Combination of atorvastatin or hydrochlorothiazide/amlodipine with *Salvia miltiorrhiza* (Danshen) and *Pueraria lobata* (Gegen) for atherosclerosis, hyperlipidaemia, and hypertension: a preclinical in vivo study (abridged secondary publication)

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KEY MESSAGES

1. Water extract of Danshen and Gegen (DG) was found to be a potent alternative medicine for atherosclerosis, hyperlipidaemia, and hypertension.
2. Combination of DG and atorvastatin could prevent atherosclerosis and reduce hyperlipidaemia.
3. Combination of DG and hydrochlorothiazide/amlodipine could ameliorate hypertension.
4. DG could help to attenuate adverse effects resulting from atorvastatin or hydrochlorothiazide/amlodipine medication.

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Introduction

Most cardiovascular diseases are caused by atherosclerosis secondary to build-up of fatty streaks on the innermost layer. Risk factors include hypertension and hyperlipidaemia. Medications for cardiovascular diseases may have adverse effects. Atorvastatin (AS) is a lipid-lowering drug that has been reported to induce liver problems (indicated by elevated aspartate aminotransferase and alanine transaminase levels) and damage to skeletal muscles (indicated by elevated creatine kinase level). Medications for hypertension are mainly diuretic drugs (ie, hydrochlorothiazide [HCT]) and calcium channel blocker (ie, amlodipine [ADP]). Nonetheless, HCT may cause hypokalaemia, hyperuricaemia, hyperglycaemia, and hyperlipidaemia. ADP may induce peripheral oedema, fatigue, dizziness, nausea, palpitations, and abdominal pain. A herbal formula containing water extract of Danshen and Gegen (DG) [7:3, w/w] has pleiotropic beneficial effects on coronary patients and menopausal women with hypocholesteraemia.[1,2] DG is considered safe for clinical use as no adverse effect has been reported in clinical trials. DG in combination with AS or HCT/ADP may yield better treatment effects while reducing adverse effects. This study aimed to verify this hypothesis in animal studies.

Methods

AS, HCT, and ADP were purchased from Pharmasolution (HK). The dried root and rhizome of *Salvia miltiorrhiza* Bunge (Danshen) and the dried root of *Pueraria lobata* (Willd) Ohwi (Gegen) were purchased from a local supplier. The water extract of DG (7:3, w/w) was prepared by boiling with water twice for 1 hour and 0.5 hours, respectively. The water extract was concentrated and lyophilised into powder. Ultra-performance liquid chromatography analysis of DG identified five hydrophilic compounds, including salvianolic acid B (4.367%), puerarin (2.193%), daidzin (0.554%), daidzein (0.159%), and protocatechuic aldehyde (0.047%).[3]

Male Sprague-Dawley (SD) rats, Wistar-Kyoto (WK) rats, spontaneously hypertensive (SH) rats, and C56BL/6 mice were supplied by the Laboratory Animal Service Centre of The Chinese University of Hong Kong. All protocols of animal research have been approved by the Animal Research Ethics Committee of the university. Animals were kept in a 12-hour circadian cycle and were supplied with food and water *ad libitum*.

For the restenosis study, the carotid artery of SD rats was induced by balloon injury.[4] Those rats then received drug treatments for 14 days. For the hyperlipidaemia study, C56BL/6 mice were given

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a high-fat diet (22% fat and 0.15% cholesterol) for 8 weeks before drug treatments and for another 8 weeks with a high-fat diet feeding concomitantly. For the hypertension study, systolic blood pressure (SBP) of WK and SH rats was measured weekly from 6 weeks old with tail cuffs (CODA system, Kent Scientific, USA). Drug treatments were given to those rats from 9 weeks old for 16 weeks.

Plasma was collected and biochemically assayed for levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), glucose, creatinine, albumin, urea nitrogen, uric acid, aspartate aminotransferase, alanine transaminase, and creatine kinase using commercial kits. Insulin level was determined using ELISA kits.

Carotid arteries of SD rats and livers of C57BL/6 mice were fixed with 4% paraformaldehyde and were sectioned at 5 μM. Sectioned carotid arteries were immunohistochemically stained with primary antibody against smooth muscle cell α-actin. Sectioned livers were stained with haematoxylin and eosin. Liver fat vacuoles were quantified with ImageJ computer software.

Results

After balloon injury–induced intima/media thickening of carotid arteries, an induced layer known as neointima was clearly shown in the innermost intima layer of the arterial wall (Fig 1). The neointima was mainly composed of massive proliferation of underlying vascular smooth muscle cells as vascular smooth muscle cell α-actin was seen in the neointima (Fig 1). DG 300 mg/kg reduced neointima/media ratio by 10.99%±1.72%, compared with control without medication. AS 40 mg/kg showed no treatment effect on intima/media thickening, whereas AS 80 mg/kg reduced neointima/media ratio by 1.14%±0.14%, compared with control without medication. Combination of AS with DG resulted in stronger treatment effect on reducing neointima/media ratio than did AS alone. AS 40 mg/kg plus DG 300 mg/kg or AS 80 mg/kg plus DG 300 mg/kg reduced neointima/media ratios by 12.81%±1.73% or 36.14%±3.86% more than did AS 40 mg/kg or AS 80 mg/kg alone, respectively.

The effects of combined medication of DG and AS on the lipid profile of C57BL/6 mice have been published by us.[5] Compared with normal diet, high-fat diet caused an increase in plasma TC and TG levels. Single medications of DG 600 mg/kg, AS 8 mg/kg, and AS 16 mg/kg decreased plasma TC and TG levels, compared with controls with fed high-fat diet only. Combined medications (AS 8 mg/kg plus DG 600 mg/kg and AS 16 mg/kg plus DG 600 mg/kg) had stronger effects on decreasing plasma TC and TG levels than did single medications of AS 8 mg/kg and AS 16 mg/kg.

Plasma LDL-C and HDL-C levels were higher in mice fed with high-fat diet than those fed with normal diet. Single medications (DG 600 mg/kg, AS 8 mg/kg, and AS 16 mg/kg) and combined medications (AS 8 mg/kg plus DG 600 mg/kg and AS 16 mg/kg plus DG 600 mg/kg) reduced plasma LDL-C level, compared with controls fed with high-fat diet only. Combined medications reduced plasma LDL-C level more than did single medications of AS 8 mg/kg and AS 16 mg/kg. Plasma HDL-C level slightly increased in groups of AS 8 mg/kg, AS 16 mg/kg, and AS mg/kg plus DG 600 mg/kg, compared with the high-fat diet group, but decreased in groups of DG 600 mg/kg and AS 16 mg/kg plus DG 600 mg/kg.

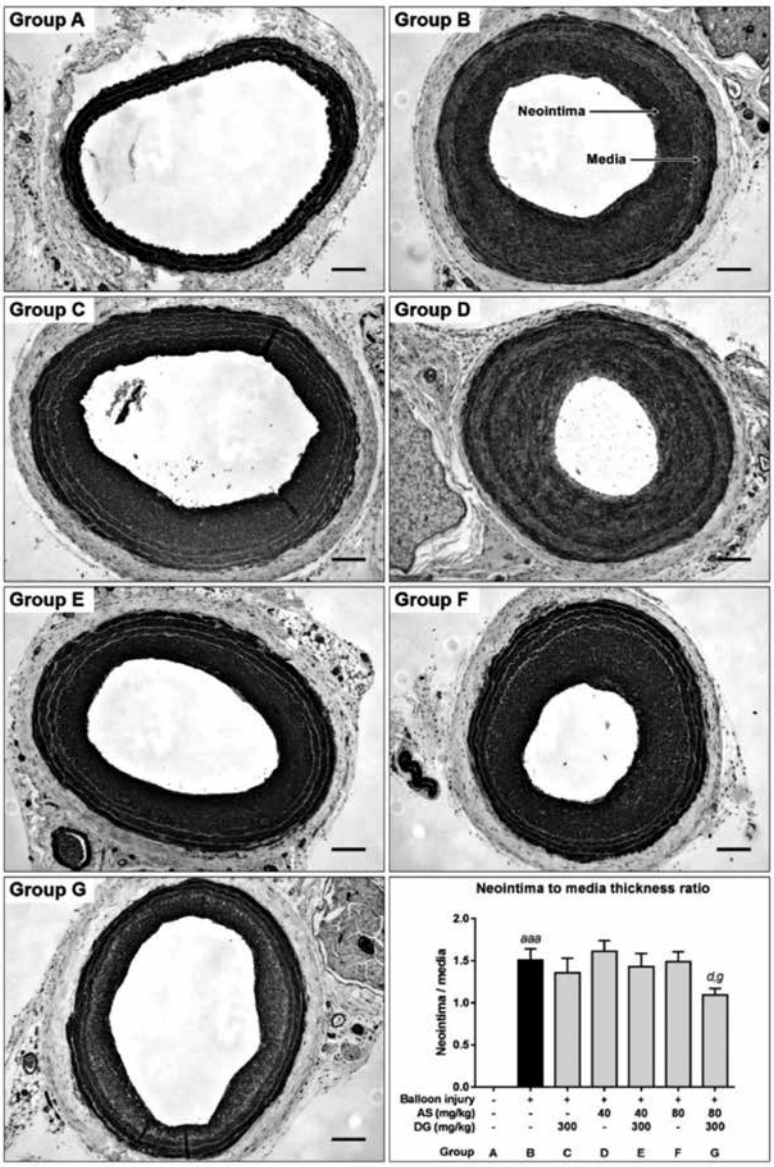

FIG 1. Micrographs showing carotid arteries of Sprague-Dawley rats with/without balloon injury surgery. Sectioned carotid arteries are stained with immunohistochemical staining with antibody against vascular smooth muscle cell α-actin (×50, scale bar = 100 μm).

Rats fed pharmacologically high doses of AS 320 mg/kg had approximately three-fold elevation in aspartate aminotransferase and alanine transaminase levels and more than four-fold elevation in creatine kinase level, compared with control without medication (Fig 2). However, rats fed AS 320 mg/kg plus DG 600 mg/kg showed lower aspartate aminotransferase, alanine transaminase, and creatine kinase levels than those fed AS alone.

At baseline, SH rats exhibited generally higher SBP than WK rats (162±2 to 177±7 mmHg vs 137±4 mmHg, Fig 2). Drug treatments were given the next day after SBP measurement in the third week. Medication with HCT 15.6 mg/kg plus ADP 6 mg/kg for 1 week significantly reduced SBP of SH rats (108±9 mmHg). Half doses of HCT and ADP in combination with DG also reduced SBP. Reduction in SBP persisted in a treatment course of 16 weeks with HCT 15.6 mg/kg plus ADP 6 mg/kg (99±5 to 123±8 mmHg), HCT 7.8 mg/kg plus ADP 3 mg/kg plus DG 600 mg/kg (101±10 to 135±13 mmHg), and HCT 7.8 mg/kg plus ADP 3 mg/kg plus DG 1200 mg/kg (101±7 to 128±11 mmHg). SBP of WK rats throughout the treatment course was 133±5 to 145±5 mmHg.

Untreated SH rats had lower TC level than WK rats (28.52±1.45 mg/dL vs 49.15±2.12 mg/dL, Fig 3). TC level was elevated in SH rats receiving HCT 15.6 mg/kg plus ADP 6 mg/kg for 16 weeks (30.97±2.65 mg/dL), compared with control. Half doses of HCT and ADP in combination with DG 600 or 1200 mg/kg resulted in decreased TC level to 19.51±1.56 mg/dL and 18.8±1.69 mg/dL, respectively. Similarly, TG level was lower in untreated SH rats than in WK rats (8.2±0.48mg/dL vs 12.88±1.12 mg/dL, Fig. 3). Medication with HCT 15.6 mg/kg plus ADP 6 mg/kg elevated TG level (9.56±1.04 mg/dL) in SH rats, compared with control. Half doses of HCT and ADP in combination with DG 600 or 1200 mg/kg resulted in decreased TG level to 7.21±1.04 mg/dL and 7.11±0.92 mg/dL, respectively.

LDL-C and HDL-C levels in SH rats were lower than in WK rats (31.55±3.24 mg/dL vs 79.16±11.05 mg/dL and 30.74±0.84 mg/dL vs 58.3±3.34 mg/dL, respectively, Fig 3). LDL-C and HDL-C levels were elevated in SH rats receiving HCT 15.6 mg/kg plus ADP 6 mg/kg for 16 weeks (44.65±3.66 mg/dL and 31.04±0.49 mg/dL, respectively). Half doses of HCT and ADP in combination with DG 600 or 1200 mg/kg decreased LDL-C level to 40.48±3.66 mg/dL and 32.01±3.7 mg/dL, respectively, and HDL-C level to 29.9±1.37 mg/dL and 28.4±1 mg/dL, respectively.

Glucose level was lower in untreated SH rats than in WK rats (118.4±4.29 mg/dL vs 135.9±3.74 mg/dL, Fig 3). HCT 15.6 mg/kg plus ADP 6 mg/kg elevated glucose level to 129.3±6.7 mg/dL, compared with control. Half doses of HCT and ADP in combination with DG 600 or 1200 mg/kg elevated glucose level to 132.6±5.78 mg/dL and 133.5±3.49 mg/dL, respectively, which were greater than that in full doses of HCT and ADP.

Insulin level in untreated SH rats and

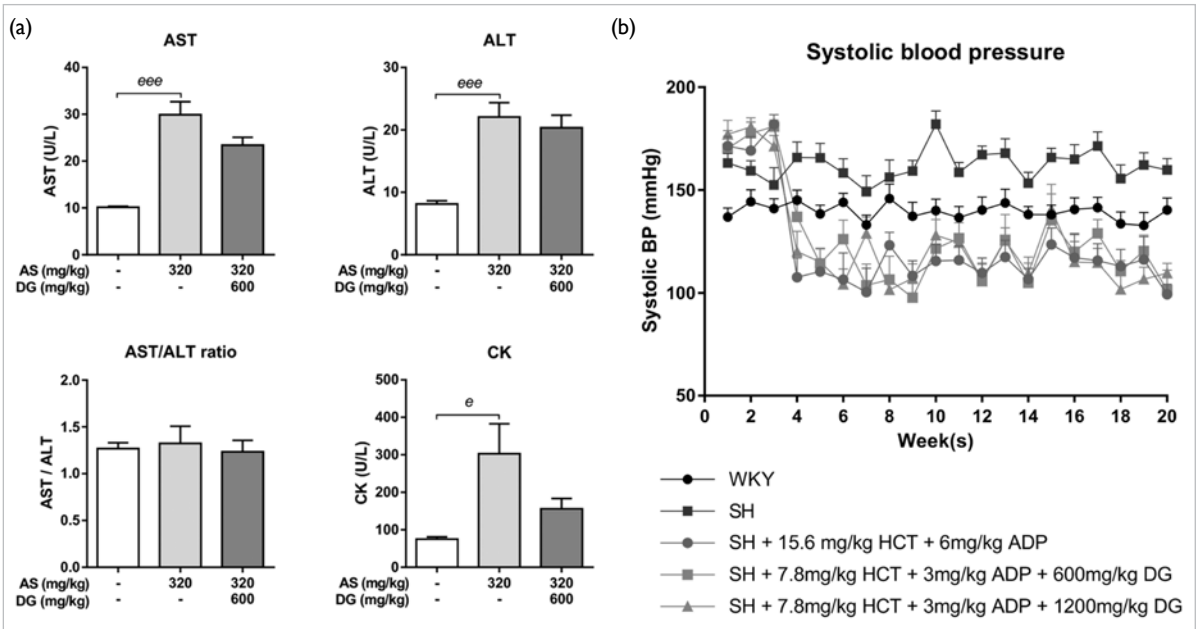

FIG 2. (a) Blood parameters of aspartate aminotransferase (AST), alanine transaminase (ALT), and creatine kinase (CK) levels of Sprague-Dawley rats fed with high doses of atorvastatin (AS), Danshen and Gegen (DG), or both, and (b) blood pressure of spontaneously hypertensive (SH) rats and Wistar-Kyoto (WK) rats.

WK rats was comparable (3.75±0.79 ng/mL vs 3.87±0.79 ng/mL, Fig 3). HCT 15.6 mg/kg plus ADP 6 mg/kg reduced insulin level to 1.9±0.85 ng/mL, compared with control. Half doses of HCT and ADP in combination of DG 600 or 1200 mg/kg resulted in elevated insulin level to 4.06±0.26 ng/mL and 6.09±0.57 ng/mL, respectively.

Creatinine level in untreated SH rats and WK rats was similar (0.59±0.06 mg/dL vs 0.6±0.02 mg/dL, Fig 3). HCT 15.6 mg/kg plus ADP 6 mg/kg increased creatinine level to 0.83±0.15 ng/mL, compared with control. However, half doses of HCT and ADP in combination of DG 1200 mg/kg reduced creatinine level to 0.5±0.04 mg/dL.

Untreated SH rats had higher albumin level than WK rats (8.21±0.32 g/dL vs 7.73±0.25 g/dL, Fig 3). All treatment groups involving HCT plus ADP or HCT plus ADP plus DG reduced albumin level in SH rats to 7.39±0.14 to 7.94±0.28 g/dL. Half dose of HCT plus ADP combining with DG reduced albumin level lower than did full dose of HCT plus ADP.

Untreated SH rats had higher urea nitrogen level than WK rats (15.77±0.84 mg/dL vs 13.83±0.74 mg/dL, Fig 3). HCT 15.6 mg/kg plus ADP 6 mg/kg increased urea nitrogen level to 17.57±0.29 mg/dL, compared with control. Half doses of HCT/ADP with DG reduced levels of urea nitrogen to 17.13±0.28 mg/dL.

Uric acid level in untreated SH rats and WK rats was similar (2.67±0.16 mg/dL vs 2.7±0.14 mg/dL, Fig 3). HCT 15.6 mg/kg plus ADP 6 mg/kg elevated uric acid level to 4±0.05 mg/dL, compared with control. Uric acid level was decreased only in half dose of HCT plus ADP with DG 1200 mg/kg to 3.81±0.03 mg/dL, compared with full dose of HCT and ADP.

Discussion

AS had a potential beneficial effect on vascular thickening, but a high dose was needed for the effect to be manifested. DG 300 mg/kg had an intima media thickness–reducing effect stronger than that of AS 80 mg/kg. AS 80 mg/kg plus DG 300 mg/kg exhibited the most remarkable intima media thickness reduction. AS plus DG (at different doses of AS 10-80 mg/kg) showed generally stronger effect on intima media thickness reduction than single medication alone. Interestingly, the reduction effect of AS 80 mg/kg plus DG 300 mg/kg (27.34%) was higher than the summation effect of AS 80 mg/kg alone (1.12%) and DG 300 mg/kg alone (9.85%), thereby suggesting a synergistic interaction. Lower doses of AS plus DG might achieve a treatment outcome better than higher doses of AS alone in terms of intima media thickness reduction. DG may be used in lieu of AS, because DG 300 mg/kg exerted a stronger effect than AS 80 mg/kg.

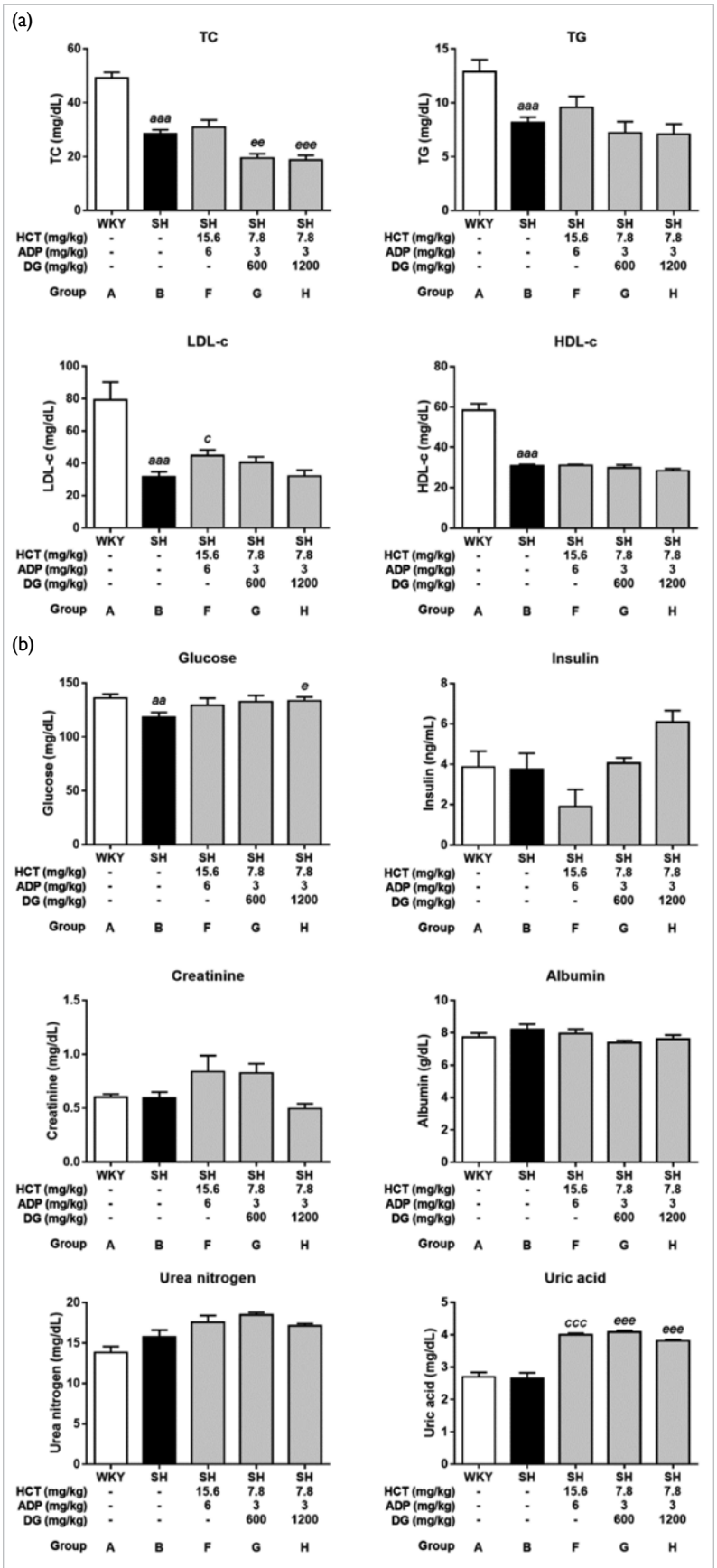

FIG 3. (a) Lipid profile of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) as well as (b) blood parameters of glucose, insulin, creatinine, albumin, urea nitrogen, and uric acid of spontaneously hypertensive (SH) rats and Wistar-Kyoto (WK) rats.

AS plus DG resulted in potentiated hypolipidaemic effects. AS plus DG exhibited stronger hypolipidaemic effects than did AS alone. Compared with AS 16 mg/kg, AS 8 mg/kg plus DG 600 mg/kg achieved stronger effects on reducing levels of TC and TG but not LDL-C and HDL-C. This observation suggested that half dose of AS (8 mg/kg) taken together with DG (600 mg/kg) may achieve a comparable hypolipidaemic effect to AS 16 mg/kg alone.

DG might be able to attenuate toxic effects caused by AS. When high doses of AS plus DG were given to SD rats, levels of aspartate aminotransferase, alanine transaminase, and creatine kinase decreased, compared with AS alone. These results suggested that DG acted to attenuate the toxicity caused by AS on the liver and skeletal muscle. Plasma examinations of aspartate aminotransferase, alanine transaminase, and creatine kinase levels showed that combined use of AS and DG was safe and beneficial.

In SH rats, DG appeared to be able to potentiate an anti-hypertensive effect of HCT and ADP, because DG 1200 mg/kg plus half effective doses of HCT and ADP was shown to reduce SBP in a magnitude comparable to full effective doses of HCT and ADP.

Medications with HCT and ADP increased the lipid profile of SH rats as evidenced in the elevation of TG, TC, LDL-C, and HDL-C. This hyperlipidaemia adverse effect significantly decreased in half doses of HCT and ADP in combination with DG in a dose-dependent manner. Reduction of HCT and ADP doses in half may contribute to the lowered lipid profile. DG played a pivotal role in lowering the lipid profile of SH rats receiving medication, as DG possesses hypolipidaemic effect on high-fat diet induced-hyperlipidaemia.

In blood parameters of elevated levels of creatinine, albumin, urea nitrogen, and uric acid, DG plus half doses of HCT and ADP was able to attenuate their elevations shown in full dose of HCT and ADP. Whether this was due to the addition of DG or reduction of doses of HCT and ADP was not known. DG was unable to lower glucose and conversely cause the glucose level to rise. Insulin level was found to increase following the increasing pattern of glucose. Increase in insulin level correlated with DG medication in combination with half dose of HCT and ADP.

Conclusion

DG combined with AS or HCT and ADP resulted in better treatment effects on preventing vascular wall thickening, reducing hyperlipidaemia, and ameliorating hypertension. In addition, DG could also attenuate adverse effects caused by AS or HCT and ADP medication, including elevations in levels of aspartate aminotransferase, alanine transaminase, creatine kinase, as well as TC, TG, LDL-C, HDL-C, creatinine, albumin, urea nitrogen, and uric acid.

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Disclosure

The results of this research have been previously published in:

1. Cheung DW, Koon CM, Wong PH, et al. Evaluating efficacy and safety of combination medication of atorvastatin and a herbal formula containing *Salvia miltiorrhiza* and *Pueraria lobata* on hyperlipidemia. Phytother Res 2017;31:1579-89.

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