

A twin herbal formula has gained sufficient clinical and laboratory evidences to be accepted as an effective cardio-vascular protective supplement

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Abstract

A two herbs formula has been successfully used as a cardiovascular supplement (tonic) to maintain a healthy vascular endothelial state in four groups of patients presenting with different symptom manifestations: coronary obstruction; hypertension; menopausal cardiovascular instability; and peripheral arterial disease. In parallel with the four clinical trials carried out separately in over ten years, laboratory platform studies to find out the mechanisms of action of the herbal formula have been carried out. The bioactivity tests demonstrated the general efficacy of the formula as being anti-inflammatory and anti-oxidative; while in addition, specific influences on vascular relaxation, cardiac protection, endothelial stability and blood lipid cholesterol adjustments were demonstrated.

Loss of arterial patency is the chief culprit for different forms of vascular accidents, and surgical interventional therapy could offer an immediate short-termed solution. The impressive relieving maneuvers yet do not take care of the causative factors like endothelial deposits in response to the changing unfavorable microenvironments provocative for inflammatory changes and lose of smooth muscle relaxation.

The herbal formula, after years of clinical applications and laboratory studies has accumulated evidences that it could serve as an effective supplement to maintain cardiovascular health through multiple bioactivities to harmonize the adverse conditions leading to the decline of cardiovascular competency.

Keywords: Cardio-vascular protection, Herbal supplement, Peripheral arterial disease

Abbreviations: D&G: Danshen and Gegen; FMD: Flow-Mediated Dilation; PAD: Peripheral Arterial Disease; IMT: Carotid Intima-Media Thickness for Cardiovascular Angiography and Interventions

Introduction

As aging has become a Public Health problem with ever rising importance, one of the most life-threatening areas involves the cardiovascular system. Age-related deteriorations of the cardiac and peripheral arteries are the culprits of coronary artery diseases and cerebral vascular accidents, both of which are common causes of mortalities [1]. For the last five decades, the risk factors leading to cardiovascular hazards have been well defined [2]. Since then, clinicians, who are uniformly energetic about surgical and pharmaceutical treatments of established pathologies, started to turn their attention towards the prevention of the risk factors leading to the hazards: which include hypertension, atherosclerosis and diabetes mellitus. Preventive measures at a perfect control of hypertension, lowering the serum cholesterol level and maintenance of normal blood sugar level [3,4].

In the past decades large series of epidemiological studies have shown that statin prophylaxis for those with high blood cholesterol resulted in fewer coronary obstructions up to a long period of over ten years. Those suffering from hypertension on top of high cholesterol similarly benefit as has been shown in other studies. When diabetes mellitus co-exists with high blood cholesterol and hypertension, the value of controlling unfavorable statins could be similarly established [5].

However, in spite of the optimism that exists, it is becoming clear that individual differences still prevail and those not responding well to preventive treatment are described to be special genome related. Genetic investigations have led to discoveries of the heritable components of cardiovascular risk factors and coronary artery diseases and genome wide association studies across populations responsible for variations in lipid-levels are discovered. Specific inflammation pathways may regulate the process of coronary atherosclerosis, challenging the expected effectiveness of standard preventive measures [6].

Marzilli in 2012 discussed the causative factors of atherosclerosis which involves the complicated microenvironment of the endothelium of the artery. Apart from simple cholesterol deposits, other conditions like inflammatory states; antioxidant and muscular relaxation states etc. are all contributing towards the progressive cardio-vascular deterioration. A logical deduction from Marzilli's "solar system theory" might be that the most ideal form of prevention of arteriosclerosis is one that would maintain an all-round favorable microenvironment [7].

While pharmaceuticals aiming at specific targets of atherosclerosis are effective, yet short of perfection, the need for supplementary measures to harmonize the different aspects of adverse bioactivities leading to cardiovascular hazards in the form of special supplements has become obvious.

Traditional Chinese Medicine May Offer the Harmonizing Supplement

Many herbs in Traditional Chinese medicine have been widely used for the promotion of "circulatory strength," which in modern terms should mean cardiovascular health. A wide variety of proprietary herbal preparations are available in market and people in the Chinese communities have been using them either in combination with pharmaceuticals like aspirin and statins or as additional prophylactic agents for blood cholesterol control and/or vascular integrity [8,9]. We decide to choose, among the many popular herbs traditionally used for cardiovascular problems, the least number to form a simple combined formula to be used as an effective cardiovascular protective supplement. The mechanisms of action need to be explored properly while the formula would be put on an evidence-based clinical trial.

Among the many medicinal herbs *Salviae Miltiorrhizae Radix et Rhizoma* (Danshen) stands out as the most frequently used one. Its clinical value and vascular protective effects have been well recognized historically and users have great confidence for its efficacy claims [10]. Given the full respect to the ancient philosophy of using multiple herbs in clinical treatment to achieve the best results, we need to identify at least one more herb to form a combined formula for the enhanced effects of synergy. Our choice appears to be supported by a respectable herbal expert and clinician. Shi Jin-mo (1882-1968), was well known for his expertise on selecting twin combinations of herbs in the formation of simple, synergistic formulae. Shi advocated the use of *Salviae Miltiorrhizae Radix et Rhizoma* (Danshen) together with *Puerariae Lobatae Radix* (Gegen) for the promotion of good vascular circulation [11]. Many proprietary manufacturers have since made other complicated formulae, based on Shi's recommendation of Danshen and Gegen (D&G).

Danshen and Gegen together (D&G), therefore, constitute a simple herbal formula suitable for further study on biological platforms to show its pharmacological effects.

Standard water extractions of the combination of the two herbs is an essential step, followed by phytochemical analysis, which provides proper records to safe-guard uniformity in subsequent laboratory and clinical studies.

Biological Activity Studies

D&G's cardiovascular protective effects should give demonstrations of anti-inflammatory, anti-oxidative, and might be anti-coagulatory effects.

Anti-inflammatory and Anti-oxidative tests include:

- [i] Inhibition of LPS—induced nitric oxide production [12].
- [ii] Inhibition of iNOS, COX2, and NFκB protein expression using Western blot [13].
- [iii] Inhibition of inflammatory cytokines using Enzyme-linked Immunosorbent Assay (ELISA) [14].
- [iv] Inhibition of NFκB translocation using electrophoretic motility shift assay (EMSA) [13].
- [v] Inhibition of iNOS and COX2 inflammatory cytokines gene expressions using real-time PCR [14].
- [vi] Inhibition of foam cell formation using macrophages (RAW 264.7) acetylated low-density lipoprotein uptake [15].

Vascular protection tests include:

- [i] Effect of D&G on blood pressure, using spontaneous hypertensive rats [SHRs] [14].
- [ii] Effect of D&G on vasodilation using ex vivo aortic ring of rats [16].
- [iii] Effect of D&G on balloon injury-induced neointimal media thickness [16,17].
- [iv] Effect of D&G on cerebral blood flow using the middle cerebral artery occlusion rat model to evaluate neurological deficit, brain infarct, and anti-oxidative effects on brain tissues [18-21].
- [v] Effect of D&G on myocardium [22-24].

Functional genomic studies include:

- [i] Using rat cardiac myoblast cell line H9c2 exposed to different doses of D&G to check cell proliferation and cell cycles, and using cDNA microarray analysis to identify the 5 categories of genes, namely, cardiovascular, apoptosis, cell proliferation, cytokine and inflammation, and anti-oxidants.
- [ii] Variations were induced through hypoxia treatment and pretreatment with D&G.
- [iii] Tissue specific gene expression pattern, protein expression profiles, and signaling pathways involved were also studied [24-26].

Results of Biological Activity and Genomic Studies were not conclusive but offered useful information for further study.

Additional Platform

A special test platform to investigate effects of D&G on peripheral arteries is designed to support a clinical trial on peripheral vascular insufficiency.

Clinical Trials

Clinical trials were designed as randomized, double-blind, placebo-controlled clinical studies.

Trial 1: The clinical trial was designed to evaluate the efficacy and safety of *Salvia miltiorrhiza* (Danshen) and *Pueraria lobata* (Gegen) in secondary prevention. One hundred (100) eligible coronary patients were randomized to take 6 capsules of the D&G preparation (3 g) or 6 capsules of placebo capsules daily, in a double-blind and parallel fashion for 24 weeks. Brachial flow-mediated dilation (FMD) and carotid intima-media thickness (IMT) were measured using ultrasound technology [27] (Table 1).

Trial 2: Atherosclerosis commonly occurs in patients with hypertension. We hypothesized that Danshen and Gegen (D&G) have beneficial effects on a thermogenesis of high-risk hypertensive subjects. 90 patients with essential hypertension (SBP 160/90 mmHg before treatment) were studied. All subjects were randomized

to receive either oral D&G capsules 1 g/day, D&G capsules 2 g/day, or placebos, in a double-blind parallel fashion for 12 months. Brachial flow mediated dilation [endothelium-dependent dilation, FMD) and carotid intima-media thickness (IMT) were measured using ultrasound technology [28] (Table 2).

Trial 3: This clinical study was designed to demonstrate the safety and effectiveness of D&G in the prevention of atherosclerosis in postmenopausal women with early hypercholesterolemia. 165 postmenopausal women were randomized to take the D&G preparation (2 capsules) or placebo capsules (2 capsules) daily, in a double-blind and parallel fashion for 12 months. Carotid intima-media thickness (IMT) was measured using ultrasound technology. The lipid profile was also tested [29] (Table 3).

Trial 4: Patients with atherosclerosis may present with progressive difficulties in walking manifested as Intermittent Claudications.

49+49 patients with known peripheral arterial disease (PAD) were

	D&G (n=45)		Placebo (n=47)	
	Baseline	Mth 6	Baseline	Mth 6
SBP (mm Hg)	128.8 ± 15.1	128.7 ± 16.0	127.3 ± 15.3	121.2 ± 13.9
DBP (mm Hg)	78.3 ± 7.6	77.5 ± 6.8	76.5 ± 7.0	74.2 ± 8.4
Total cholesterol (mmol=L)	4.8 ± 0.9	4.6 ± 0.8*	4.7 ± 0.9	4.5 ± 0.9
LDL cholesterol (mmol=L)	2.7 ± 0.9	2.6 ± 0.7*	2.8 ± 0.8	2.5 ± 0.7*
HDL cholesterol (mmol=L)	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	1.2 ± 0.2
Triglyceride (mmol=L)	1.7 ± 1.0	1.7 ± 1.0	1.6 ± 0.8	1.7 ± 1.2
Folate (nmol=L)	39.7 ± 23.4	31.0 ± 20.9	35.0 ± 17.8	32.8 ± 17.5
B ₁₂ (pmol=L)	319.6 ± 109.1	374.7 ± 229.0	316.8 ± 124.5	350.4 ± 148.0
Homocysteine (m mol=L)	9.6 ± 2.4	10.7 ± 2.4	10.1 ± 2.7	10.9 ± 3.2
sICAM-1 (ng=mL)	482.9 ± 125.9	510.6 ± 167.8	486.2 ± 156.4	479.6 ± 154.0
sVCAM-1 (ng=mL)	820.5 ± 205.4	833.9 ± 248.0	833.6 ± 239.8	847.0 ± 244.2
E-selectin (ng=mL)	24.1 ± 11.7	24.5 ± 13.4	27.0 ± 13.7	25.0 ± 13.0
hs-CRP (mg=L) ^a	1.1 (2.5)	1.3 (1.7)	1.2 (1.8)	1.1 (2.1)
Fibrinogen (g=L)	4.3 ± 1.1	4.2 ± 0.6	4.3 ± 0.9	4.2 ± 1.1

*p<0.05 when compared with baseline, after Bonferroni adjustment. ^aData were expressed as median (interquartile range) for skewed distributed variables. D&G: Danshen and Gegen; B₁₂: Vitamin B12; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; Hs-CRP: High-sensitivity C-Reactive Protein; sICAM-1: soluble Intercellular Adhesion Molecules-1; sVCAM-1: soluble Vascular Cell Adhesion Molecules-1.

	D&G (n=45)		Placebo (n= 47)	
	Baseline	Mth 6	Baseline	Mth 6
Hyperemia (%)	899 ± 106	901 ± 97	887 ± 160	883 ± 148
FMD (%)	5.35 ± 1.21	5.91 ± 0.95*** ^b	5.33 ± 1.13	5.48 ± 0.97*
IMT (mm)	0.98 ± 0.30	0.96 ± 0.32* ^c	0.98 ± 0.34	0.98 ± 0.34
GTN (%)	15.9 ± 2.6	16.6 ± 2.4**	15.8 ± 2.6	16.1 ± 2.6

*p<0.05, **p<0.01, ***p<0.001 when compared with baseline. ^cp<0.05, ^bp<0.001 when compared with placebo group; D&G: Danshen and Gegen; FMD Flow-Mediated Dilation of brachial artery; IMT: carotid Intima-Media Thickness; GTN: Glyceryltrinitrate induced dilation of brachial artery.

$$\text{Hyperemia} = \frac{\text{Velocity} - \text{time integral}_2 \times \text{heart rate}_2}{\text{Velocity} - \text{time integral}_1 \times \text{heart rate}_1} \times 100$$

Table 1: Results of Clinical Trial 1: Patients with Coronary Insufficiency (Summary) [27].

	Placebo (n=29)		D&G (1 gm/day) (n=31)		D&G (2 gm/day) (n=30)	
	Baseline	12 months	Baseline	12 months	Baseline	12 months
SBP (mmHg)	124.4 ± 17.8	136.0 ± 24.0	132.3 ± 13.5	127.3 ± 11.0	134.9 ± 18.8	140.3 ± 14.6
DBP (mmHg)	78.7 ± 13.7	81.90 ± 12.50	88.0 ± 7.6	84.1 ± 8.3	82.9 ± 11.0	84.4 ± 7.1
TC (mmol/L)	4.8 ± 0.9	4.7 ± 1.0	5.4 ± 1.1	5.1 ± 1.0	5.2 ± 0.9	5.0 ± 0.9
TG (mmol/L)	2.0 ± 1.4	2.0 ± 1.5	2.2 ± 1.7	1.8 ± 1.3	1.7 ± 0.8	1.6 ± 0.9
HBA1C (%)	7.5 ± 2.6	7.3 ± 2.1	6.8 ± 2.0	7.0 ± 2.4	6.4 ± 1.0	6.6 ± 0.9
Creatinine (mol/L)	110.0 ± 40.6	109.4 ± 50.3	127.9 ± 58.0	130.0 ± 64.0	110.1 ± 49.3	110.4 ± 83.2
Reactive hyperemia (%)	543 ± 192	485 ± 105	615 ± 208	606 ± 154	535 ± 206	505 ± 133
FMD (%)	5.5 ± 1.3	6.2 ± 1.1*	5.4 ± 1.9	7.0 ± 2.1**	5.0 ± 1.5	6.2 ± 1.6**
NTG (%)	15.2 ± 2.5	15.9 ± 3.0	18.3 ± 11.9	16.7 ± 3.3	15.4 ± 3.3	16.1 ± 3.1
IMT (mm)	0.80 ± 0.18	0.81 ± 0.17	0.79 ± 0.20	0.74 ± 0.16**	0.85 ± 0.16	0.82 ± 0.15**

Compared with baseline within group: *P=0.001; **P<0.0001. SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; TC: Total Cholesterol; TG: Triglyceride; HBA1-C: Hemoglobin A1-C; FMD: Flow-Mediated Dilatation; NTG: Nitroglycerin-induced dilatation; IMT: Intima-Media Thickness; Reactive hyperemia = (velocity - time integral₂ × heart rate₂) / (velocity-time integral₁ × heart rate₁) × 100^o.

Table 2: Results of Clinical Trial 2: Patients with Hypertension (Summary) [28].

Percent changes of IMT from baseline stratified by age			Percent changes of lipid profile from baseline after 12- month treatment				
Group	6-month (v3)	12-month (v5)	Group	LDL	HDL	TG	CHOL
D&G (n=85)	-0.57%	-1.52%	D&G	-6.92%	-2.88%	4.33%	-5.85%
<55 years (n=31)	0.82%	-1.07%	SD	14.24	15.12	43.41	9.94
>55 years (n=54)	-1.37%	-1.78%	Placebo	-3.21%	-3.71%	9.32%	-3.42%
Placebo (n=38)	-0.03%	-1.13%	SD	14.1	13.5	43.71	9.29
<55 years (n=38)	-0.04%	-1.73%	P value ^a	0.159	0.682	0.283	0.165
>55 years (n=42)	-0.01%	-0.59%					
p-value	0.028	0.181					
<55 years	0.282	0.557					
>55 years	0.069	0.197					

D&G: Danshen and Gegen; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; TG: Triglyceride; CHOL: Cholesterol; IMT: Carotid Intema-Media Thickness, ^aMann-Whitney test between the two groups.

Table 3: Results of Clinical Trial 3: Post-menopausal Patients with Borderline Hypercholesteraemia (Summary) [29].

Patients	Twin Formula Group	Control Group	P Value
Age	66.2 ± 9.4	68.5 ± 7.5	0.187
Index (kg/m ²)	24.8 ± 4.2	24.2 ± 3.5	0.420
Systolic BP	144.7 ± 16.9	150.5 ± 21.5	0.142
Diastolic BP	71.9 ± 10.4	73.6 ± 11.9	0.460
IMT (mm)	1.159 ± 0.624	1.083 ± 0.343	0.456
Max. Walking Distance	↑21.8%	↑7.2%	0.0499

IMT: Carotid Intima-media Thickness

Table 4: Results of Clinical Trial 4: Patients with Peripheral Arterial Disease (Summary) [30].

treated with the twin formula or placebo for 24 weeks. Assessment using ultrasonography showed thinning down of the carotid intima (2.67%) only in the treatment group. Maximal walking distance also increased by 21.8% in the treatment group compared with 7.2% in the placebo group ($p=0.499$) [30,31] (Table 4).

Summation of Laboratory and Clinical Results

The comprehensive approach to the creation of an evidence based simple herbal formula for cardiovascular health has taken nearly twenty years to reach the present state of maturity. In the laboratory, through a variety of in vitro platforms, we have demonstrated the multiple biological activities of D&G, namely, anti-inflammation, anti-oxidation, and anti-foam cell formation in the vascular endothelium. The different mechanistic channels leading to these favorable cardiovascular protective events have also been demonstrated in the extensive cytokine studies. Through a variety of animal studies, D&G has been demonstrated to provide control on hypertension, atherosclerosis, and smooth muscle activity leading to vasodilatation. D&G appears to be both cardiac protective and vascular protective.

In the genomic study, the genomic and proteomic signatures of D&G treated samples either in vitro or in vivo were investigated using cDNA microarray and iTRAQ labeled LC/MS/MS techniques, respectively, which provide better understanding of the mechanism of action of Danshen-Gegen. Our future challenge is to integrate the information to give a more complete picture of the interaction between the herbal formula and the living organisms.

Four randomized controlled clinical trials have been conducted on four groups of patients with different pathological orientations using the same surrogate markers. The clinical results have been promising and safety have been confirmed.

Discussion

Herbal medicine started in the old days as “Folk-practices”. The over two thousand years of practice has offered no scientific explanations apart from clinical observations. Clear indications for clinical applications have not been given. Instead, the acquisition of general well-being through the harmonization of existing adversities has been the aim. Since cardiovascular problems must have been affecting all human beings ever since history started, rich experiences should have accumulated to facilitate researchers to select the ideal herbal items for modern studies.

When we started this research project in 2003, we carefully selected two widely used medicinal herbs in Traditional Chinese Medicine to form the twin formula, with the aim of giving additional support to the prevention of cardiovascular hazards. We assumed that the current orthodox preventive therapies might be deficient and might not be effective for some patients because the specific target orientations might be too narrow and unable to give comprehensive preventive effects. We assumed that the twin formula could be taking care of multiple problems leading to the cardiovascular hazards [32,33], through combined anti-inflammation, anti-oxidation, vascular relaxation and cardio protective effects.

While we started planning the clinical trials for the efficacy of the twin-formula, we performed a series of laboratory experiments with the aim of showing multiple bioactivities of the formula, which should indicate its pharmacological effects.

The bioactivity studies included [i] Anti-inflammatory and anti-oxidative tests [25,26]; [ii] Vascular protection tests [27,28]; [iii] Myocardial effects [29,30]; and Genomic studies. Results of the bioactivity studies allowed us to speculate that the twin formula should be able to create a favorable microenvironment with good control of inflammatory activities in the promotion of an effective prevention program along a holistic direction [34].

We chose four areas of major concern related to cardiovascular hazards viz. coronary obstruction; hypertension, perimenopausal stress; and peripheral arterial disease, for separate clinical trials under randomized control directions. The objectives were uniform and the major outcome measure was Intimal Medium Thickness (IMT) which is a well-accepted surrogate marker for the study of arterial patency. Uniformity of trial data with comparable value could be expected. The blood lipid profile was selected as a supportive outcome measure.

The individual clinical trials all showed positive effects using the twin formula. The pooled data of the four trials are being analyzed.

Future Work

In the completed trials, patients on specific anti-coagulants like Warfarin were excluded for fear of interference and safety. To continue the proper development of the twin formula, whether its application would interfere with the usual anti-coagulant therapies needs to be carefully worked out [35,36]. In the strict sense, D&G could be considered an effective supplement. Nevertheless, the series of carefully planned biological tests and randomized controlled trials should have given the impression of a “Drug”. D&G has been utilized as a gross herbal extract concentrate. Its efficacy could be further enhanced through phytochemical procedures of extract separations to screen out the chemical sections that are of high potency [37].

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Author Contributions Statement

Each author has been responsible for one clinical trial. Corresponding author initiated the herbal supplement project and compiled the whole text. KS Woo supervised the coronary and hypertension project; Kwok Chi Yui Timothy supervised the menopausal project; and Yan Ping Yen Bryan supervised the peripheral arterial project.

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