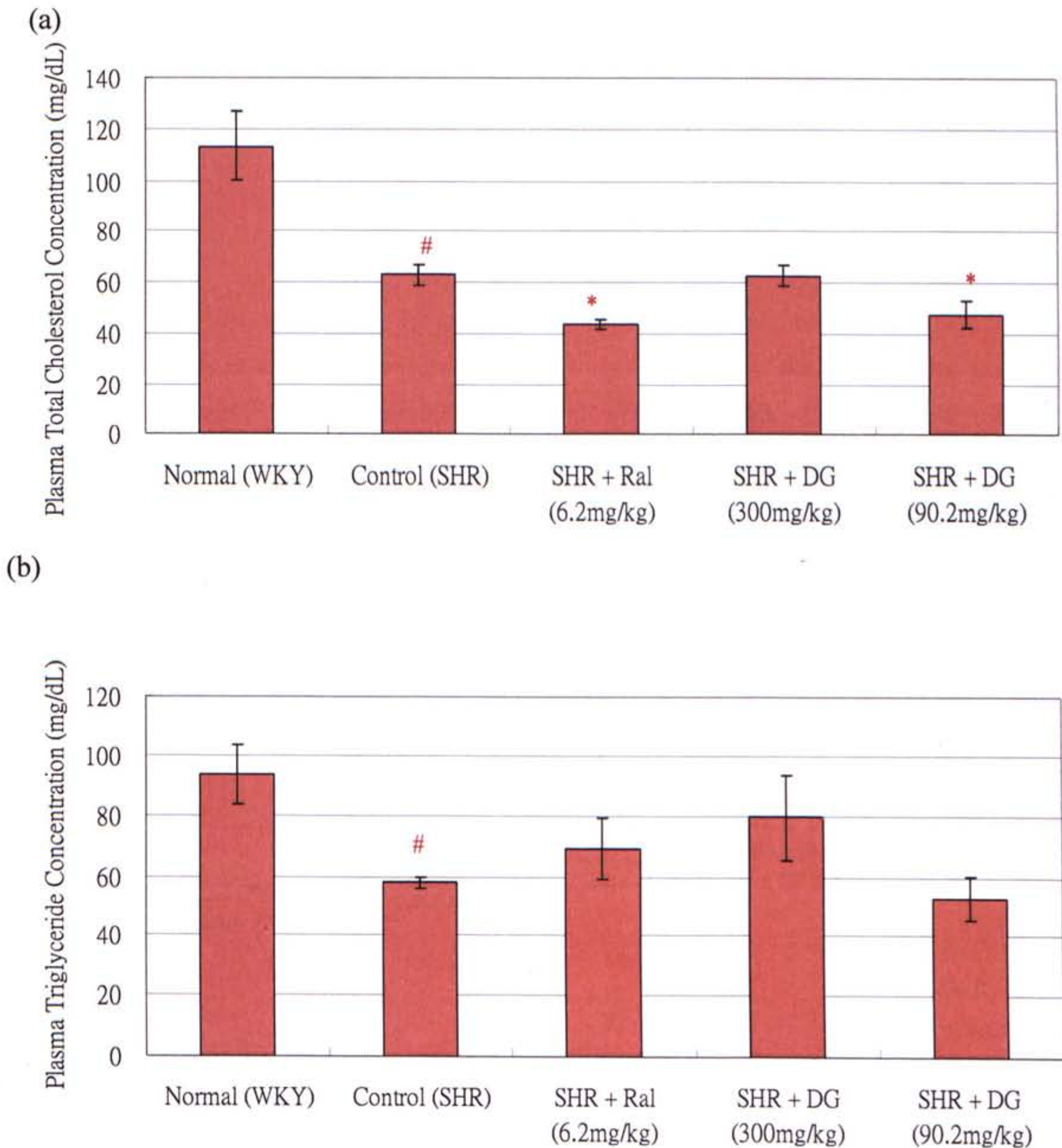


Fig 3.7 Effect of Danshen-Gegen 7:3 water extract on plasma lipid level in SHR rats (Therapeutic Study). (a)Plasma total cholesterol level (b) Plasma triglyceride level. SHR group is compared with WKY groups. All treatment groups are compared with SHR group. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. WKY vs SHR using student's t-test, # $p < 0.05$; SHR vs treatment groups using ANOVA, * $p < 0.05$.

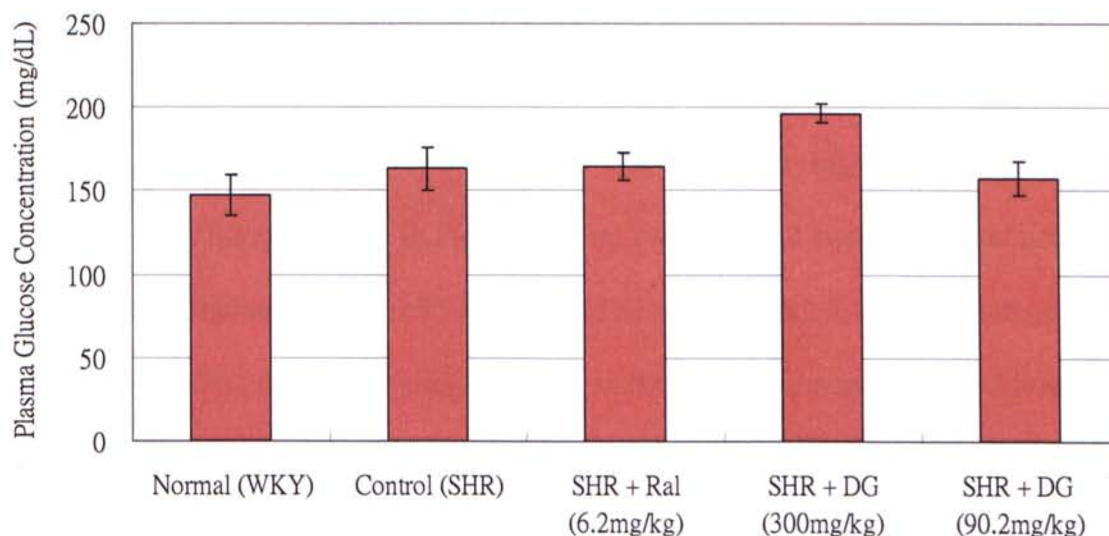


Fig 3.8 Effect of Danshen-Gegen 7:3 water extract on plasma glucose level in SHR rats (Therapeutic Study). All data do not show a significant difference. All data are presented in mean ± SEM (n=6). WKY vs SHR using student's t-test, # $p < 0.05$; SHR vs treatment groups using ANOVA, * $p < 0.05$.

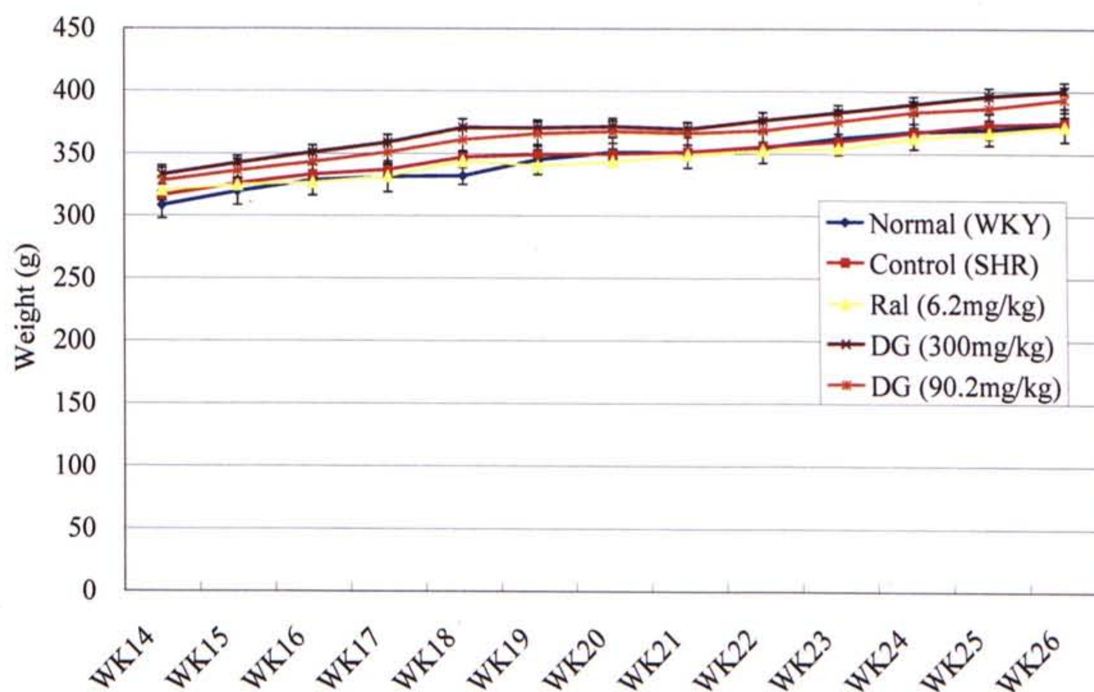


Fig 3.9 Effect of Danshen-Gegen 7:3 water extract on the weight of SHR rats (Therapeutic Study). All data do not show a significant difference. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. * $p < 0.05$.

3.3.2 Detailed Underlying Mechanistic Studies

3.3.2.1 DG extract-induced Vasodilation

DG extract was tested for the vasodilative effect on aorta rings isolated from SD rats, which were normotensive. In Fig 3.10, aorta rings without any DG extract added had 26% of relaxation. With 0.25 mg/ml of DG extract added, the percentage of relaxation was 27%, which was similar to that without DG treatment. With addition of 0.5 mg/ml of DG extract, the relaxation percentage was 46% and showed a significant relaxation. The addition of 1 and 2 mg/ml DG extract caused a 96% and 110% relaxation, respectively. These doses of DG extract were used in the vasodilation study, as 0.25mg/ml DG extract induced no relaxation and 2mg/ml of DG extract induced a full relaxation. This ensured the full coverage of different relaxation extents in our test.

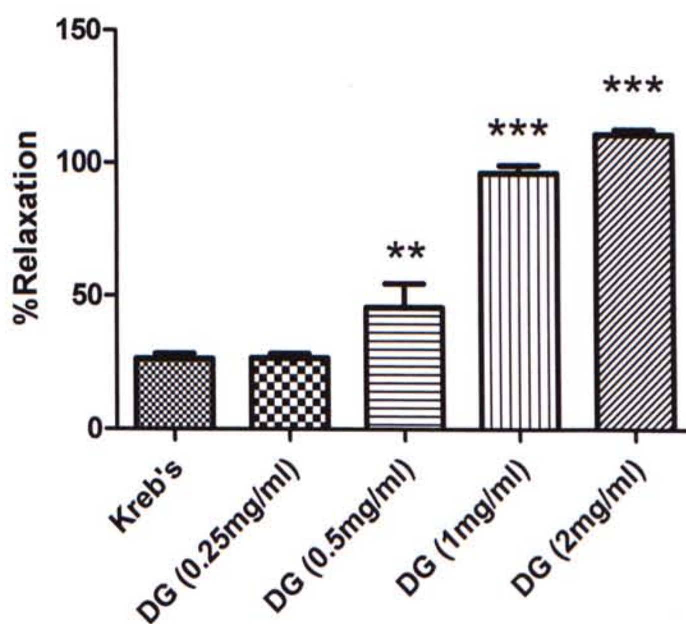


Fig 3.10 Effect of Danshen-Gegen extract on dilation of intact rat aorta rings. All data are presented in mean + SEM (n=7-12). ANOVA was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$.

3.3.2.2 Endothelium-independent Vasodilation

The endothelium of aorta rings were denuded by gentle rubbing against a wooden toothpick to remove endothelium. The denuded aorta rings were tested and compared against the intact ones. In Fig 3.11, aorta rings without any DG extract added had 19% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 32%, 42%, 94% and 113% relaxation, respectively. The relaxation was not significantly different from intact aorta.

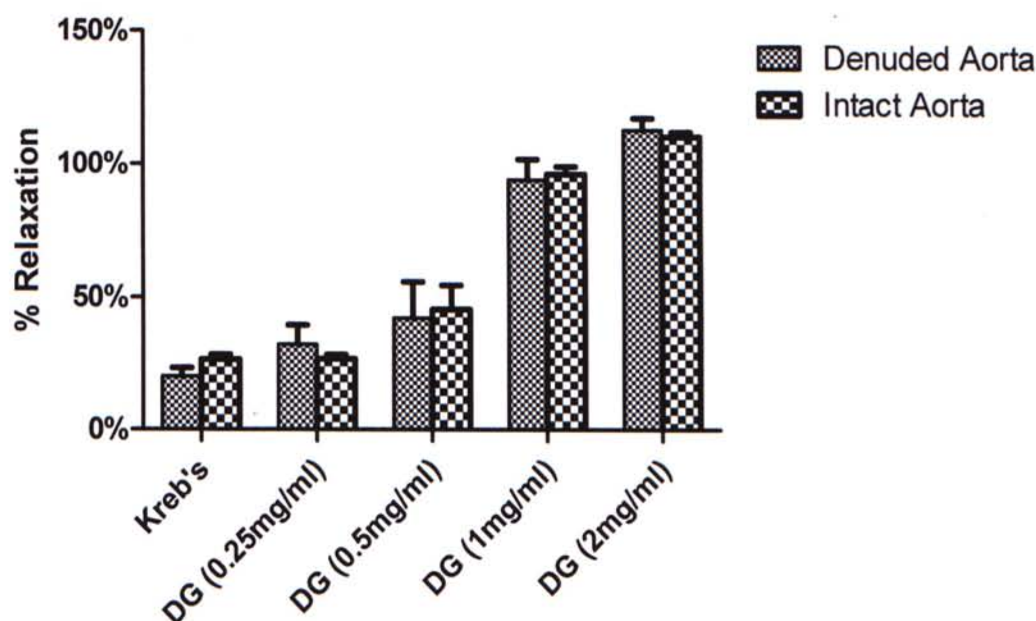


Fig 3.11 Effect of Danshen-Gegen extract on dilation of denuded rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. * $p < 0.05$.

3.3.2.3 Nitric Oxide-mediated Vasodilation

The intact aorta rings were treated with L-NAME before addition of DG extract. The treated aorta rings were tested and compared against the untreated ones. In Fig 3.12, aorta rings without any DG extract added had 24% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 27%, 82%, 99% and 110% relaxation, respectively. The relaxation was not significantly different from intact aorta.

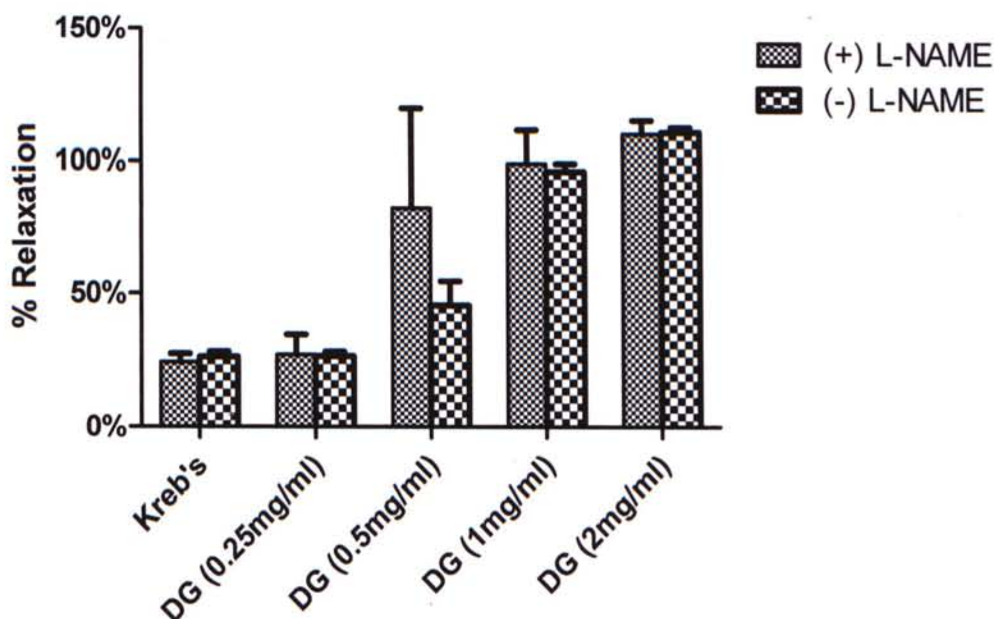


Fig 3.12 Effect of L-NAME on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=3-12). Student's t-test was used for statistical analysis of each pair. * $p < 0.05$.

3.3.2.4 Prostacyclin-mediated Vasodilation

The intact aorta rings were treated with indomethacin before addition of DG extract. The treated aorta rings were tested and compared against the untreated ones. In Fig 3.13, aorta rings without any DG extract added had 34% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 35%, 60%, 97% and 111% relaxation, respectively. The relaxation was not significantly different from intact aorta, except the relaxation with indomethacin was significantly higher at 0.25mg/ml DG extract.

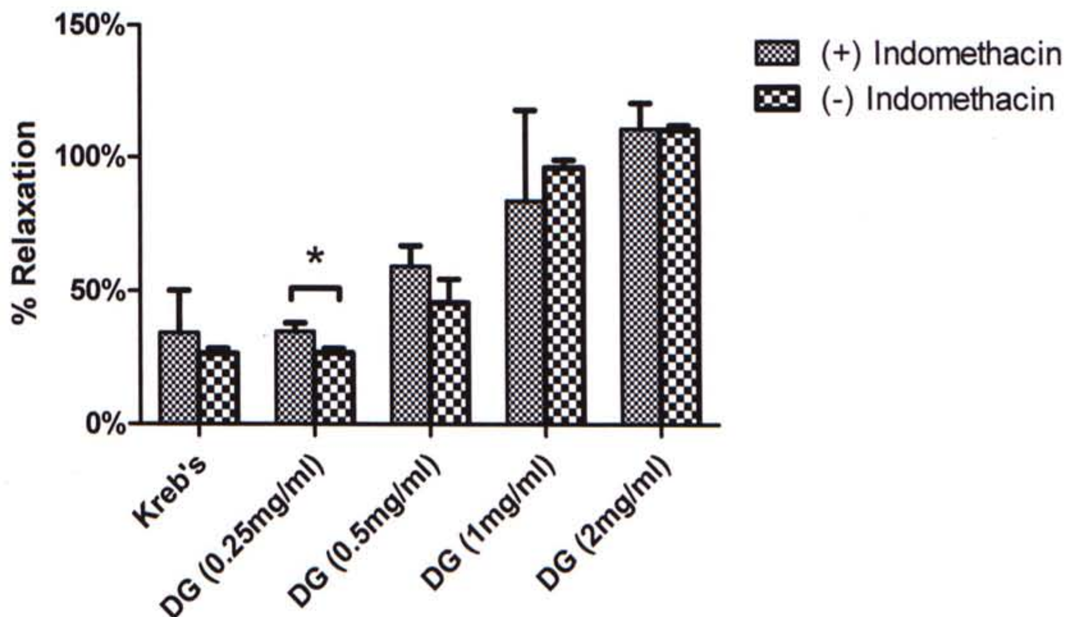


Fig 3.13 Effect of indomethacin on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. * $p < 0.05$.

3.3.2.5 Hyperpolarization-mediated Vasodilation

The intact aorta rings were treated with TEA, $BaCl_2$, 4-AP, IbTX or glibencamide before addition of DG extract. The treated aorta rings were tested and compared against the untreated ones. In Fig 3.14, aorta rings were treated with TEA, a non-selective potassium channel blocker. Aorta rings without any DG extract added exhibited 29% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 32%, 47%, 58% and 104% relaxation, respectively. The relaxation with addition of 1mg/ml DG extract was significantly lower with TEA.

In Fig 3.15, aorta rings were treated with $BaCl_2$, a Kir channel blocker. Aorta rings without any DG extract added had 31% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 29%, 31%, 41% and 95% relaxation, respectively. The relaxation with addition of 1mg/ml DG extract was significantly lower with $BaCl_2$.

In Fig 3.16, aorta rings were treated with 4-AP, a voltage-dependent potassium channel blocker. Aorta rings without any DG extract added had 27% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 35%, 38%, 64% and 106% relaxation, respectively. The relaxation with addition of 1mg/ml DG extract was significantly lower with 4-AP.

In Fig 3.17, aorta rings were treated with IbTX, a BK_{Ca} channel blocker. Aorta rings without any DG extract added had 41% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 37%, 47%, 92% and 99% relaxation, respectively. The relaxation without DG extract added with addition of 0.25mg/ml DG extract was significantly higher with IbTX.

In Fig 3.18, aorta rings were treated with Glibencamide, a K_{ATP} channel blocker. Aorta rings without any DG extract added had 14% of relaxation. With 0.25, 0.5, 1 and 2 mg/ml of DG extract added, the percentage of relaxation was 14%, 23%, 19% and 58% relaxation, respectively. The relaxations of all groups were significantly lower with addition of Glibencamide. Severe inhibition was shown in the DG extract dosages of 1 and 2mg/ml.

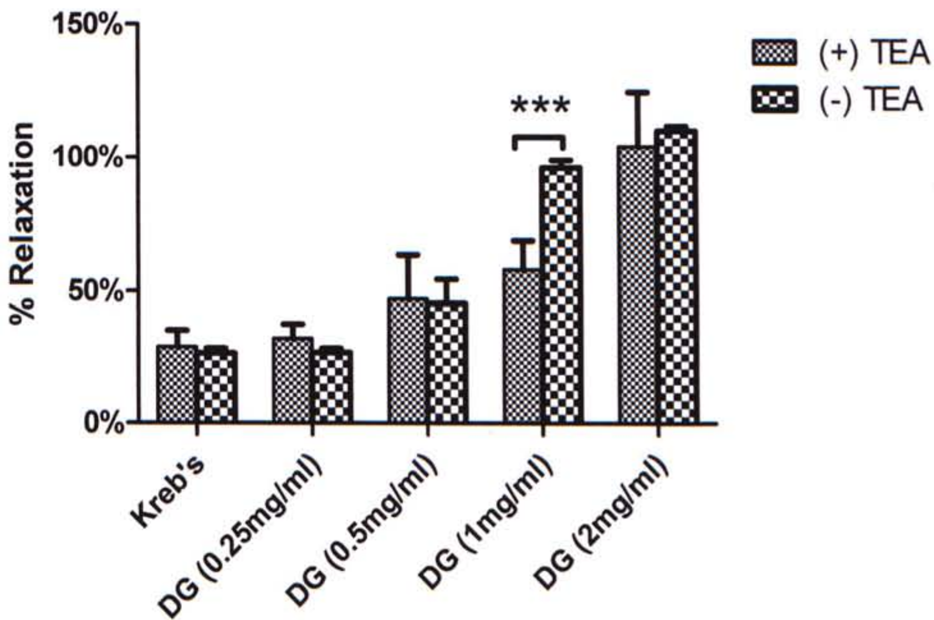


Fig 3.14 Effect of tetraethylammonium (TEA) on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=3-12). Student's t-test was used for statistical analysis of each pair. *** $p < 0.001$.

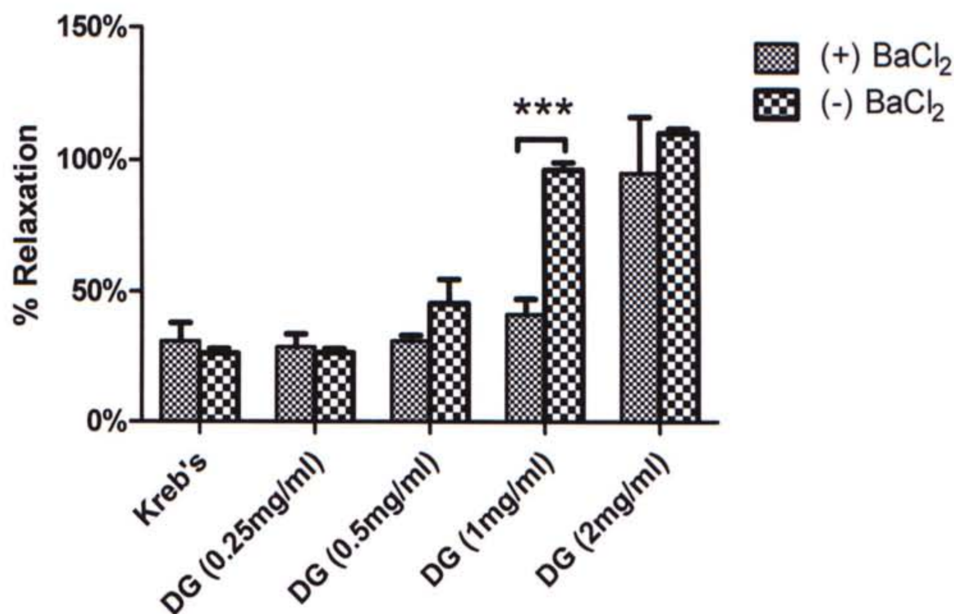


Fig 3.15 Effect of barium chloride ($BaCl_2$) on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. *** $p < 0.001$.

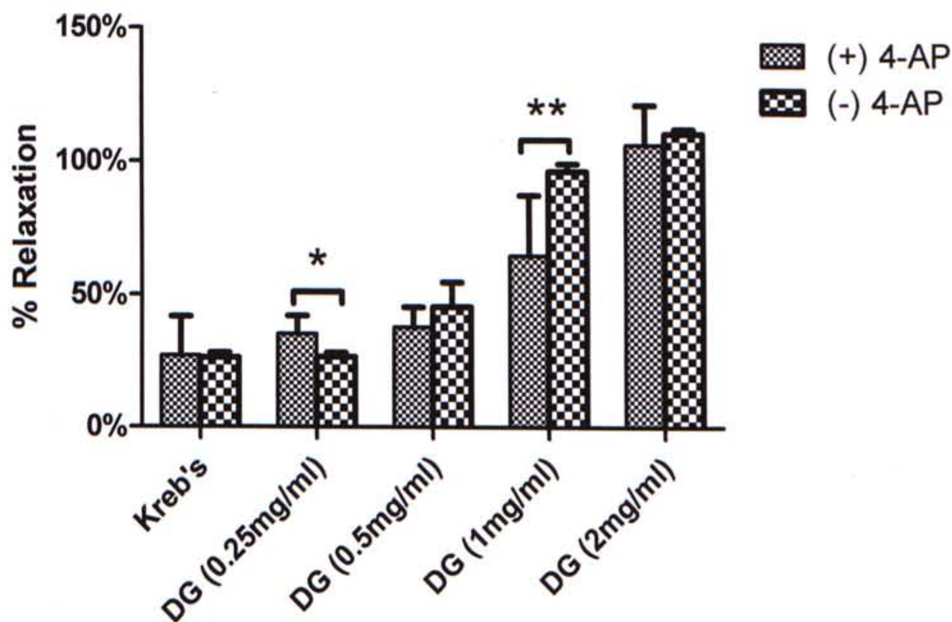


Fig 3.16 Effect of 4-aminopyridine (4-AP) on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. *** $p < 0.001$.

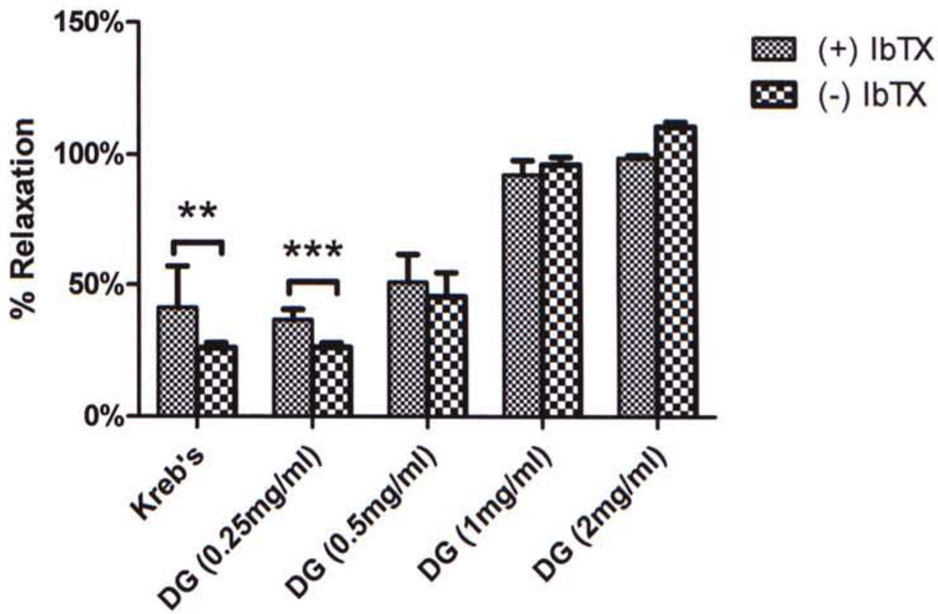


Fig 3.17 Effect of iberiotoxin (IbTX) on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. ** $p < 0.01$; *** $p < 0.001$.

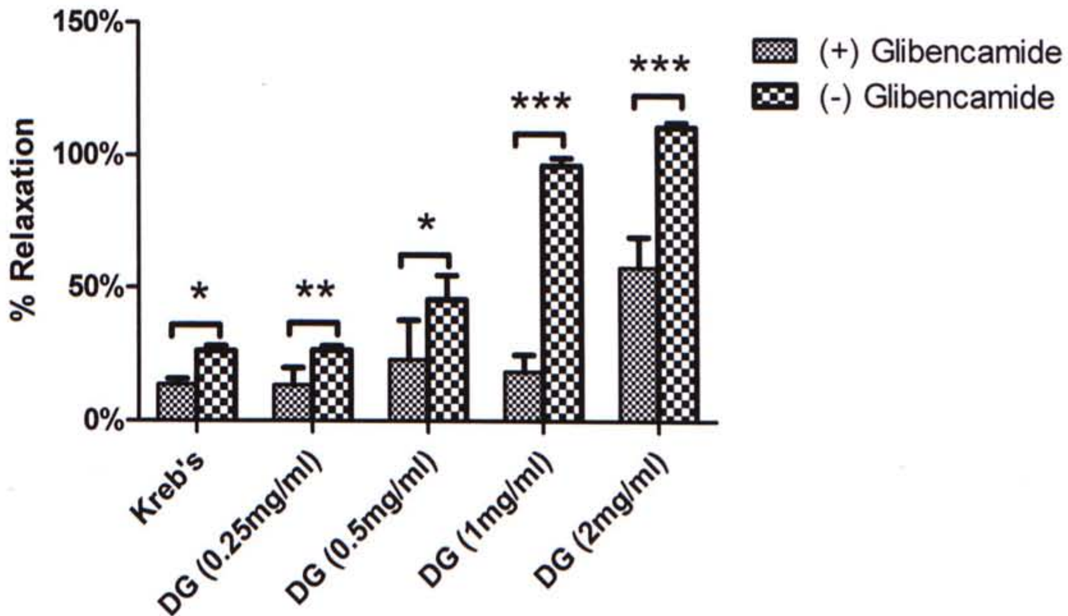


Fig 3.18 Effect of glibencamide on DG extract-induced vasodilation on intact rat aorta rings. All data are presented in mean ± SEM (n=4-12). Student's t-test was used for statistical analysis of each pair. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.3 Long Term Underlying Mechanistic Studies

From Fig 3.19, the initial systolic pressures for different groups of SHR rats were similar, while the systolic blood pressures of SHR rats were significantly higher than WKY rats at 14 weeks old. After 4 weeks of treatment, raloxifene, 90.2 and 300 mg/kg of DG extract could significantly lower the systolic blood pressure comparing with SHR rats control. Systolic blood pressures of SHR rats remained higher than WKY rats.

From Fig 3.20, DG extract- or Raloxifene-treated and untreated aorta did not show any significant difference in relaxation in any dosages of ACh, with or without addition of inhibitors of different pathway. WKY rats showed a significant less relaxation than SHR rats by addition of low concentration of ACh (10^{-8} to 10^{-9}M) in the absence of blocker or with indomethacin.

Fig 3.21 showed the maximum relaxation of aorta rings from different groups of rats. The relaxations of SHR rats were significantly lower than those of WKY rats for about 10%. The treated groups showed slight decrease in relaxation percentage. However, no significance was reached. Contractile force was significantly reduced from about 0.8g to 0.6g by Raloxifene, 300 or 90.2 mg/ml DG extract-treated groups when comparing with untreated SHR group (Fig. 3.22)

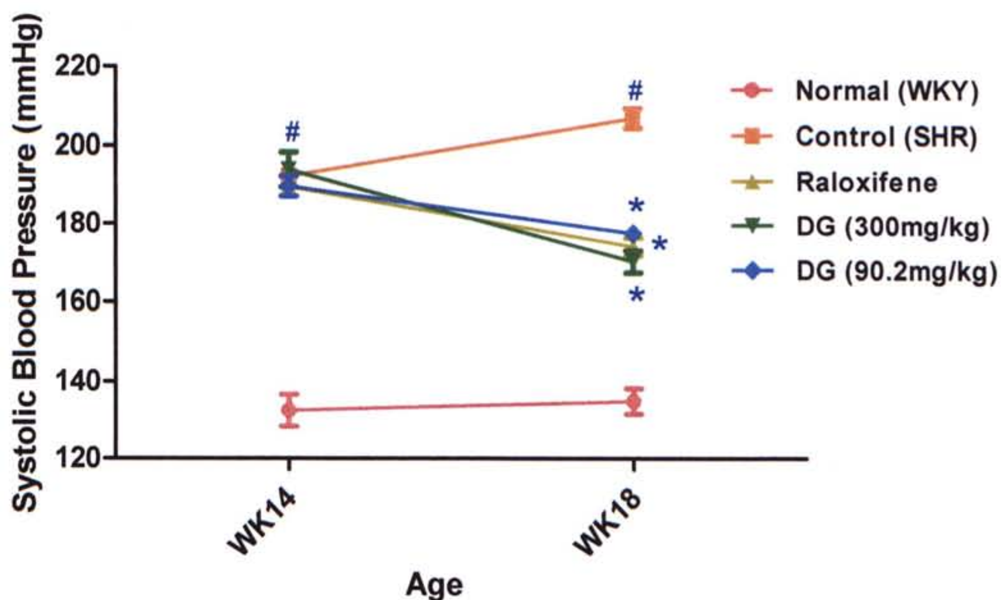
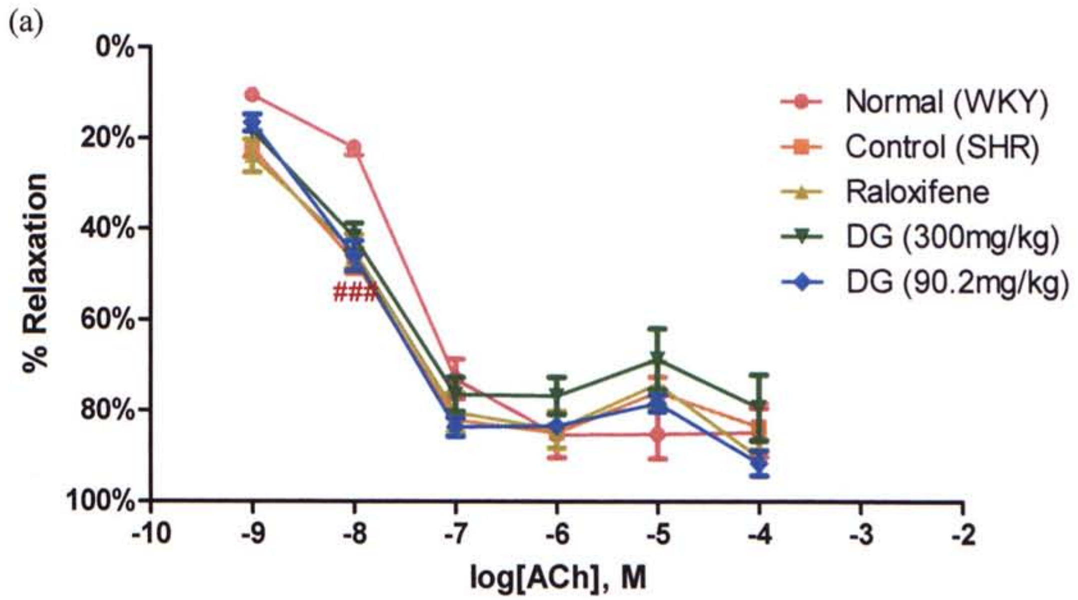
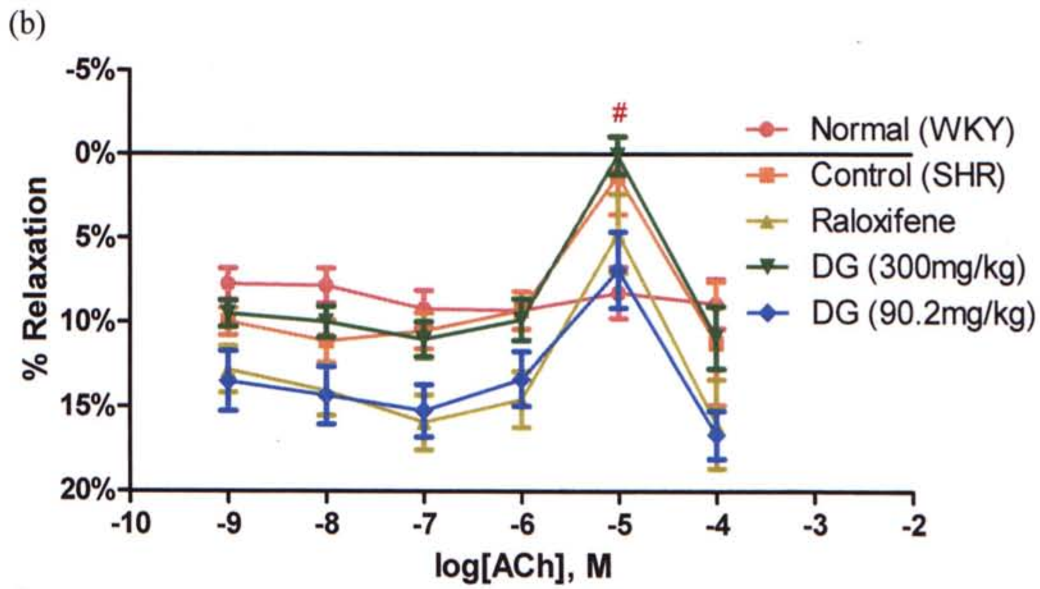


Fig 3.19 Effect of Danshen-Gegen 7:3 water extract on hypertension in SHR rats (Mechanistic study). All extracts/drug/water were administered daily by oral gavage. All data are presented in mean ± SEM (n=6). ANOVA was used for statistical analysis. WKY vs SHR, # $p < 0.05$; SHR vs treatment groups, * $p < 0.05$.

(a)



(b)



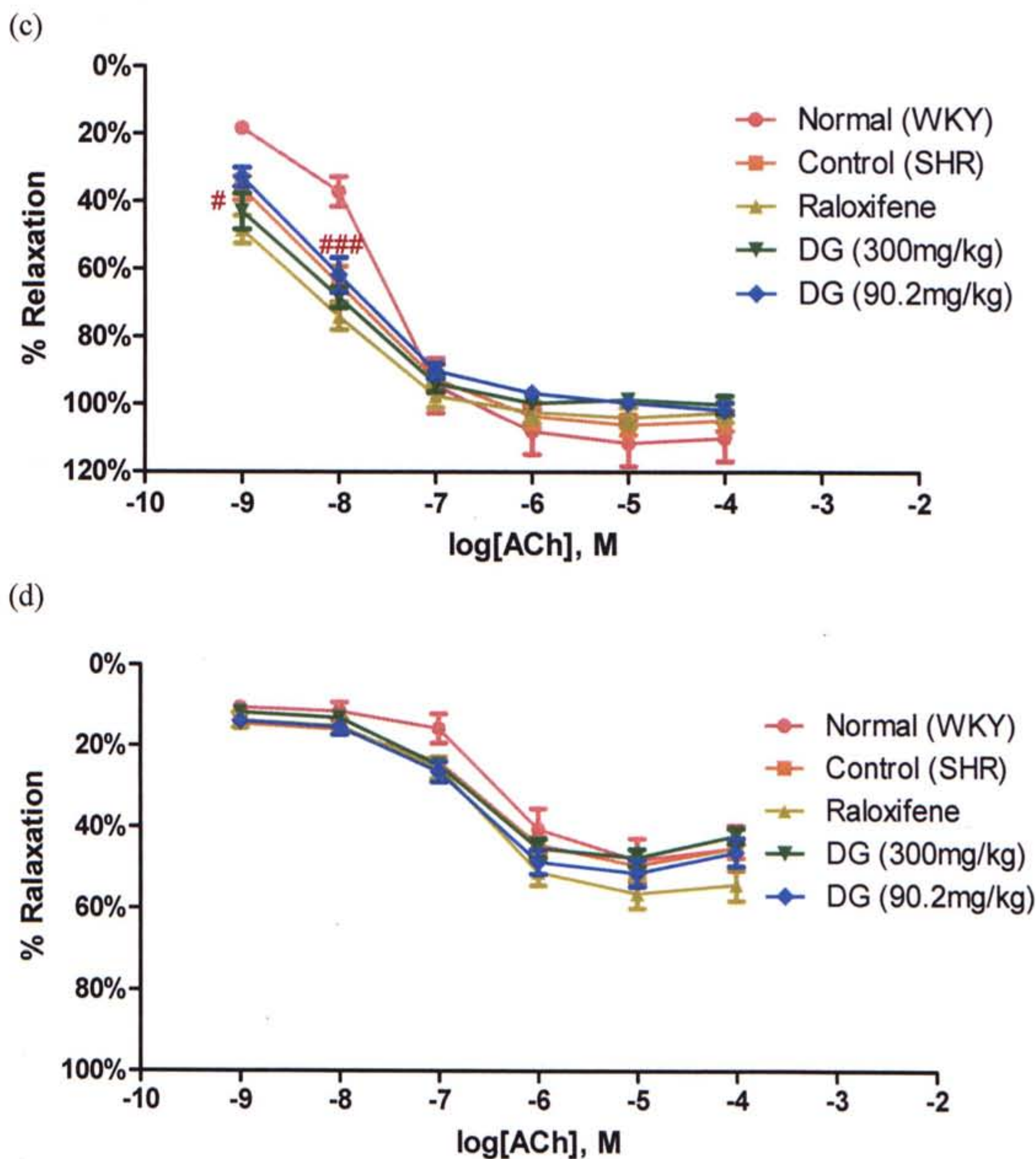


Fig 3.20 Effect of different concentration of acetylcholine (ACh) on phenylephrine-contracted aorta rings. The aorta rings were (a) not treated, or treated (b) L-NAME, (c) indomethacin and (d) TEA. All data are presented in mean ± SEM (n=4-6). ANOVA was used for statistical analysis. WKY vs SHR, # $p < 0.05$; ### $p < 0.001$.

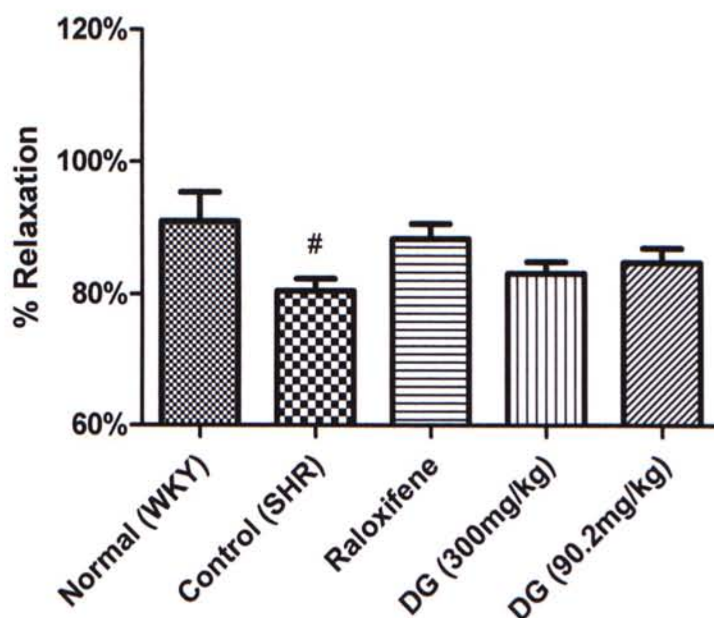


Fig 3.21 Maximum relaxation induced by 0.3μM acetylcholine (ACh) on 0.3μM phenylephrine-contracted aorta rings. All data are presented in mean ± SEM (n=4-6). ANOVA was used for statistical analysis. WKY vs SHR, # $p < 0.05$

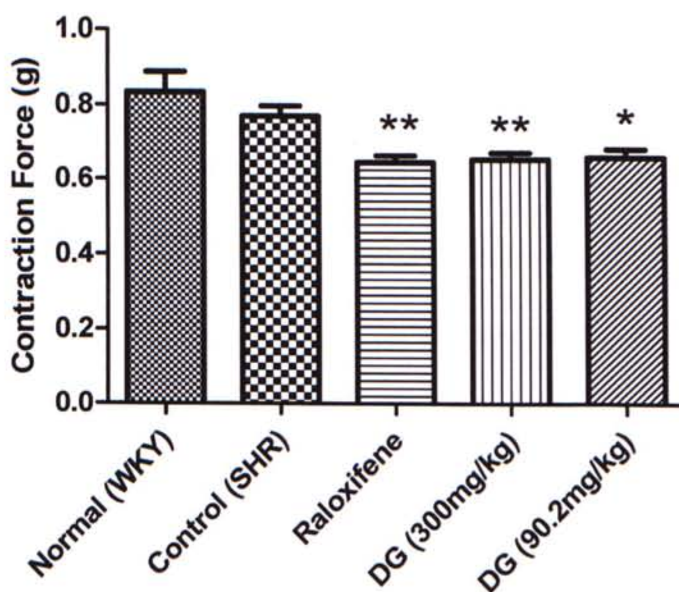


Fig 3.22 Maximum contraction force induced by 0.3μM phenylephrine. All data are presented in mean ± SEM (n=4-6). ANOVA was used for statistical analysis. SHR vs treatment groups, * $p < 0.05$; ** $p < 0.01$.

3.4 Discussion

In order to investigate the anti-hypertensive effect of DG extract, the change in systolic blood pressure was compared between SHR rats with and without DG treatments. The positive control raloxifene was used due to its effect in reducing blood pressure, which was validated in both preventive and therapeutic studies. The results were consistent with what we have mentioned in section 3.1.1 that raloxifene was able to reduce plasma cholesterol level but the effect was not related to triglyceride, glucose and weight. Preventive effect of DG extract was tested on the SHR rats of 6 weeks old, which were in the pre-hypertensive state. Severe hypertension would gradually develop in adults. The effect of DG extract on the development of hypertension, i.e. the ability to delay, prevent or reduce the hypertensive progression, was the scope we want to examine. In present study, DG extract was unable to delay or prevent hypertension. However, it had the ability to stably lower the plateau systolic blood pressure.

Therapeutic effect of DG extract was tested on the SHR rats of 14 weeks old, which bear severe hypertension. The aim of study became the ability of DG extract to reduce blood pressure. In this study, daily administration of DG extract caused decline in systolic blood pressure within 2 weeks, and no rebounce was observed in later weeks.

About the lipid profile, the higher cholesterol level in the preventive study and the lower cholesterol level in the therapeutic study could be explained by the diurnal cycle of the rat, which has higher cholesterol in early morning (6 to 8 a.m.) and lower in the afternoon (1 to 3 p.m.) (Rand and Quackenbush, 1965). On the other hand, the triglyceride levels were higher in the preventive study than those in the therapeutic

study. These might be due to the individual differences in these two studies, as the trends were similar in both studies, except a general increase in readings for all groups.

In both preventive and therapeutic studies, the blood pressure after DG extract treatments were similar, and no dose-dependent effect was shown. The possible explanation can be that the dose of DG extract was already in excess even at the low dose studied. Therefore, the hypotensive effects in both cases were already at maximum and the increase in doses could not further lower the blood pressure. For prevention of hypertension, a drug should be able to avoid hypertension from developing or slowing down the progression of hypertension. However, DG extract could not achieve either of them. Whilst the only effect of DG extract found was reducing blood pressure from ~200mmHg to ~180mmHg, suggesting that the effect of DG extract was therapeutic instead of preventive in hypertension management. After confirming the anti-hypertensive effect of DG extract, the next step was to identify its action mechanisms. As discussed before, the drug treatment of hypertension includes diuretics, ACE inhibitors, angiotensin receptor antagonists, calcium channel blockers, β-adrenergic receptor blockers, and vasodilators. These drugs are having different action target and mechanisms. In our study, Danshen and Gegen were found to have vasodilative functions. Therefore, it is highly suspected that DG extract would also act as a vasodilator. Thus, we evaluated the vasodilative activity in isolated aorta to support the DG extract-induced blood pressure alleviation in hypertensive rats.

Vasodilators mainly act through three pathways as mentioned: nitric oxide-mediated, prostacyclin-mediated, or hyperpolarization- mediated vasodilation. The commonly used method to identify the mechanism is the vascular reactivity study.

Blocker or inhibitor of above pathways may change the degree of relaxation of aorta ring, which indicates its participation in DG extract action.

SD rats were used instead of WKY rats in this part of the study because the concentrations of blockers, vasoconstrictors and vasodilators used were based on previous studies. Since SD rats were used in most of the previous studies and that different strains of rats may have different responses to the blockers, vasoconstrictors and vasodilators, hence SD rats were chosen in our study.

The major findings of the present study indicate that DG extract caused full relaxation in rat aorta rings in a dose-dependent manner. The vasodilation was not affected by endothelium denudation, reflecting the effect is endothelium-independent. Theoretically, endothelium is responsible for the expression of eNOS and cyclooxygenase, which produce NO and prostaglandin H_2. NO and prostaglandin I_2 are the mediator of NO-mediated or prostacyclin-mediated vasodilation. Thus, an endothelium-dependent vasodilation should not have NO-mediated or prostacyclin-mediated vasodilation involved. The results of this study show that the vasodilation is not abolished by L-NAME and indomethacin. That reflects DG extract induced vasodilation is not NO-mediated or prostacyclin-mediated, which match the theory.

Other major findings are the involvement of hyperpolarization in vasodilative effect of DG extract, which presented by the inhibition of relaxation by a non-selective potassium channel blocker, TEA. The finding is reasonable as hyperpolarization-mediated vasodilation is endothelium independent, which is proved to be responsible for DG extract's action. So, individual types of potassium channels

are investigated for their involvement. It is found that the response to 1mg/ml of DG extract was markedly reduced by $BaCl_2$, 4-AP, and glibencamide, which are blockers of Kir channel, voltage-dependent potassium channel and K_{ATP} channel, respectively. However, the inhibition is recovered in high concentration of DG extract. This indicates that the vasodilative action of DG extract is not a single pathway process.

From other studies, it is found that tanshinone II_A, a lipophilic active ingredient of Danshen, induce relaxation on rat coronary arteries and aorta, through NO-mediated vasodilation (Kim, *et al.*, 2007, Wu, *et al.*, 2009) and K_{ATP} channel-related (Chan, *et al.*, 2009) respectively. Aqueous extract of Danshen and its active components, Danshensu and salvianolic acid B, are able to cause relaxation in rat coronary arteries. The relaxation can be partially inhibited by TEA and is potassium channels-related. The relaxation is also tested to be related to inhibition of calcium channels (Lam, *et al.*, 2007, Lam, *et al.*, 2006). Gegen active component, Daidzein, is showned to improve endothelial function in many studies (Colacurci, *et al.*, 2005, Mishra, *et al.*, 2000). Therefore, its vasodilative action is mainly described as NO-mediated (Mishra, *et al.*, 2000, Vera, *et al.*, 2005). However, daidzein is also found to be acting on rats cerebral basilar arteries and thoracic aorta through large-conductance calcium-activated potassium channels (Sun, *et al.*, 2007, Zhang, *et al.*, 2010), K_{IR} channels and Na^+/K^+-ATPase (Sodium-Potassium Pump) (Woodman and Boujaoude, 2004). In addition, daidzein also found to be reducing the endothelial release of prostaglandin H_2, a cyclooxygenase-derived vasoconstrictor (Vera, *et al.*, 2005). Vasodilative action of puerarin, another active component of Gegen, was also found to be involved in the large-conductance calcium-activated potassium channels (Sun, *et al.*, 2007). The above studies showed that a large variety of pathways are involved in the vasodilation process, and the action mechanism remains controversial.

As shown in Table 3.5, we observed that the findings in the present study have some discrepancies from our previous findings (Chan, 2006). A possible explanation is the difference in active components in the aqueous and ethanolic extracts. Also, the findings among studies differed from each other when different arteries were used (Chan, *et al.*, 2009; Kim, *et al.*, 2007; Mishra, *et al.*, 2000; Sun, *et al.*, 2007; Woodman and Boujaoude, 2004; Wu, *et al.*, 2009; Zhang, *et al.*, 2010). Moreover, the other studies were using pure compounds in which their doses were much higher than the crude extract. The insufficient doses of pure compounds in the crude extract would also explain the absence of involvement of some pathways in our study, which found to be involved in other studies. The vasodilation effect of DG extract, in its effective dose, should act endothelium-independently through Kir channel, voltage-dependent potassium channel, K_{ATP} channel, and one or more mechanisms that were not tested in this study. The comparison is summarized in Table 3.5.

After testing the direct effect of DG extract on rat aorta rings, the effect of chronic administration of DG extract was assessed. Hypertensive, 14 weeks old SHR rats were orally fed with DG extract for 4 weeks. The aorta was isolated and test for vascular reactivity. Commonly used blockers for NO-mediated, prostacyclin-mediated and hyperpolarization-mediated, L-NAME, indomethacin and TEA, were added to test for the involvement of the pathways.

At 14 weeks, the SHR rats were hypertensive with similar systolic blood pressure. The hypertension was significantly alleviated by 4 weeks of raloxifene or DG extract treatments. However, the isolated aorta rings of different groups shows similar relaxation patterns during serial addition of increasing concentrations of ACh.

The observation is very different from the other studies, which SHR rats show a lower

Table 3.5 Summary of the comparisons between results of present study and previous studies in vasodilation

Herbs	Extract and (or) "purified compound"	Biological preparations	Endo	NO	PGI_2	K^+ C	Kir C	K_{ATP} C	K_v C	BK_{Ca} C	Ca^{2+} C	References
Pueraria lobata + *Salvia miltiorrhiza*	Aqueous extract	Rat aorta	-	-	-	+	+	+	+	-	NA	Present study
Pueraria lobata + *Salvia miltiorrhiza*	80% ethanol extract	Rat aorta	-	-	-	+	+	NA	-/+	NA	NA	Chan, 2006
Pueraria lobata + *Salvia miltiorrhiza*	"Salvianolic acid B"	Rat aorta	-	+	-	+	+	NA	+	NA	NA	Chan, 2006
Pueraria lobata + *Salvia miltiorrhiza*	"Daidzein"	Rat aorta	+	+	+	+	+	NA	+	NA	NA	Chan, 2006
Salvia miltiorrhiza	"Tanshinone II(A)"	Hamster cheek pouch	NA	+	NA	NA	NA	NA	NA	NA	NA	Kim, *et al.*, 2007
Salvia miltiorrhiza	"Tanshinone II(A)"	Mouse cremaster muscle	NA	+	NA	NA	NA	NA	NA	NA	NA	Kim, *et al.*, 2007
Salvia miltiorrhiza	"Tanshinone II(A)"	Rat coronary arteriole	+	+	-	-	-	-	-	+	NA	Wu, *et al.*, 2009
Salvia miltiorrhiza	"Tanshinone II(A)"	SHR rat aorta	-	NA	NA	NA	NA	+	NA	NA	NA	Chan, *et al.*, 2009
Salvia miltiorrhiza	"Danshensu"	Rat coronary artery	-	NA	NA	+	NA	NA	NA	NA	+	Lam, *et al.*, 2007
Salvia miltiorrhiza	"Salvianolic acid B"	Rat coronary artery	-	NA	NA	+	NA	NA	NA	NA	+	Lam, *et al.*, 2006

Herbs	Extract and (or) "purified compound"	Biological preparations	Endo	NO	PGI_2	K^+ C	Kir C	K_{ATP} C	K_v C	BK_{Ca} C	Ca^{2+} C	References
Pueraria lobata	"Daidzein"	Human brachial artery diameter and flow	NA	+	NA	NA	NA	NA	NA	NA	NA	Colacurci, *et al.*, *2005*
Pueraria lobata	"Daidzein"	Rat aorta	+	+	-	NA	NA	NA	NA	NA	NA	Mishra, *et al.*, 2000
Pueraria lobata	"Puerarin"	Rat aorta	NA	NA	NA	NA	NA	NA	NA	+	NA	Sun, *et al.*, 2007
Pueraria lobata	"Daidzein"	Rat basilar artery	NA	NA	NA	+	NA	NA	NA	+	NA	Zhang, *et al.*, 2010
Pueraria lobata	"Daidzein"	Rat aorta	NA	NA	-	NA	+	NA	NA	-/+	NA	Woodman and Boujaoude, 2004

Note: NA - not available; + dependency; - independency; -/+ slight trend of dependency and further investigation needed;
Endo - endothelium; NO - nitric oxide; PGI_2 - prostacyclin; K^+ C - potassium channel; Kir C - inward rectifier potassium channel;
K_{ATP} C - ATP-sensitive potassium channel; K_v C - voltage-dependent potassium channel;
BK_{Ca} C - large-conductance Ca^{2+}-activated potassium channel; Ca^{2+} C - calcium channel

vascular reactivity than WKY rats (Gomez-Roso, *et al.*, 2009, Jednakovits, *et al.*, 2000, Romero, *et al.*, 2010). This possibly due to the young age of the rats, as generally, rats are sacrificed at 24 weeks old or more in this type of blood pressure measurement plus vascular reactivity test (Gomez-Roso, *et al.*, 2009, Romero, *et al.*, 2010), as supported by the increase in vascular stiffness along with aging. As there was no observable difference between SHR rats and WKY rats, it was normal that the chronic intake of raloxifene or DG extract did not modify the relaxation profile of SHR rats. Nevertheless, NO-mediated, prostacyclin-mediated and hyperpolarization-mediated vasodilation are not affected either.

As a follow-up investigation, maximum relaxation on aorta rings by single 0.3μM of ACh were compared, and the relaxations of SHR rats were significantly smaller than the WKY rats, but the difference was only about 10%. The chronic intake of raloxifene or DG extract has a dose-dependent trend to increase the relaxation. However, no significance was reached, possibly due to the small basal difference between relaxation of SHR rats and WKY rats. The basal difference of the other studies would be 30-40% (Gomez-Roso, *et al.*, 2009; Jednakovits, *et al.*, 2000; Romero, *et al.*, 2010).

Carotid arteries from 1-wk-old SHR rats exhibited narrower lumen and greater intrinsic stiffness than those from their WKY and SD counterparts (Arribas, *et al.*, 2008). This characteristic may also present in adult rats. The increased stiffness can explain the reduced contractile force in SHR rats than WKY rats, while the narrower lumen maintains the high blood pressure. The contractile force was also smaller in SHR rats after chronic intake of raloxifene or DG extract. Deducing from that, raloxifene and DG extract may possibly reduce vasoconstriction, in addition to

its vasodilative effect.

Conclusion

In conclusion, this study provides experimental evidence that DG extract treatments reduce the systolic blood pressure of SHR rats. The reduction of systolic blood pressure may be contributed by the vasodilative effect of DG extract. The vasodilative effect of DG extract is endothelium-independent and partially mediated by hyperpolarization. Possibility of NO-mediated and prostacyclin-mediated vasodilation are eliminated. Long term treatment of DG extract have a trend of increasing vascular relaxation percentage, and reduce the contraction force.

Chapter 4

Anti-atherosclerosis Studies of Danshen-Gegen Formula in Rabbits

4.1 Introduction

From our previous clinical studies (Tam, 2004), long term intake of Danshen-Gegen 7:3 formula showed an anti-atherosclerotic effect in human assessed by carotid Doppler ultrasound. In order to show morphological changes induced by DG extract on atherosclerosis, a rabbit model was applied. Atherosclerosis was induced in rabbits by a high cholesterol diet, which is also a main inducing factor in human situation. The treatment started once after the plaque was formed. The treatment aimed to test DG extract's therapeutic effect instead of preventive effect. This mimics the real situation of giving the medical treatment after diagnosis of atherosclerosis in human. DG extract was given daily by oral gavage. The dosages of DG extract was equivalent to 1X, 3X, and 9X of the current clinical dose that is being used in the current clinical trial. Blood lipid profile and oxidative status were measured for deduction of possible underlying mechanism that causes the anti-atherosclerotic effect. Most importantly, the intima-media thickness, which reflects the extent of atherosclerosis, was shown histochemically in the aorta section (Boger, *et al.*, 1998, Choe, *et al.*, 2001).

4.1.1 Intima-Media Thickening

Intima-media thickening is a main morphological change on blood vessels in atherosclerosis (Cobble and Bale, 2010). Atherosclerosis is caused by the inflammatory process. A high plasma cholesterol level, especially low-density lipoprotein (LDL) cholesterol, can cause the formation of plaque. LDL cholesterol can be oxidized and become oxidized LDL cholesterol (oxLDL). On the other hand, endothelial cells would express more adhesion molecules that recruit leukocytes from

the bloodstream to the endothelium. One of the major adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1), binds with monocytes and T-lymphocytes, which actively participate in initial stage of atherosclerosis. After the recruitment, the monocytes enter the sub-endothelial space, and differentiate into macrophages. The macrophages process scavenger receptors to bind and endocytose oxLDL continuously. The macrophages would accumulate oxLDL and subsequently fill with lipid droplets. This leads to the formation of foam cells. The foam cells accumulate under endothelium and form fatty streak, which show an initiation of intima-media thickening. The foam cells would release cytokines, which worsen the inflammatory process. As the vicious cycle continues, smooth muscle cells (SMC) migrate from the media to the sub-endothelial space. As the SMC proliferate, it would secrete extracellular matrix proteins, turning fatty streak to a fibrous plaque. The intima-media thickness would increase and the lumen size is further reduced. Therefore, intima-media thickness can reflect the stage and extent of atherosclerosis (Bots, *et al.*, 1997).

In worst situation, the atheroma would rapture and the content would form a thrombus, which travel along the bloodstream. The thrombus would block the blood flow of small vessels, leading to an infarction of the destination organ. This is also the main clinical complication seen in atherosclerosis.

4.1.2 Effect of High Cholesterol Diet in Rabbit

New Zealand White rabbits were chosen to be the subject of our study. From previous researches, a few days of high cholesterol diet can induce hypercholesterolaemia in rabbits, but not in rats or mice (Bocan, *et al.*, 1993). The normal level of cholesterol in rabbits is very low, and it is able to increase several fold

rapidly. The reason may due to the inability in increasing excretion of sterols (Kroon, *et al.*, 1985, MacKinnon, *et al.*, 1985, Thompson and Zilversmit, 1983). The high plasma cholesterol would promote the formation of foam cells and intima-media thickening. However, long term of high cholesterol diet causes high hepatotoxicity and extensive inflammation throughout the body (Prior, *et al.*, 1961). Therefore, the 1% cholesterol diet can only sustain for 12 weeks in the experiments. In some experiments, in order to produce advanced lesions that are similar to human, balloon injury would be done in the thoracic and abdominal aorta (Chen, *et al.*, 2001, Hegyi, *et al.*, 2004, Ma, *et al.*, 2008). However, considering the high risk and stress on the animals, the surgery was not applied in our study.

Like in human, female rabbits would produce female sex hormone, making the accumulation of cholesterol much slower (Haarbo and Christiansen, 1996). Therefore, male rabbits were used to avoid the preventive effect of estrogen on atherosclerosis. Our rabbit model design was modified from another study (Zhang, *et al.*, 2005). However, the uniqueness of our study was the beginning time of DG extract treatment was after the formation of plaque, instead of the beginning of cholesterol diet. The time for the plaque formation was determined in the pilot study.

4.1.3 Thiobarbituric Acid Reactive Substances

The plasma samples from the rabbits contain thiobarbituric acid reactive substances, including lipid hydroperoxides and aldehydes, which are the side-products of lipid peroxidation. During lipid peroxidation, polyunsaturated fatty acids react with free radicals to form fatty acid radicals. The fatty acid radicals would react with oxygen molecules to form peroxyl-fatty acid radicals. The peroxyl-fatty acid radicals would in turn react with another fatty acid to form fatty acid radicals and a lipid

peroxide, and hence the above steps repeat again (Meagher and FitzGerald, 2000).

In the assay, lipid hydroperoxides were decomposed in the presence of acid and metal to form Malondialdehyde (MDA), in which the concentration was determined. MDA reacted with thiobarbituric acid (TBA) to form an adduct, which could be detected spectrophotometrically at 532nm (Meagher and FitzGerald, 2000). The amount of TBARS was the indicator of the oxidative stress inside the plasma, which should be reduced if DG extract possessed anti-oxidative effect.

4.2 Methods

4.2.1 Pilot Study for Establishment of Experimental Protocol

The aims of the pilot study were to establish the effective dose of vitamin E as positive control, and the duration for the plaque to form after taking high cholesterol diet. These data would help us to design the experimental protocol for the actual animal study. Male New Zealand white rabbits of 3.0 to 4.0kg were used. After 1 week of acclimatization and modification of diet (from the usual diet in Laboratory Animal Services Centers to the customized diet without supplement of cholesterol), rabbits were randomly divided into 5 groups. All groups were given *at libitum* assess to 1% cholesterol rabbit chow and water. Two groups of rabbits were daily fed with 150mg/kg or 300mg/kg of vitamin E (serve as positive control) orally. The other three groups were given assess to 1% cholesterol diet for 4, 5, and 6 weeks (Li, *et al.*, 1993), respectively, without vitamin E treatments (Table 4.1).

Table 4.1 Duration of treatment and sample size of each group in the pilot study

Duration of Treatments	Treatments	Sample size
4 weeks	Water (2ml/kg)	2
5 weeks	Water (2ml/kg)	3
6 weeks	Water (2ml/kg)	2
6 weeks	150mg/kg Vitamin E	2
6 weeks	300mg/kg Vitamin E	2

Tissue Sampling and Analysis of Effect of High Cholesterol Diet and Vitamin E

After 4th, 5th, and 6th week from the beginning of 1% cholesterol diet, one group of untreated rabbits were sacrificed by ketamin-xylazine anesthesia followed by heart puncture blood drainage. The vitamin E treated rabbits were sacrificed by the same method at the end of 6th week. The aorta was isolated from ascending aorta to the

celiac branch, clear of fat and excess adventitial tissue, rinsed with saline buffer and immersed into 1% Sudan III (w/v) in 70% alcohol (Zhang, *et al.*, 2005) overnight at room temperature. After staining, the aorta was washed using distilled water and cut in the mid-line to become a "Y" shape. The intima side of aorta was scanned with an Epson Perfection 1260 image scanner (Epson, Shinjyuku, Tokyo).

Images were analyzed using Adobe Photoshop CS3 (Adobe, California, U.S.) by quantifying the total area and the Sudan III stained area (Red: 255, Green: 0, Blue: 0). Percentage of atheroma represented the extent of atherosclerosis.

$$\textbf{\% of Atheroma} = \frac{\textbf{Sudan III Stained Area (Atheroma Area)}}{\textbf{Total Area}}$$

At 4th, 5th, and 6th week, blood was collected before sacrifice. All rabbits were fasted for 12 hours before blood collection. Plasma was prepared from whole blood for total cholesterol, triglyceride and glucose measurements as stated in protocol 2.4.2.

Results and Implications on the Experimental Design

Our results showed that the cholesterol diet induced atherosclerotic plaque formation throughout the duration of cholesterol diet. There was not much progression from week 4 to week 6 (Fig. 4.1 and 4.2). All dosages of vitamin E tested could reduce the plaque formation. 300mg/kg of vitamin E exhibited slightly higher effect than 150mg/kg of it (Fig 4.2). Therefore, the treatment of DG extract began at the earliest time for plaque to emerge – 4th week. The dose of vitamin E of 300mg/kg was found to be effectively inhibiting plaque formation and would be use as the positive control.

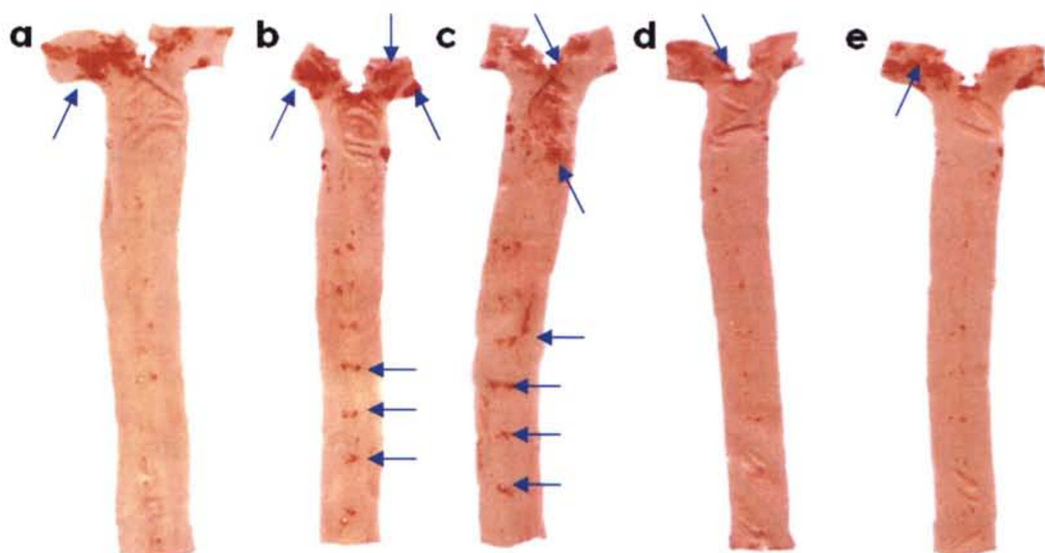


Fig 4.1 Representative photograph showing the effect of duration of cholesterol diet and vitamin E supplement on plaque formation. The patches stained in red by sudan III were the atherosclerotic plaque (indicated by arrows). Rabbits were fed with 1% cholesterol diet only for with (a) 4 weeks; (b) 5 weeks; (c) 6 weeks; and rabbits fed with 1% cholesterol diet and (d) 150 mg/kg; (e) 300mg/kg of vitamin E supplement.

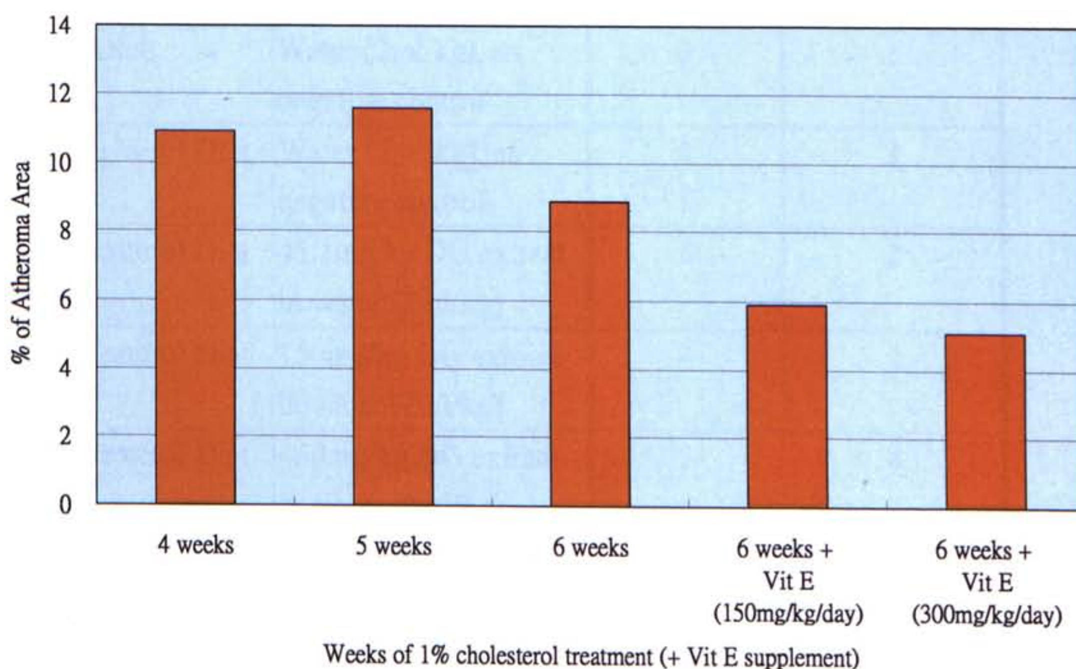


Fig 4.2 The effect of duration of cholesterol diet and vitamin E supplement on aorta sections. Rabbits were fed with (a) 4 weeks; (b) 5 weeks; (c) 6 weeks of 1% cholesterol diet, and 1% cholesterol diet with (d) 150 mg/kg; (e) 300mg/kg of vitamin E (n=2-3). Due to small sample size, no statistical analysis was conducted.

4.2.2 Effect of DG extract on Intima-media Thickening

Male New Zealand white rabbits of 3.0 to 4.0kg were used. After 1 week of acclimatization and modification of diet (switched from the usual diet in Laboratory Animal Services Centers to the customized diet without supplement of cholesterol), rabbits were randomly divided into 6 groups. "Normal Diet" group and "Cholesterol Diet" groups were given *at libitum* assess to respective normal and 1% cholesterol rabbit chow and water. After 4th week, rabbits were fed daily with distilled water by oral gavage. The other 5 groups of rabbits were fed daily with distilled water, 45.1mg/kg, 150mg/kg or 450mg/kg of DG extract dissolved in distilled water, or 300mg/kg of vitamin E (as positive control) by oral gavage (Table 4.2).

Table 4.2 Diet, treatment and sample size of each group

Diet	Treatments	Sample size in 1st Run	Sample size in 2nd Run
Normal Diet	Water (2ml/kg), as negative control	3	1
1% Cholesterol Diet	Water (2ml/kg), as negative control	3	3
1% Cholesterol Diet	45.1mg/kg DG extract in water (2ml/kg)	4	2
1% Cholesterol Diet	150mg/kg DG extract in water (2ml/kg)	4	2
1% Cholesterol Diet	450mg/kg DG extract in water (2ml/kg)	3	4
1% Cholesterol Diet	300mg/kg Vitamin E, as positive control	3	3

Blood Sample Collection and Analysis

Before the 1st week, and after the 4th and 8th week, the rabbit was restrained with a drape. 3ml of blood was drawn from rabbit's marginal ear vein with the use of 10ml

syringe and 22G needle (Fig 4.3). Plasma was prepared as stated in protocol in section 2.4.2. Total cholesterol, triglyceride and glucose assays were performed for first three samples. After 12th week, blood was collected by cardiac puncture blood drainage (Fig 4.3) under anesthesia. Total, HDL- and LDL-cholesterol, triglyceride, glucose and TBARS assay were done for the 12th week samples. All rabbits were fasted for 12 hours before blood collection.

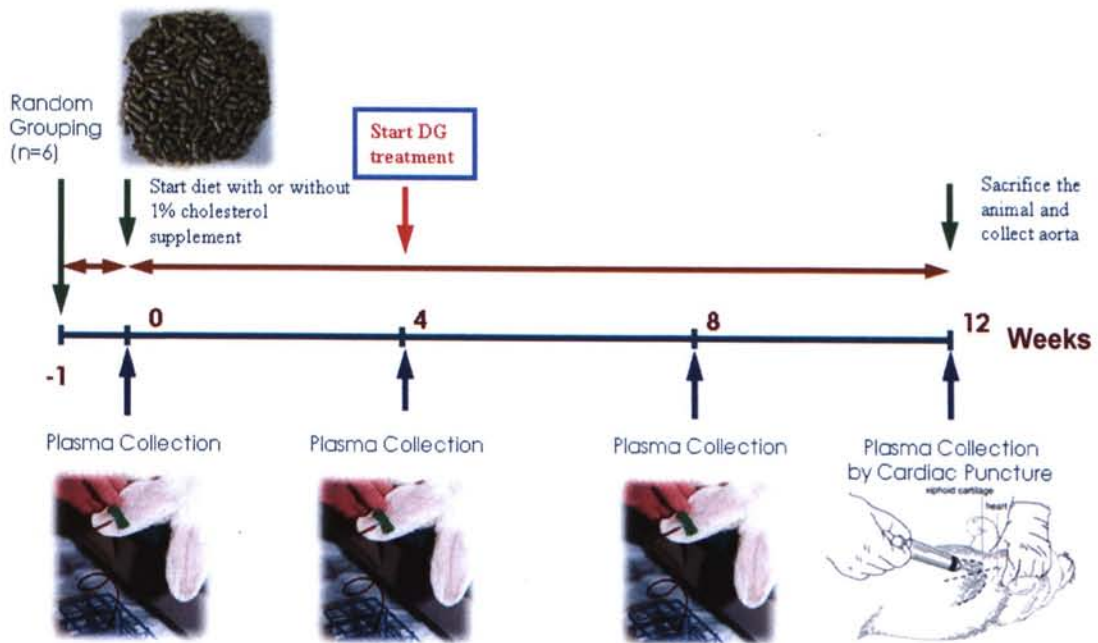


Fig 4.3 Timeline for the rabbit IMT study.

Sampling of Animal Tissues

After 12 weeks of treatment, the rabbits were sacrificed by ketamine-xylazine anesthesia followed by heart puncture blood drainage. The heart and liver tissue were collected and snap frozen in liquid nitrogen. The carotid arteries were isolated out and kept in 4% paraformaldehyde. The aorta was isolated from ascending aorta to the celiac branch, clear of fat and excess adventitial tissue, rinsed with saline buffer and divided into 5mm segments, which were then stored in 4% paraformaldehyde (w/v) in

PBS or snap frozen in liquid nitrogen.

Quantification of Intima-media thickness

The aorta sections that were 10mm proximal to heart were routinely dehydrated and paraffinized in an upright position. Serial sectioning was done, and 4 sections per aorta block were obtained. The sections were stained histochemically by routine haematoxylin and eosin (H&E) staining and mounted in permount.

Images were digitized with a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan), equipped with Nikon Digital Sight camera and image analysis software NIS Elements F 3.0 (Magnification: 40X Exposure time: 200ms).

The aorta image was merged using Photomerge function using Adobe Photoshop CS3 (Adobe, California, U.S.). The merged image was analyzed using Image J (NIH). The margin of intima and media were drawn manually, according to the colour difference. The area of intima and media were quantified. The intima-media ratio was calculated and used as the parameter to assess the degree of atherosclerosis.

$$\textbf{Intima-media ratio} = \frac{\textbf{Intima area}}{\textbf{Media area}}$$

4.2.3 Statistical analysis

In blood profile assays, Mann-Whitney test was used to compare the data for 1% cholesterol diet group and normal diet group. Kruskal-Wallis test was used for comparison between the data for the 1% cholesterol diet groups with treatments and that of untreated 1% cholesterol diet group.

4.3 Result

4.3.1 Study of the Anti-atherosclerosis Effect of DG extract – First Run

Danshen-Gegen 7:3 water extract was tested for the therapeutic effect in intima media thickening in cholesterol-fed rabbit. In the first run (Fig 4.4 and Fig 4.5), 1% cholesterol diet induced a significantly higher intima-media (I/M) ratio. 300mg/kg vitamin E significantly reduced the I/M ratio. Thus, both negative and positive controls were validated. There was a dose-dependent declining trend of I/M ratio after DG treatments and a significant difference was reached for treatment of 450 mg/kg DG extract.

1% cholesterol diet had a trend of increasing plasma triglyceride (Fig 4.6 a), and reached a significant difference at 12th weeks, comparing with normal diet group. It also increased the plasma total cholesterol, reaching a significant different from 4th weeks onwards (Fig 4.6 b). 1% cholesterol diet induced a significantly higher plasma LDL- and HDL-cholesterol at the end of the experiments (Fig 4.6 c,d, respectively). The plasma glucose level was significantly increased from 4th weeks to 8th weeks (Fig 4.7). The amount of thiobarbituric acid reactive substances (TBARS), which reflects the lipid peroxidation, was in very low concentration (0.1 to 0.4 μM) and did not show significant changes in all groups (Fig 4.8).

The positive control, 300mg/kg vitamin E, caused a slight reduction in plasma triglyceride, and total cholesterol (Fig 4.6). However, significant difference was only reached at 8th week total cholesterol level (Fig 4.6 b). On the other side, 300 mg/kg of vitamin E slightly decreased the plasma glucose level, from 8th week (Fig 4.7).

The formula under investigation, DG extract, showed an interesting trend. 45.1

mg/kg and 450 mg/kg of DG extract had a trend of reducing the plasma lipid level (Fig 4.6). Meanwhile, 150 mg/kg of DG extract did not modify the plasma lipid profile of the rabbits (Fig 4.6). All dosages of DG extract were not significantly affecting plasma glucose level (Fig 4.7).

For the weight of the rabbits, all groups experienced a slight elevation in weight at the beginning. The weight continued increasing in normal diet group, remained stable in 45.1 mg/kg and 450 mg/kg of DG extract treatment groups and declined in 150 mg/kg of DG extract treatments, vitamin E treatment and 1% cholesterol diet group (Fig 4.9).

For the amount of diet intake, average daily intake (ADI) was recorded. Normal diet group had a higher ADI (range from 160-180g daily) than other groups. The ADI of the other groups were similar (range from 60-120g daily) with each other throughout the experiment (Fig 4.10).

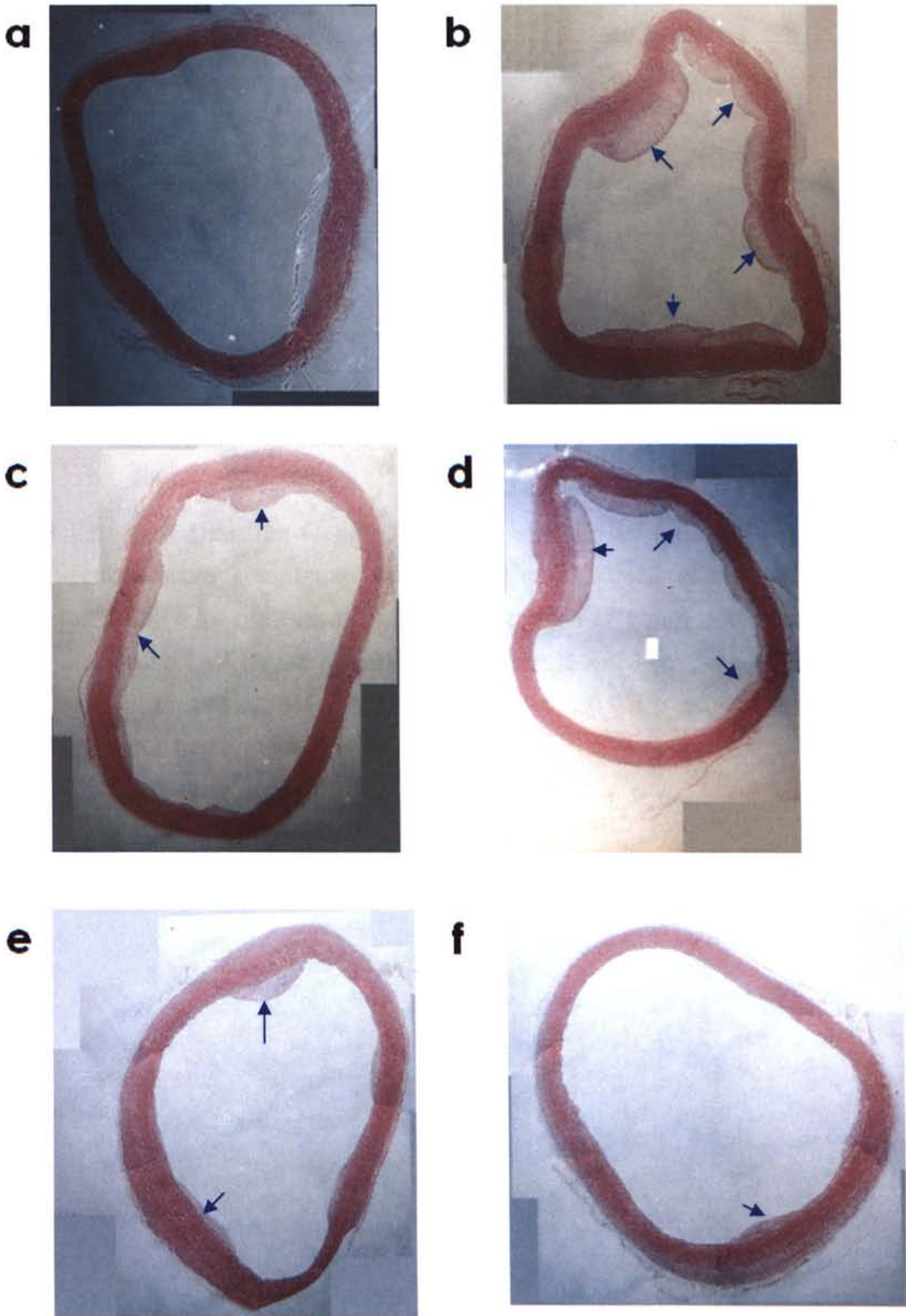


Fig 4.4 Representative photographs showing effect of Danshen-Gegen 7:3 water extract on aorta intima thickness (1st Run). Smooth muscle cells are stained in deep pink and plaque were stained in light pink (indicated by arrows). All sections were 10mm away from heart. The rabbits were fed with (a) normal diet; (b) 1% cholesterol diet; 1% cholesterol diet with DG extract of (c) 45.1mg/kg; (d) 150mg/kg, and (e) 450mg/kg daily; and 1% cholesterol diet with (f) vitamin E of 300mg/kg daily.

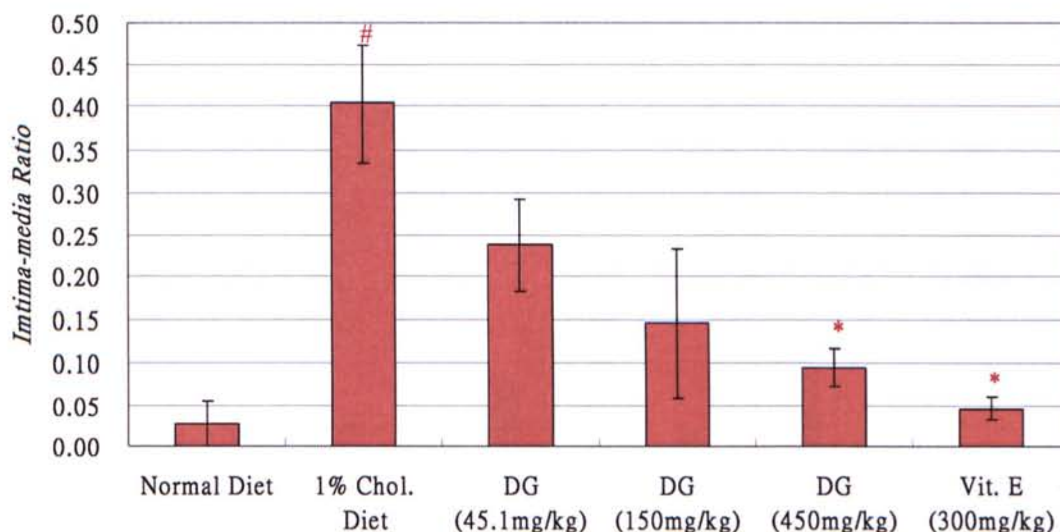
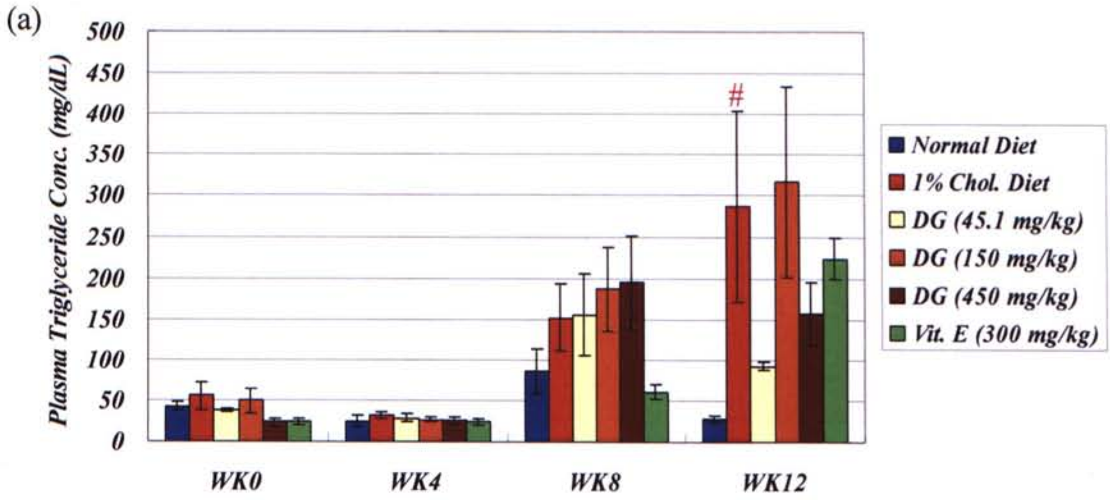
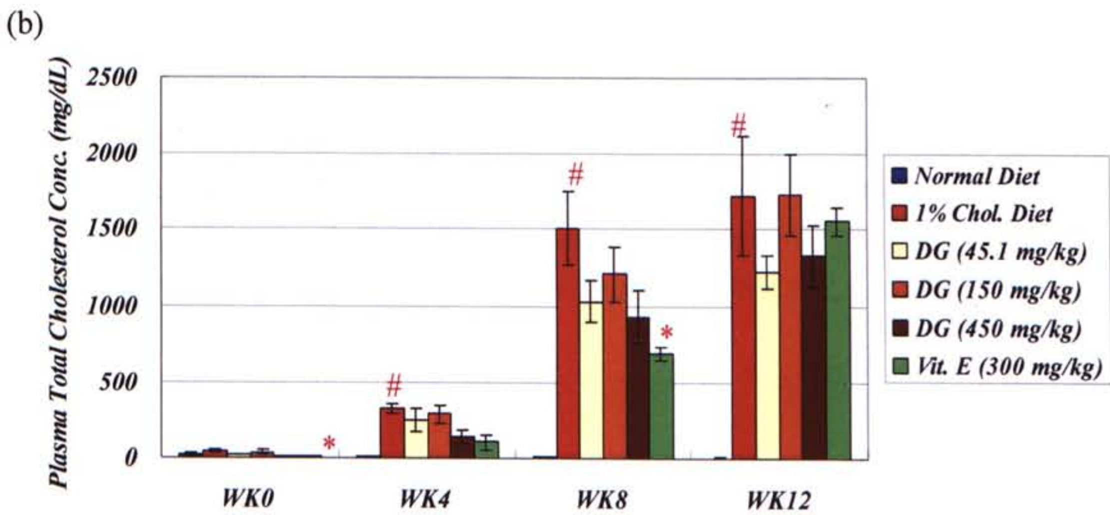


Fig 4.5 Effect of Danshen-Gegen 7:3 water extract on intima-media ratio (1st Run). Sections were 10mm away from heart. All extracts/vitamin E/water were administered daily by oral gavage. 1% cholesterol diet group was compared with normal diet group. All data are presented in mean ± SEM (n=3-4). 1% Cholesterol diet vs Normal diet using Mann-Whitney test, # $p < 0.05$; treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

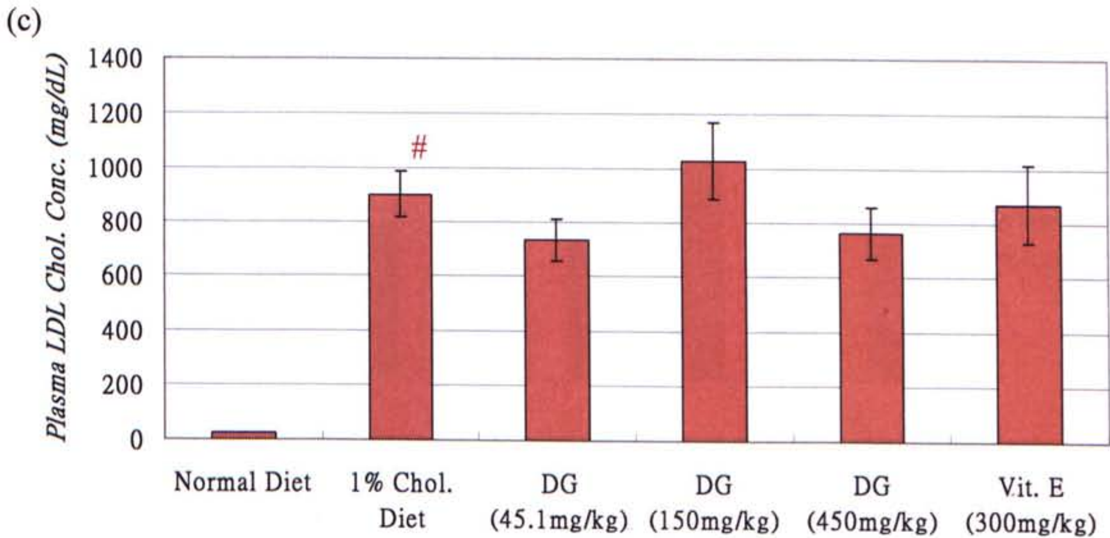
(a)



(b)



(c)



(d)

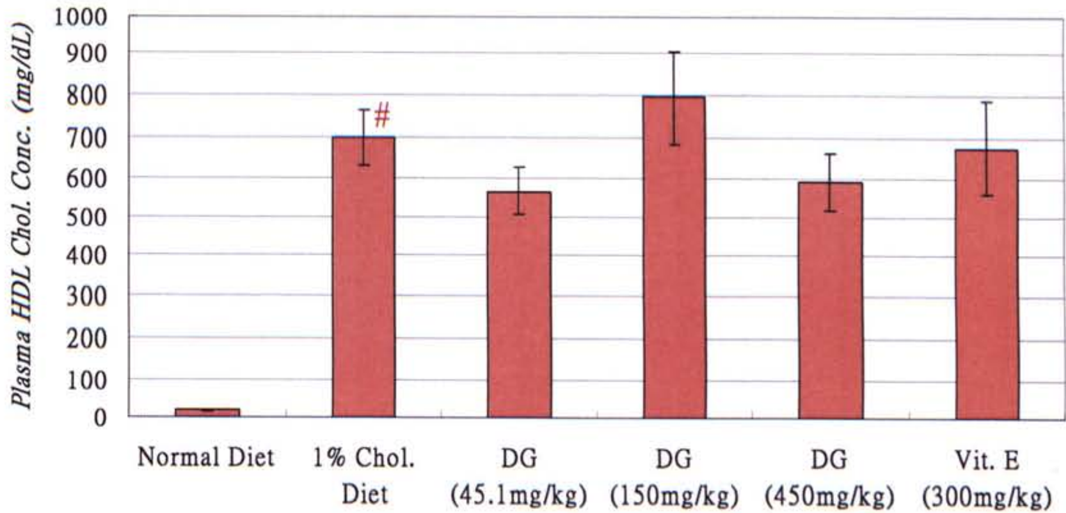


Fig 4.6 Effect of Danshen-Gegen 7:3 water extract on plasma lipid level in cholesterol-fed rabbits (1st Run). Plasma level of (a) triglyceride and (b) total cholesterol from 1st week to 12th week is shown. Plasma (c) LDL cholesterol and (d) HDL cholesterol for 12th week is shown. All data are presented in mean ± SEM (n=3-4). 1% Cholesterol diet vs Normal diet using Mann-Whitney test, # $p < 0.05$; treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

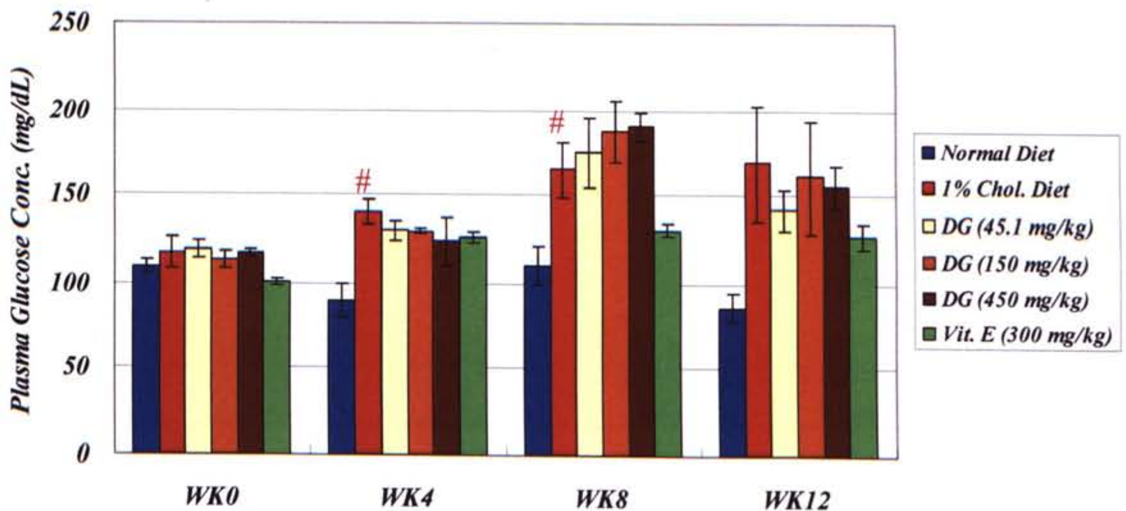


Fig 4.7 Effect of Danshen-Gegen 7:3 water extract on plasma glucose level in cholesterol-fed rabbits (1st Run). All data are presented in mean ± SEM (n=3-4). 1% Cholesterol diet vs Normal diet using Mann-Whitney test, # $p < 0.05$; treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

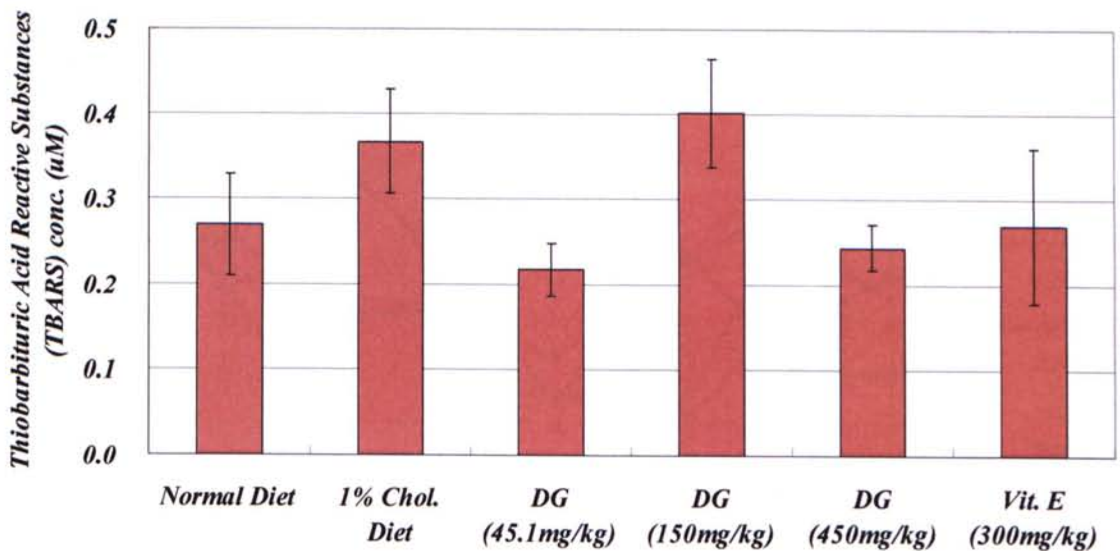


Fig 4.8 Effect of Danshen-Gegen 7:3 water extract on plasma TBARS level in cholesterol-fed rabbits (1st Run). All data do not show a significant difference. All data are presented in mean ± SEM (n=3-4). 1% Cholesterol diet vs Normal diet using Mann-Whitney test, # $p < 0.05$; treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

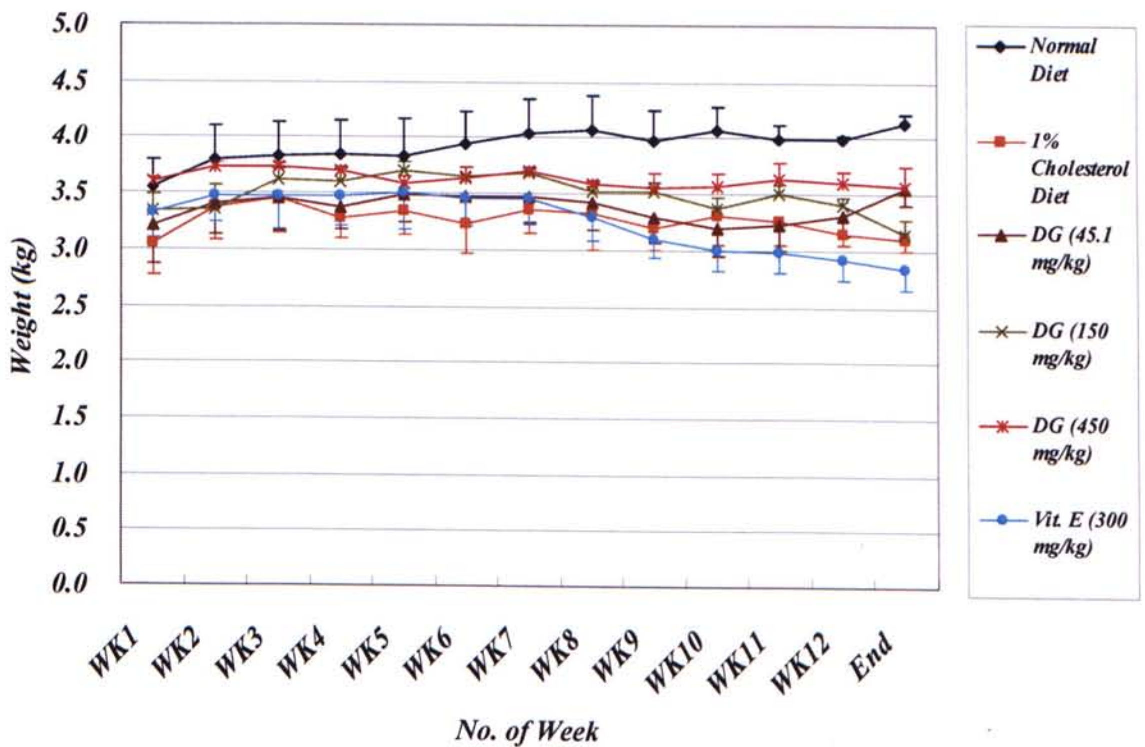


Fig 4.9 Effect of Danshen-Gegen 7:3 water extract on weight in cholesterol-fed rabbits (1st Run). All data were presented in mean +/- SEM (n=3-4).

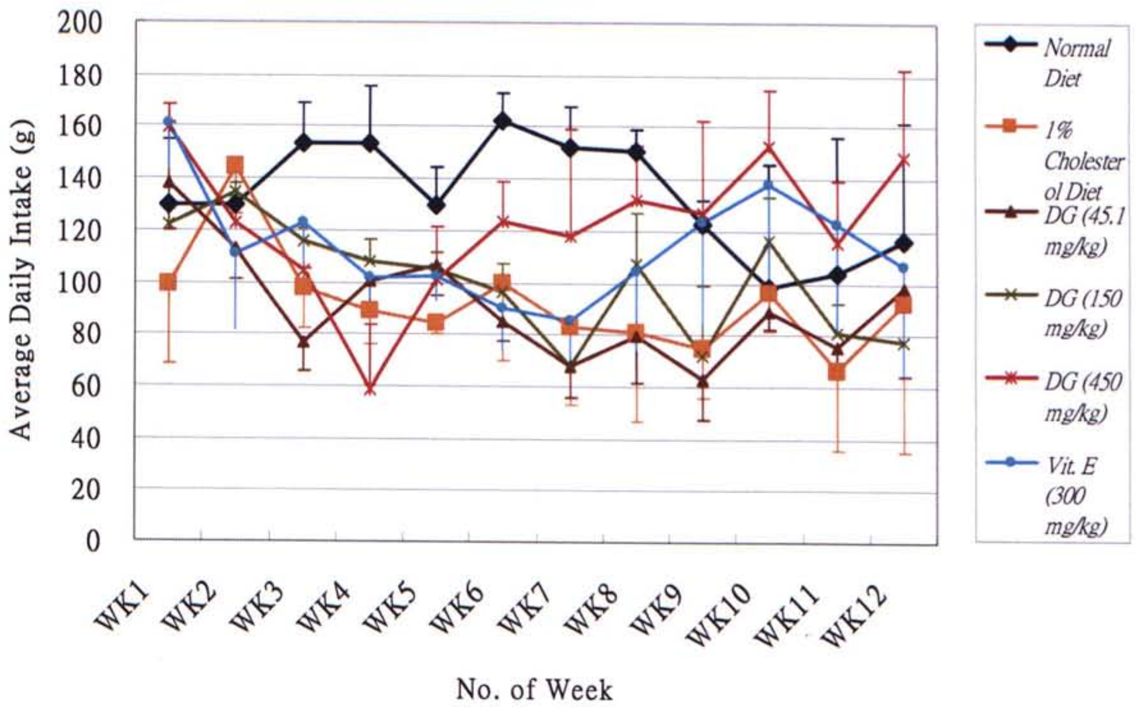


Fig 4.10 Effect of Danshen-Gegen 7:3 water extract on food intake in cholesterol-fed rabbits (1st Run). All data were presented in mean +/- SEM (n=3-4).

4.3.2 Study of the Anti-atherosclerosis Effect of DG extract – Second Run

In order to confirm the result obtained from the first run, we started the second run of the study. In the second run (Fig 4.12 and Fig 4.13), 1% cholesterol diet was however unable to induce a significantly higher intima-media (I/M) ratio. Thus, the cholesterol diet control cannot be established and the results of this run should be invalid. The intima-media ratio for cholesterol diet control is further investigated in Fig 4.11. Every section of aorta of rabbit 2 has plaque. However, I/M ratio in every section are not comparable to the results in 1st run. For rabbit 1 and 3, most of the sections did not have any plaque. In section 2 of rabbit 1 and 3, the I/M ratio was also unexpectedly low. These sections were 0mm, 10mm, 20mm, etc. away from heart according to the ascending number. Nevertheless, we still analyze the data and investigate the possible explanation for that.

A declining trend of I/M ratio with the increasing dose of DG extract was no longer observed. However, when comparing with the 1st run cholesterol diet control. 150mg/kg and 450mg/kg of DG extracts could still lower the I/M ratio. The positive control, vitamin E, was also having a low I/M ratio Fig 4.13.

In Fig 4.14, cholesterol diet control was having a higher plasma triglyceride and cholesterol and 45.1mg/kg and 450mg/kg of DG extract was able to reduce that, but no significant difference was seen. No elevation of plasma glucose was seen in cholesterol fed groups and no significant difference among groups were seen in 2nd run (Fig 4.15). The TBARS concentration remained very low in the 2nd run (Fig. 4.16). The trends in the weight were similar in both runs (Fig 4.17), with slight increase in weight at the beginning in all groups. About last 4 weeks, the weight remained stable for normal diet, 45.1mg/kg and 450mg/kg of DG treatment groups, while declined for

cholesterol diet, 150mg/kg of DG treatment and 300mg/kg of vitamin E treatment groups. Lastly, the ADI in the 2nd run is more stable than in the 1st run (Fig 4.18). Normal diet group had a higher ADI than other groups, while the ADI of the other groups were similar with each other throughout the experiment. These groups were having declined intake from week 5 to 9, a sudden increase in week 10 and dropped again after that. Overall, the blood profile and other trends (except I/M ratio) were similar to 1st run for all groups, although the trends were having less significance differences in 2nd run.

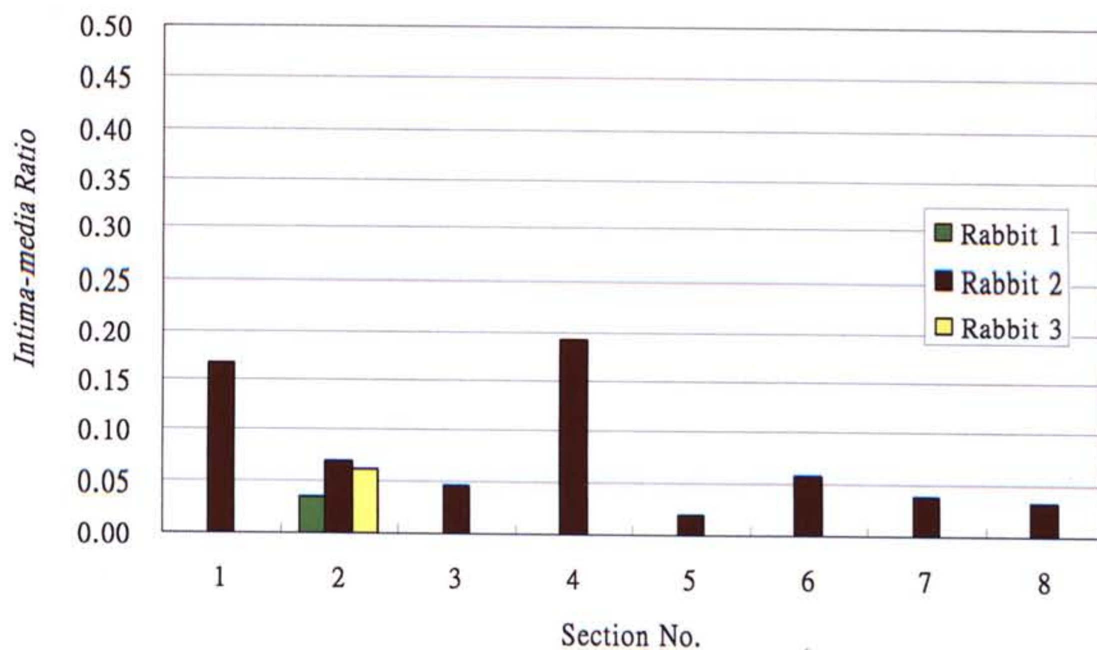


Fig 4.11 The intima-media ratio of serial sections of aorta of rabbit fed with 1% cholesterol diet (2nd Run).

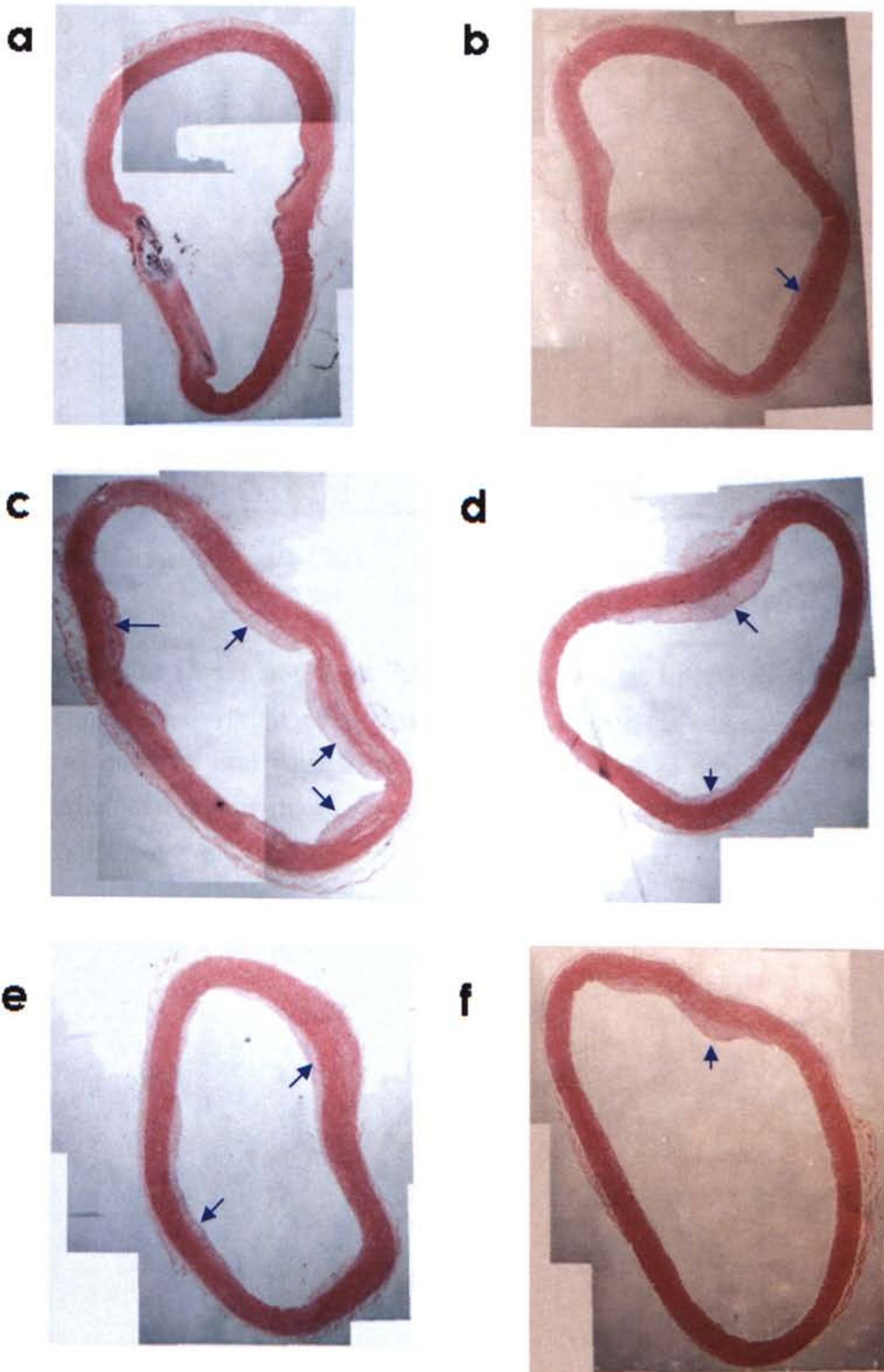


Fig 4.12 Representative photographs showing effect of Danshen-Gegen 7:3 water extract on aorta intima thickness (2nd Run). Smooth muscle cells were stained in deep pink and plaque were stained in light pink (indicated by arrows). All sections were 10mm away from heart. The rabbits were fed with (a) normal diet; (b) 1% cholesterol diet, 1% cholesterol diet with DG extract of (c) 45.1mg/kg; (d) 150mg/kg; and (e) 450mg/kg daily; and 1% cholesterol diet with (f) vitamin E of 300mg/kg daily.

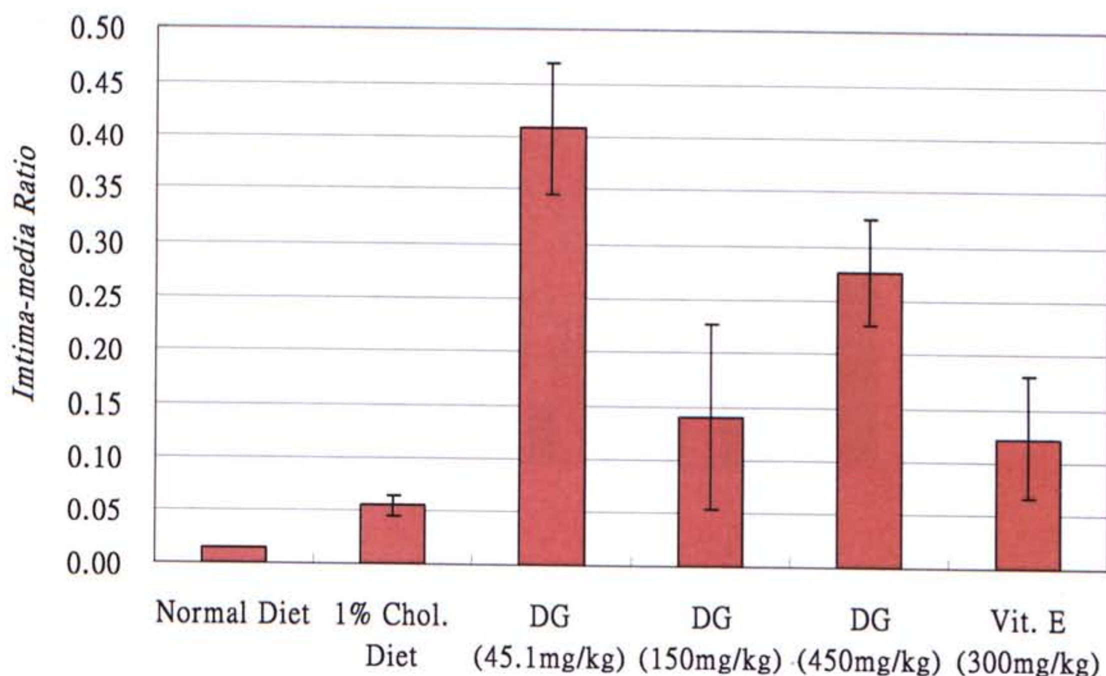
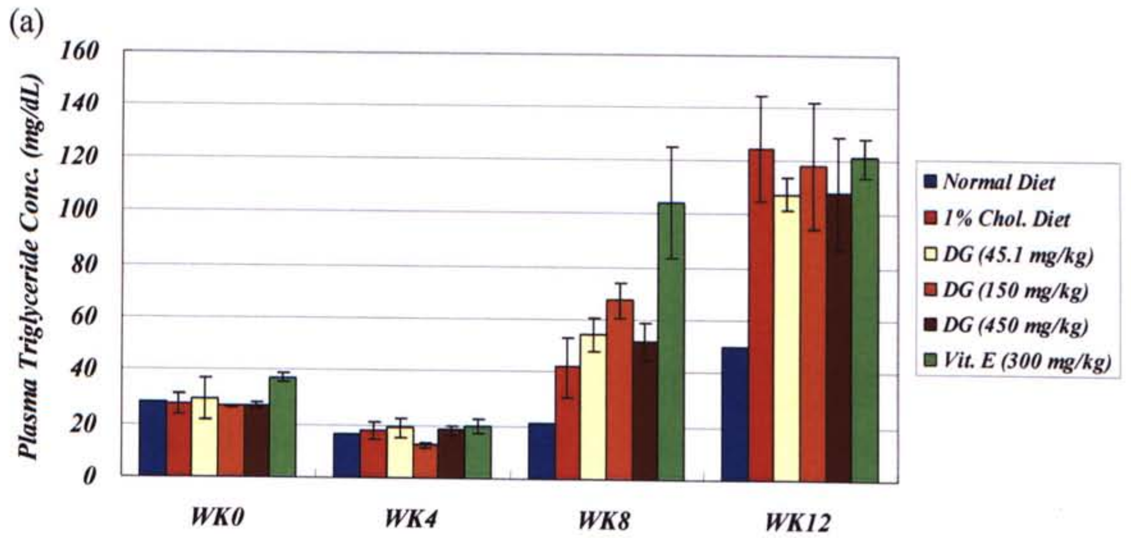
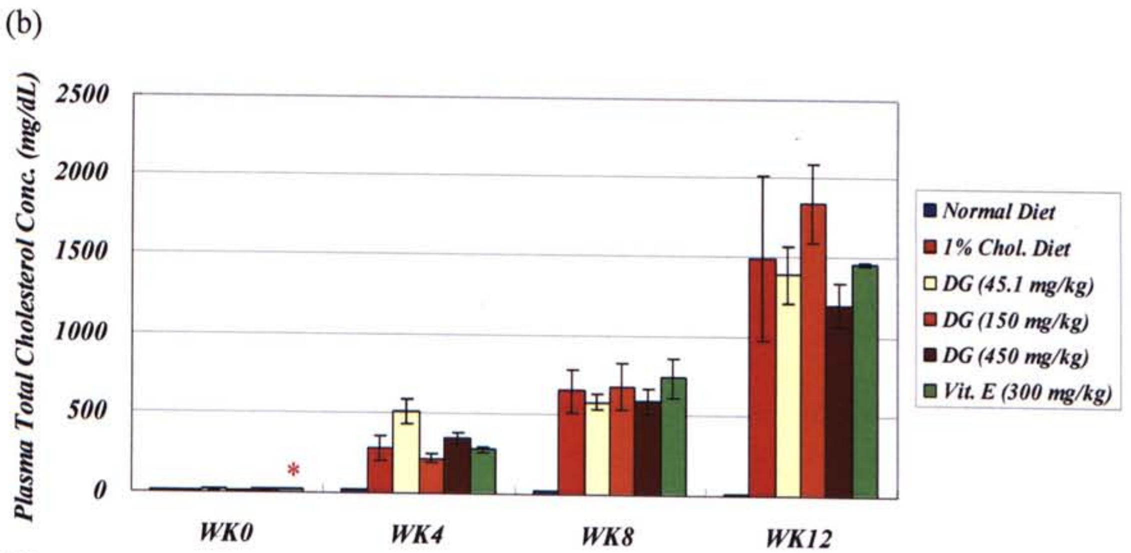


Fig 4.13 Effect of Danshen-Gegen 7:3 water extract on intima-media ratio (2nd Run). Sections were 10mm away from heart. All extracts/vitamin E/water were administered daily by oral gavage. All data are presented in mean ± SEM (n=2-4) except normal diet group (n=1). Treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

(a)



(b)



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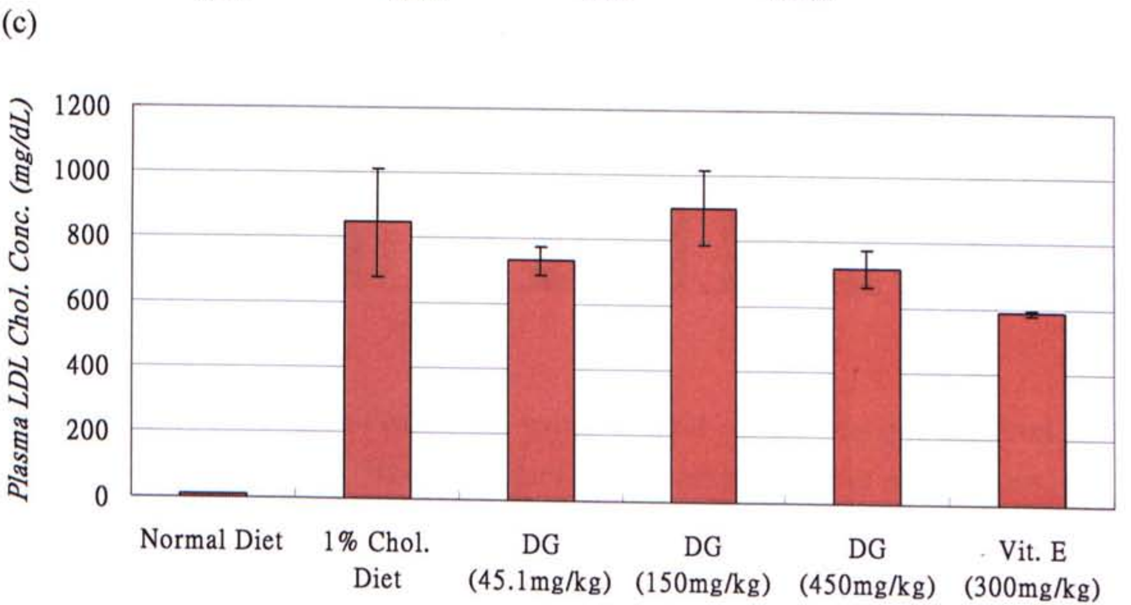

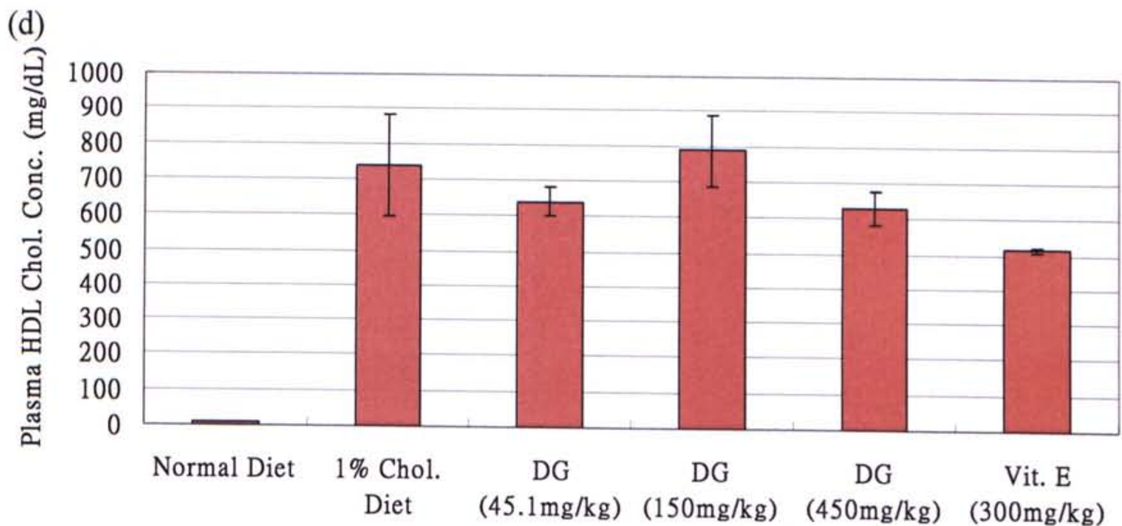

Fig 4.14 Effect of Danshen-Gegen 7:3 water extract on plasma lipid level in cholesterol-fed rabbits (2nd Run). Plasma level of (a) triglyceride and (b) total cholesterol from 1st week to 12th week is shown. Plasma (c) LDL cholesterol and (d) HDL cholesterol for 12th week is shown. All data are presented in mean ± SEM (n=2-4) except normal diet group (n=1). Treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

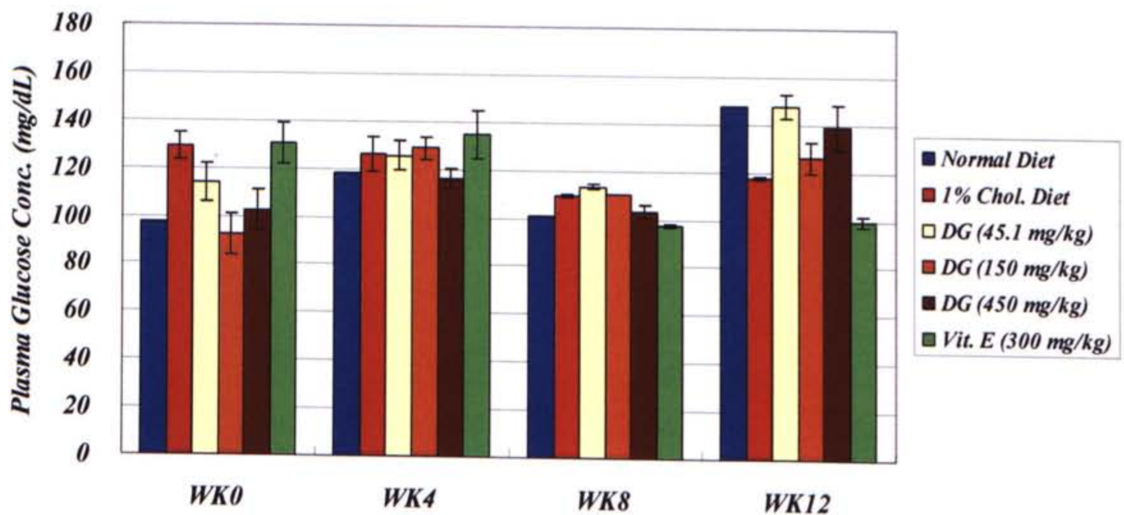

Fig 4.15 Effect of Danshen-Gegen 7:3 water extract on plasma glucose level in cholesterol-fed rabbits (2nd Run). All data are presented in mean ± SEM (n=2-4) except normal diet group (n=1). Treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

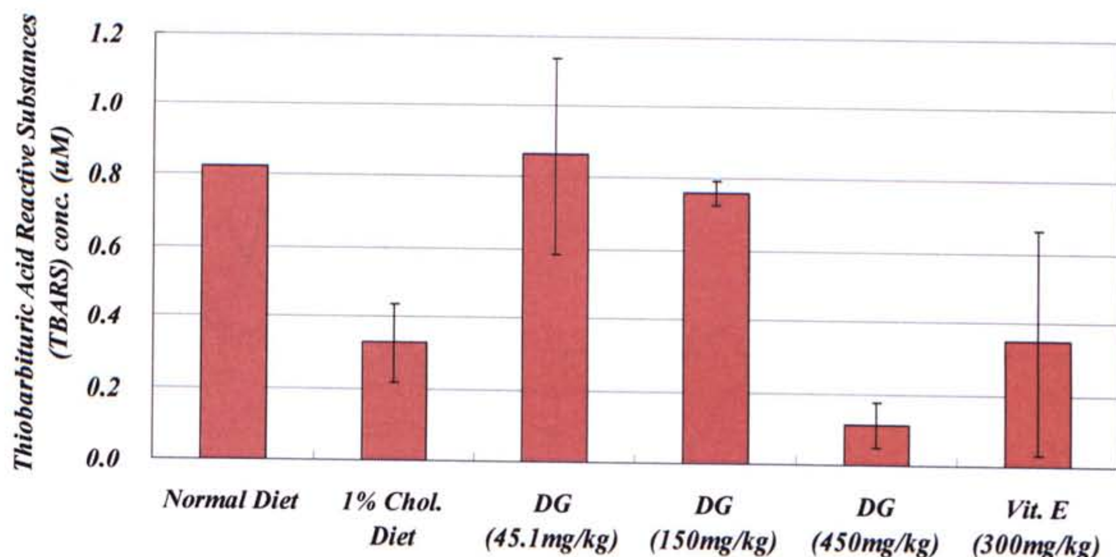

Fig 4.16 Effect of Danshen-Gegen 7:3 water extract on plasma TBARS level in cholesterol-fed rabbits (2nd Run). All data do not show a significant difference. All data are presented in mean ± SEM (n=2-4) except normal diet group (n=1). Treatment groups vs 1% Cholesterol diet using Kruskal-Wallis test, * $p < 0.05$.

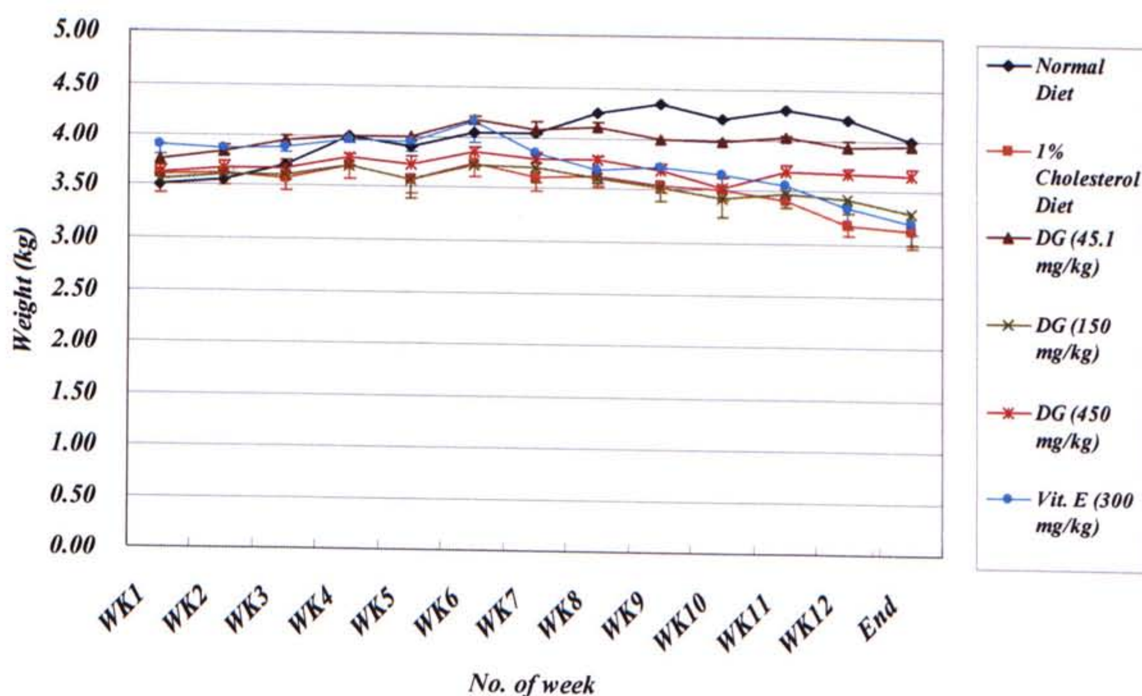

Fig 4.17 Effect of Danshen-Gegen 7:3 water extract on weight in cholesterol-fed rabbits (2nd Run). All data were presented in mean +/- SEM (n=2-4) except normal diet group (n=1).

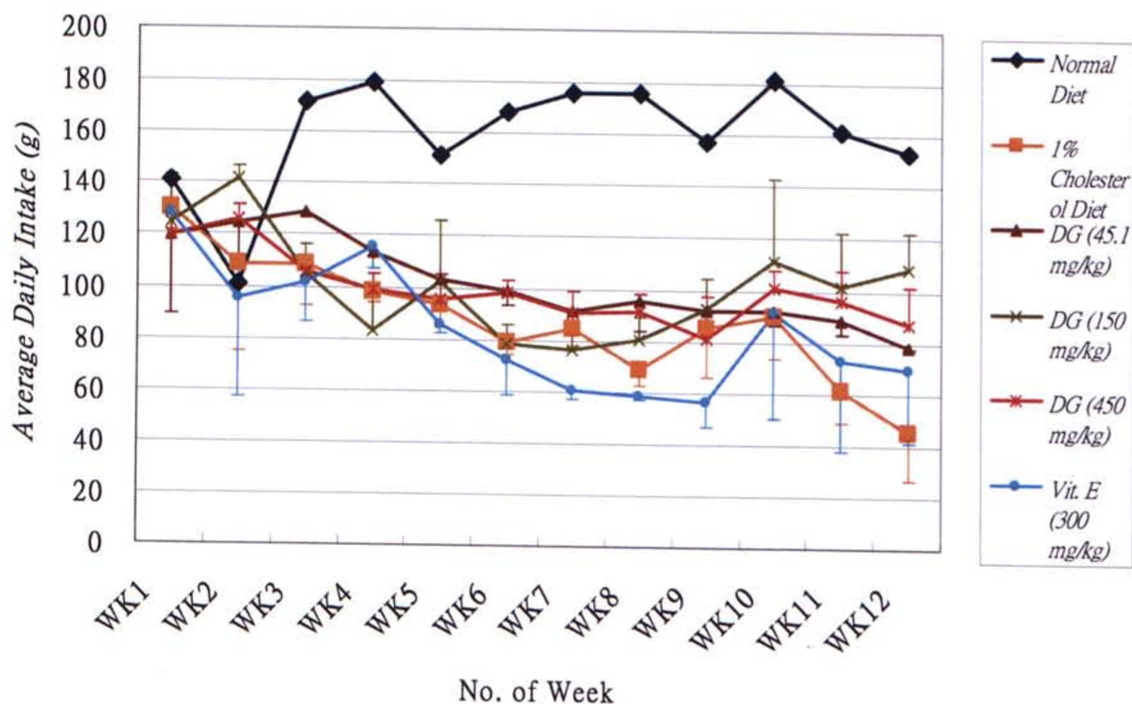

Fig 4.18 Effect of Danshen-Gegen 7:3 water extract on food intake in cholesterol-fed rabbits (2nd Run). All data were presented in mean +/- SEM (n=2-4) except normal diet group (n=1).

4.4 Discussion

Atherosclerosis is a continuous pathological process involving stages of development and years to progress. To speed up the process and enable researcher to sample the tissue for further investigation, high fat diet-induced atherosclerosis in rabbit was developed.

Results from the pilot study indicated that the plaque was formed before 4th week of 1% cholesterol diet. Therefore, treatment could start on the 4th week of high cholesterol diet. In other studies, vitamin E was commonly used in the control of the plaque formation (Hayashi, *et al.*, 2005, Hegyi, *et al.*, 2004, Okolie and Iroanya, 2003). Our results provided experimental evidence to that. Vitamin E functions as an antioxidant, which can scavenge the reactive oxygen species (ROS) (Chiu and Taylor, 2007, Halliwell, *et al.*, 1992). The reduced amount of ROS causes a reduced oxidation of LDL cholesterol. As the level of oxLDL decline, the progression of atherosclerosis would also slow down. Moreover, vitamin E can help attenuating the progression of atherosclerosis through inhibition of vascular SMCs proliferation (Boscoboinik, *et al.*, 1991), inhibition of platelet aggregation (Murohara, *et al.*, 2002), and the reduction of the expression of soluble cell adhesion molecules (van Dam, *et al.*, 2003). It can also reduce the risk of atherosclerosis by inhibition of leukotrienes production and promotion of prostacyclin production (Tran and Chan, 1990). Most importantly, the dose used in our study did not exert toxic effect on the rabbits. The results supported the use of vitamin E as the positive control.

In our experiments, the main finding was the significant and dose dependent reduction in the intima-media ratio by treatment of different concentrations of DG extract. The sections were positioned at the aortic arch. The sections in 1st run were

verified to locate at the aortic arch by their curvature. In Fig 4.19, the curvature of the section is shown. This should due to the fact that atherosclerotic lesion initiates at branching points of arteries and regions with disturbed blood flow (Cunningham and Gotlieb, 2005). DG extract-treated rabbits exhibited dose dependent reduction in intima-media thickness. The effect of 450mg/kg DG extract was comparable with that of 300mg/kg vitamin E. In addition, the plasma total, HDL- and LDL- cholesterol levels were lowered in 45.1 or 450 mg/ml DG extract-treated rabbits comparing with those on cholesterol diet only.

Fig 4.19 Representative photo for aortic arch and descending aorta (Left) the section 10mm away from heart, which is curve in nature; (Right) the section 70mm away from heart, which is straight in nature.

45.1 or 450 mg/ml DG extract-treatment could maintain the weight of the rabbits during the last few weeks, while vitamin E treatment or cholesterol diet without treatment would cause reduction in weight in the last few weeks. Since the amounts of diet intake were similar, the weight reduction should due to deterioration of general health condition of the rabbits. During the experiment, two groups of rabbits, one

from cholesterol diet group and one from vitamin E treatment group, lost over 25% of their initial weight. The wasting was too serious, below the ethical standard and were euthanatized. The health maintenance effect of DG extract was possibly due to the reduced intima-media thickness, as atherosclerosis is secondary to many body disorders or conditions like hypertension, diabetes, propensity to form thrombi and level of homocystine. However, the situation may also be vice versa. Reduced atherosclerosis can be the cause of a better health condition. Other study revealed that better, less resistant blood flow is critical to the health condition (Morris and Blumgart, 1957). But no matter which is the truth, the quality of life of the DG extract-treated rabbits was improved. This improvement was even not observed in vitamin E-treated group.

TBARS include lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. Our results showed that there was no significant differences in TBARS among all tested groups. The concentration of TBARS was only 0.2 to 1.0μM, which is very low in the assay. Therefore, the results might not have been correctly reflected the real situation, as bioactive compound in Danshen (Niu, *et al.*, 2000, Wang, *et al.*, 2003, Zhao, *et al.*, 1996) and Gegen (Finking, *et al.*, 1999, Huang and Shi, 1996, Rimbach, *et al.*, 2008) were commonly reported for their anti-oxidative function. The comparisons are summarized in Table 4.3.

In the second run of the rabbit study, 1% cholesterol diet without drug treatment was unable to induce intima-media thickening, while 1% cholesterol diet with drug treatment are having intima-media thickening, is completely opposite to the results observed in the first run. The blood profiles of these rabbits however match the first run of rabbits with cholesterol level increased dramatically, which implied that the

Table 4.3 Summary of the comparisons between results of present study and previous studies in atherosclerosis

Herbs	Extract and (or) "purified compound"	Biological Preparation	Anti-atherosclerosis	Anti-oxidation	Hypolipidemic	Reference
Pueraria lobata + *Salvia miltiorrhiza*	Aqueous extract	Aorta section + plasma	+	-	-/+	Current Study
Pueraria thomsonii + *Salvia miltiorrhiza*	Aqueous extract	Whole aorta	+	NA	+	Koon, 2006
Pueraria thomsonii + *Salvia miltiorrhiza*	Aqueous extract	*In vitro* assays	NA	+	NA	Koon, 2006
Salvia miltiorrhiza	"Tanshinone II(A)"	*In vitro* LDL assays	NA	+	NA	Niu, *et al.*, 2000
Salvia miltiorrhiza	Injectable *Salvia miltiorrhiza*	*In vitro* assays	NA	+	NA	Wang, *et al.*, 2003
Salvia miltiorrhiza	Injectable *Salvia miltiorrhiza*	Ischemia-reperfused heart	NA	+	NA	Zhao, *et al.*, 1996
Pueraria lobata	"Daidzein"	Plasma lipoprotein	NA	+	+	Finking, *et al.*, 1999
Pueraria lobata	"Daidzein"	*In vitro* assays	NA	+	NA	Rimbach, *et al.*, 2008

Note: NA – not available, + dependency; - independency; -/+ slight trend of dependency, further investigation needed

cholesterol diet was working normally. The trend of ADI and body weights were also matched with those of the first run of rabbits. Therefore, the difference should not due to the difference in intake of cholesterol diet. The slides were also checked to be match in shape with the paraffinized samples. So, the slides were named correctly. In order to check the possibility of displaced plaque, all eight sections of the affected rabbits were stained and have I/M ratio calculated. No large extent of intima-media thickening was seen in any section. From other studies, the sections were also taken from the aortic arch but not the other locations (Alfon, *et al.*, 1999, Boger, *et al.*, 1998, Choe, *et al.*, 2001, Maranhao, *et al.*, 2008). Thus, the reason of the absence of intima-media thickening is unknown.

Conclusion

In conclusion, DG extract could protect the New Zealand white rabbits from the progression of atherosclerosis in a dose-dependent manner. Hypocholesterolemic effect of DG extract on these hypercholesterolemic rabbits was also shown. DG extract was also found to improve the health condition in terms of reduced weight drop. However, anti-oxidative effect of DG extract was not found in this study. Due to the inability of cholesterol diet to induce intima-media thickening in the control group of the second run of the rabbit study, verification of the results can be included in the further studies. The same study should be repeated in the near future in order to confirm the results.

Chapter 5

General Discussion and Conclusion

5.1 Significance of the Study

Cardiovascular diseases (CVD) are one of the most prevalent diseases in the world. It is also one of the top mortality causes. Treatment and management of CVD pose a heavy burden to the economy. Hence, prevention or suppression of the diseases in an economical way becomes a target of research. Although progression of CVD is a complex and inter-related process, hypertension and atherosclerosis are the common risk factors for CVD. Hypertension is also a main risk factor for atherosclerosis. Therefore, the study starts from the investigation of the anti-hypertensive effect of DG extract, and proceed to the research of anti-atherosclerotic effect of DG extract.

In international research field, compound formula seldom becomes the target for research, due to its complexity and multiple compound nature. However, most of the Chinese medicine formulations include multiple herbs, and these herbal formulae were used in China for thousands of years for treatment of diseases. Danshen and Gegen were commonly used in the treatment of cardiovascular diseases. Therefore, investigation of DG compound formula become our research target and is exclusively investigated by our research team. In the previous studies (Leung, 2003), different ratio combination of DG (*Pueraria thomsonii* as Gegen) was tested using red blood cell hemolysis assay, which mainly test for the anti-oxidation power of DG extract. The ratio of DG in 7:3 was determined to be the best combination and used in the later studies, which DG 7:3 performed better than the same dose of Danshen or Gegen. After a series of *in vitro*, *ex vivo* and *in vivo* biological assays, DG (*Pueraria thomsonii* as Gegen) compound formula was found to be effective in antioxidant (Chan, 2006, Koon, 2006), cardioprotection, vasodilation (Chan, 2006),

anti-hypertension and anti-atherosclerosis (Koon, 2006, Tam, 2004).

Previously, anti-hypertension experiments were carried out to test for the effect of DG extract on systolic blood pressure. However, the related species *Pueraria thomsonii* was used as Gegen in some of the previous studies. The ongoing studies are using the other species *Pueraria lobata* as the standardized Gegen, as mention in the most recent Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010). The present study also tested for the efficacy of the dose of DG formula (Human equivalent dose (HED) = 45.1mg/kg in rats) that is used in the current clinical trial. It was found that the new dose, which is only one-third of the previous dose (HED = 150mg/kg in rats), can still achieve the same efficacy as the previous dose in both preventive and therapeutic experiments. These findings consolidate the anti-hypertensive effect of DG extract and reconfirm the use of *Pueraria lobata* as Gegen would not adversely affect the effectiveness of DG extract. The positive effect of the new dose of DG extract gives a solid evidence to support the current clinical trial.

The vasodilative actions of DG extract on aorta rings isolated from the DG extract-fed SHR rats were tested. Their ACh-induced relaxation profiles were similar to those of SHR rats of control group under the situation without inhibitors added, or with NO-pathway inhibitor, or prostacyclin-pathway inhibitor, or with hyperpolarization-pathway blocker added. Our results are consistent with the results of previous studies where no inhibitors added, or with NO-pathway inhibitor added. However, the results also showed that the maximum relaxation percentage of SHR rats is lower than those of the normotensive WKY rats, and that DG extract treatments have a trend to increase the relaxation. In addition, the DG extract treatments

alleviated the contractile force during Phe induction.

Comparing with the previous vasodilation studies (Chan, 2006), the main difference of the present study is the inclusion of different concentrations (0.25 to 2mg/ml) of DG extract, whereas a single concentration was used previously, and also tested for the involvement of BK_{Ca} and K_{ATP} channels. The significance of different concentrations of DG extract is to ensure the results are not underestimated by insufficient dose used. Besides, the vasodilative effect of DG extract is thought to involve multi-mechanisms. Our study prevents the overdose of DG extract shading the inhibitory effect of the inhibitor or blocker added, which may cause a false negative result for an involved mechanism. The new pathways investigation is based on the findings that BK_{Ca} and K_{ATP} channels were found to be related to actions of the bioactive components of Danshen and Gegen (Chan, *et al.*, 2009, Sun, *et al.*, 2007). To be consistent with the other experiments in this study, same batch of DG extract was used in the entire study.

The results of present studies showed that the DG extract-induced relaxation is endothelium-independent, neither NO-mediated nor prostacyclin-mediated. In fact, the relaxation depended on the hyperpolarization. Both Kir channels and K_v channels were involved in the vasodilation in these concentrations. These findings were consistent with the previous studies (Chan, 2006) (see Table 3.5), which focused on a single concentration of DG (*Pueraria lobata* as Gegen) formula. Our study also demonstrated that the relaxation involves of K_{ATP} channels and was unrelated to BK_{Ca} channels. Our study also suggested that the vasodilative action has multi-targets. As the vasodilative action was endothelium-independent, explanations involving endothelium were excluded. Some other possible mechanisms, such as inhibition of

calcium influx through calcium channels, inhibition of myosin light chain kinase, or activation of myosin light chain phosphatase, are still warrant further investigations. Detailed elucidation of these mechanisms will be the target of further research.

Apart from the current study, we have previously tested the effect of DG extract on diet-induced atherosclerosis in rabbits (Koon, 2006). The comparisons are summarized in Table 4.3. However, in the previous study, DG extract was mixed to the diet, which was susceptible to denaturation. Besides, the amount of DG extract intake depended on the diet intake of rabbits, which was shown to be decreasing in the later stage of the study. Thus, the DG extract doses were not accurately controlled. Furthermore, only quantity of the atherosclerotic plaque was determined. But in the real situation, stenosis and blockage of blood flow were the main cause to the complications. Hence, improvements were made in the current study by using a daily oral gavage of freshly prepared DG extract in aqueous solution form. This method mimics the intake practice of human and gives a precise intake of DG extract to rabbits, ensuring an accurate dose of treatment. In addition, instead of quantity of plaque, the thickness of the plaque, namely intima-media thickness, was investigated with histochemical methods.

Our results showed that DG extract could reduce intima-media thickening in a dose dependent manner. The effect of 450mg/kg DG extract was comparable to that of 300mg/kg vitamin E, which is a positive control. Combining with our previous results, we concluded that DG extract reduces the atherosclerotic plaque in both quantity and thickness, which could be partially explained by the slight hypocholesterolemic effect of DG extract. Moreover, base on our previous *in vitro* studies of our institute, DG extract also reduced endothelial-monocyte adhesion (Koon, 2006), which is critical

for the initiation of atherosclerosis. The protein responsible for the penetration of monocytes through the endothelium and remain in intima, MCP-1, was also found to have reduced production with the addition of DG extract previously (Koon, 2006). As for the later stage of atherosclerosis, SMC proliferation and migration plays an important role in thickened, fibrous intima. Inhibitory effect of DG extract on vascular SMC was previously verified by ^{3}H-thymidine uptake assay and cell cycle analysis (Koon, 2006). The migration of vascular SMC was also inhibited by DG extract in transwell assay (Koon, 2006). All these parameters would be responsible for the anti-atherosclerosis effects of DG extract. Therefore, further studies can be performed on the action mechanisms of DG extract on anti-atherosclerosis, which is expected to be a complex process involving cytokines, adhesion molecules and different proteins.

5.2 Limitations and Future work

For the therapeutic effect study on SHR rats in hypertensive state, the main limitation of our study is the extremely high systolic blood pressure of about 200mmHg, which is not close to the reality in human. Another limitation is the small difference between the relaxation of WKY and SHR rats' aorta. As mentioned before, older rats perform better in this assay since older SHR rats were known to have increased stiffness in blood vessels (Safar and Laurent, 2003). This could result in a larger difference in stiffness in aorta between WKY and SHR rats. The stiffness in aorta would affect its performance on vasodilation in the organ bath. Therefore, further study should be performed using older rats of above 24 weeks old at the time of sacrifice. Their aorta could be sampled for the vascular reactivity test.

Another limitation is the doses of DG extract used in organ bath for mechanism elucidation have a weak linkage with the clinical dose. The DG extract was directly added to the aorta ring. Therefore, the dose should be linked to the plasma dose in DG extract *in vivo*. To investigate that, the bioavailability should be considered. The plasma concentration of markers of DG extract, like salvianolic acid B, Tanshinone II A, puerarin and daidzin, can be determined by HPLC after oral gavage of a known dose of DG extract. With the information on the bioavailability, the linkage between the dose of DG extract used in organ bath and the clinical dose can be estimated.

There are various follow up studies that can be done in the future. In investigation of mechanism of DG extract-induced vasodilation, unidentified pathways are the main target in the future. Other studies showed the possibility of involvement of myosin light chain kinase (MLCK) or myosin light chain phosphatase (MLCP) in the action of another herbal extract (Park, *et al.*, 2009). Inhibitors for

MLCK and MLCP, which are ML-9 and Calyculin A, respectively, can be included to test if DG extract affects their expression or actions. The other possibility includes the blocking effect on calcium channels. After depolarization, influx of calcium ions through voltage dependent calcium channels causes increase of intracellular calcium level. Intracellular calcium binds with myosin light chain and mediates vasoconstriction. The blockage of calcium channels by DG extract, if happens, can reduce the intracellular calcium and inhibit vasoconstriction. The inhibitory effect can be verified by testing $CaCl_2$-induced vasoconstriction (Lam, *et al.*, 2006) in different concentrations of DG extract.

With the setup of blood pressure measurement on SHR rats for studying anti-hypertensive effect, *ex vivo* vascular reactivity assay for mechanism elucidation and *in vivo* confirmation of the mechanism, we have now established a platform with *in vivo* and *ex vivo* assay for investigation of anti-hypertensive effect and mechanism of potential herbs or drugs. This platform can be used to assess the vasodilation mechanism of Danshen, Gegen and their individual active components. This can certainly help in the mechanism elucidation of this formula and reveal the potential involvement of the bioactive components in this formula in vasodilation. In the future research of other vasodilative herbs, the same platform can also be used. The standardized platform enables us to assess and compare the vasodilation efficacy of different formula, herbs or single compound easily. This comparison can help us to find a better formula and any potential herbs for anti-hypertension, which should be the final goal of the research.

In order to verify the effect of positive control raloxifene experiment, a better setup of the study is to include a group of WKY rats treated with raloxifene. This can

ensure raloxifene has no adverse effects on rats. Moreover, a better choice of positive control is to use the commonly prescribed western medication for anti-hypertension, like the ACE inhibitors. Another improvement is to include the assays of the plasma salts, such as potassium, sodium and calcium levels. As depletion or retention of salt is often seen in anti-hypertensive drugs, e.g. diuretics and calcium channel blockers, the tests of contents of these salts determine whether DG extract exhibits any effect in salts content.

Besides, hypertension may also be related to the heart. A more rapid heart rate or a larger contractile force of the heart can also lead to larger blood flow, resulting in hypertension. Therefore, the effect of DG extract on heart rate and beating pattern can be monitored by electrocardiogram *in vivo*, while the contractile force and relaxation can be monitored by Langendorff *isolated heart* perfusion system *ex vivo* in rats.

For the anti-atherosclerosis study, the main limitation is rabbits' inability to form advanced lesions without balloon surgery. Therefore, if treatment of advanced lesions is the future research target, the study should be repeated with balloon surgery performed on thoracic or abdominal aorta. Moreover, although DG extract showed a dose-dependent anti-atherosclerosis effect in the first run of rabbit study, the high cholesterol-diet control in the second run showed unexpected low intima-media thickness. There was also no displacement of plaque in rabbits in the second run. Due to time-limitation, another run of the rabbit study is not available. However, the same study will be repeated in the future to confirm the observation. Nevertheless, as an improvement of the study, infiltration of macrophages, migration and proliferation of smooth muscle cells (Djahansouzi, *et al.*, 2001, Hegyi, *et al.*, 2004, Rosenfeld and Ross, 1990), expression of adhesion molecules (ICAM-1 and VCAM-1) (Broisat, *et*

al., 2007, Choe, *et al.*, 2001, Iiyama, *et al.*, 1999) can also be considered. Thiobarbituric acid reactive substances (TBARS) assay is an index of lipid peroxidation (Buege and Aust, 1978). The previous test on TBARS did not provide adequate information for the anti-oxidative effect induced by DG extract even if it has that effect. As a follow-up study, the anti-oxidative effect can be tested on copper-mediated oxidation of isolated LDL (Burstein, *et al.*, 1970) or serum in the future. Formations of conjugated diene and lipid hydroperoxides (Barnes, *et al.*, 2002, Visavadiya, *et al.*, 2009), which are the better indicators for lipid oxidation can also be examined. By doing these, it should give a full picture on the anti-oxidative effect of DG extract on preventing lipid oxidation *in vitro* and *in vivo*. If the results are still negative, then anti-oxidation is unlikely to be the mechanism responsible for reduced IMT caused by intake of DG extract.

For the anti-atherosclerotic study, we have so far covered the therapeutic effect of DG extract. Future study can also look at the preventive effect of DG extract on intima-media thickening, i.e. by giving DG extract and 1% cholesterol diet together at the beginning of the study before the plaque was formed. The combined results would enable us to have a complete picture on whether DG extract possessed the anti-atherosclerotic effect in both preventive and therapeutic aspects.

Alternative animal models can also be considered. The main limitations of current SHR rat include 1) too high pressure; and 2) fixed period and long duration for generation of hypertension. For angiotensin-induced hypertensive model, the rats have the advantage of having relatively lower blood pressure of about 170mmHg (Kane, *et al.*, 2010, Walter, *et al.*, 2008), which is closer to the reality as in human. Dahl salt-sensitive hypertensive rat is another choice. The rat can be used if a more

rapid increase in blood pressure, or the research is target specifically on salt-sensitive hypertension (Garrett, *et al.*, 2000). Both models can have hypertension induce at the desired time. However, these rats need extra intake of other drugs or salt for induction of hypertension and the successful induction may also be affected by individual response. For atherosclerotic model, ApoE-deficient mice are also commonly used as an alternative of rabbits (Meir and Leitersdorf, 2004). The use of mice enables a larger sample size, as rabbits are space occupying. Intragastric intake of water extracts for rabbits is also time-consuming and has a higher risk of aspiration pneumonia. However, rabbit model for studying IMT was established for much longer time. Rabbits also have the advantage of having larger quantity of tissue available for sampling than mice. Therefore, other animal models may be considered for further studies.

5.3 Clinical Implication of the Use of the DG Preparations for Patients with CVD

The previous clinical study found that DG extract could have beneficial effect on atherosclerosis-related parameters in patients with coronary artery disease, such as reduction in total and LDL-cholesterol, improvements in vasodilation (flow-mediated or nitroglycerin-induced) and reduction in IMT (Tam, 2004). In our studies, DG extract exhibited anti-hypertensive effect in the *in vivo* rat model and *ex vivo* organ bath models, and possibly anti-atherosclerotic effect in *in vivo* rabbit model, through vasodilative and hypocholesterolemic action.

We would recommend the use of DG extract as alternative medication in patients diagnosed as higher normal to mild hypertension (Table 1.1) based on the clinical and non-clinical experimental results. The extract is also recommended for patients with atherosclerosis as DG extract can reduce the intima-media thickness in clinical trial and possibly in animal study. In addition, the hypocholesterolemic effect of DG extract observed in clinical study and the trend in our animal study also suggest the recommendation of its use in the control of hypercholesterolemia.

Although the anti-hypertensive and anti-atherosclerotic effects of DG extract is promising, the interactions of DG extract with other commonly used conventional cardiovascular medications like statin, warfarin or other vasodilators are unknown. If synergistic effect exists, the adverse events can be serious. Hence, the use of DG preparation together with other drugs should be with caution. The interactions of DG extract with these drugs should be investigated in details in the future studies.

5.4 Conclusion

The present study shows that DG extract induces a dose-dependent, endothelium-independent vasodilation on isolated aorta of SD rats. Potassium channels, especially Kir, K_v and K_{ATP} channels, are likely to be involved in the aortic vasodilation, while the involvement of extra mechanisms are also expected. This pharmacological effect, combining with a lower contractile force, can explain the anti-hypertensive effect of DG extract in SHR rats in this study. In another part of present study, DG extract is able to slow down IMT progression. Hypocholesterolemic effect of DG extract is likely to be involved in the anti-atherosclerosis process. As DG extract can have positive effects on hypertension and atherosclerosis, which are the main risk factors for CVD, our study provide scientific evidence to support the previous and current clinical trial on DG extract's anti-atherosclerosis effect.

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