Effect of combined use of *Fructus Schisandrae* and statin on high-fat-diet-induced metabolic syndrome in rats

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KEY MESSAGES

1. The combined use of *Fructus Schisandrae* aqueous extract (FSE) and atorvastatin (AS) exerts no further synergistic effect on diet-induced non-alcoholic fatty liver disease in rats, compared with rats given AS alone.
2. The use of FSE can protect high-fat-fed rats from AS-induced liver toxicity.
3. Preliminary pharmacokinetic studies suggest that FSE can enhance plasma metabolites of AS but reduce the liver metabolites of AS.

Hong Kong Med J 2016;22(Suppl 6):S24-7
HHSRF project number: 09100241

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Introduction

3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors, also known as statins, are the best-selling class of prescription drug in the world. Statins inhibit the reduction of 3-hydroxy-3-methyl-glutaryl-coenzyme A to mevalonic acid, thus reducing cholesterol production. They are beneficial to patients of different ages and genders with moderate and high cardiovascular disease risk; nonetheless no drug is without potential adverse effects.[1]

Fructus Schisandrae, the fruit of *Schisandrae chinensis*, is a traditional Chinese herb believed to be a liver tonic. Extracts isolated from *Fructus Schisandrae* can alleviate hepatic cholesterol and triglyceride levels, and attenuate the development of fatty liver in rodents fed a high-fat diet.[2] Preliminary pharmacokinetic study has been performed to determine whether the combined use of *Fructus Schisandrae* aqueous extract (FSE) and atorvastatin (AS) affects the plasma or liver concentration of AS and/or its metabolites in high-fat-fed rats.

The present study, conducted from January 2012 to December 2013, aimed to determine whether the combined use of FSE and AS would be hepatoprotective by (1) improving high-fat diet-induced non-alcoholic fatty liver, and (2) reducing the side effects of taking statin alone, including increased incidence of elevated liver enzymes and liver toxicity.

Methods

Fructus Schisandrae and statin

Fructus Schisandrae was purchased from 致信 in Guangzhou, China. Chemical authentication was performed in accordance with the Chinese Pharmacopoeia 2010. Extraction of *Fructus Schisandrae* was by boiling in water. The aqueous extracts were combined and filtered. Filtrate was concentrated under reduced pressure at 60°C. The concentrated extract was frozen and lyophilised to dryness. The percentage yield was 48.7% w/w. Herbarium voucher specimen of *Fructus Schisandrae* was deposited at the museum of the Institute of Chinese Medicine at the Chinese University of Hong Kong (No.: 2012-3357). In addition, AS calcium (purity 99%) was purchased from ZheJiang Dankong Pharmaceutical.

Animal study 1

All experiments were carried out in accordance with the guidelines approved by the Animal Research Ethics Committee at the Chinese University of Hong Kong (No.: 11/004/MIS-5). Male Sprague Dawley rats weighing 200-210 g were purchased from the Laboratory Animal Services Centre of the Chinese University of Hong Kong.

Study 1 was performed to determine the combined effect of FSE and AS on diet-induced non-alcoholic fatty liver disease. All rats were housed in standard cages at a constant temperature of 21°C with a 12-hour light-dark cycle. Animals were randomly divided into three groups (n=8-10) and fed a high-fat diet (21% fat and 0.15% cholesterol) and one of three supplements: (1) distilled water, (2) 0.3% AS, or (3) 0.3% AS + 0.45% FSE.

After 8 weeks, all rats were sacrificed after a 16-hour overnight fast. Animals were anaesthetised

with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Blood was withdrawn by cardiac puncture. Plasma was collected and the livers excised and stored at -80°C until analysis. Plasma aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) activities were determined by a kinetic method using ALT/SGPT Liqui-UV Test and AST/SGOT Liqui-UV Test (Stanbio Laboratory, TX, USA). Total liver lipids were determined gravimetrically after extraction by the method of Bligh and Dyer.[3]

For liver glutathione peroxidise measurement, liver tissues were rinsed with PBS, pH 7.4 and homogenised in 5-10 mL cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM dithiothreitol). Homogenates were centrifuged at 10 000 g for 15 minutes at 4°C. Supernatant was removed and assayed for GPx activity using a commercially available kit (Cayman Chemical, USA) according to the manufacturer's instructions.

Mitochondrial permeability transition was assessed using mitochondrial fractions from liver samples prepared according to the commercially available kit (Millipore, USA). The mitochondrial fractions were incubated with buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES (pH 7.2), 5 mM succinate (freshly prepared), and 5 mM rotenone (freshly prepared), and were mixed with cyclosporine A (5 μM in 0.5% ethanol final concentration). The mixtures were incubated for 5 minutes at 30°C, followed by calcium chloride solution (1 μM final concentration) at 30°C for another 5 minutes. Absorbance was measured at 520 nm. The swelling reaction was started by addition of K_3PO_4 (0.5 mM, pH 7.2), and absorbance was read every 2 minutes for 30 minutes at 30°C. The extent of mitochondrial swelling was assessed by measuring the total area under the swelling reaction curve and was expressed as % relative to control group.

Reactive oxidant species were measured from mitochondrial fraction (50 μg protein/ml) using DCFDA solution (Invitrogen, USA). Fluorescence intensity was measured every 5 minutes for 60 minutes by Biorad Fluostar Optima 413-101 BMG Fluorescent Microplate Reader at 485 nm excitation and 520 nm emission. Levels were expressed as % relative to control group.

Animal study 2

To study the pharmacokinetics between AS and FSE, high-fat-fed Sprague Dawley rats were given distilled water or FSE for 1 week, followed by one dose of AS or distilled water on the day of blood and liver collection. FSE or AS were given intragastrically (instead of diet supplementation) to provide a more accurate dose. The dose of AS and FSE was calculated so that it was equivalent to the AS diet supplementation dose of 0.3% and FSE supplementation dose of 0.45%. The animals were randomly divided into three groups (n=4-6) and fed a high-fat diet and one of the three treatments intragastrically: (1) 200 mg/kg AS, (2) 0.6 g/kg FSE, or (3) 200 mg/kg AS + 0.6g/kg FSE.

To measure drug/herb concentration within the blood, blood samples (200 μl) were collected at time 0 (control), 0.5, 1, 2, 4, 6, and 8 hours. Blood samples were centrifuged and plasma samples (100 μl) were stored at -80°C until LC-MS analysis. For measurement of the liver herb/drug concentration, livers were collected at time 0 (control), 0.5, 1, and 2 hours. Liver samples were quickly snap frozen with liquid nitrogen and stored at -80°C until analysis.

Plasma and liver concentration of AS, ortho-hydroxy AS, para-hydroxy AS, schiszandrin was measured using an electrospray ionisation LC-MS, using hesperetin as the internal standard. Plasma samples containing herb/drug were prepared as previously described.[4] Extracted samples were sent for LC-MS analysis. Liver samples were homogenised in saline, followed by mixing with acetonitrile, vortexing, and sonication (5 minutes). Samples were centrifuged at 3000 rpm (5 minutes) and supernatant was sent for LC-MS analysis. LC-MS was conducted according to the conditions previously described, using AS, AS metabolites: ortho-hydroxy AS, para-hydroxy AS, and FSE markers: schizandrin as the markers.

Statistical analysis

The differences between treatment and control groups were compared using one-way analysis of variance followed by the post-hoc Bonferroni multiple comparison test. All statistical analyses were performed using GraphPad Prism Version 5.0c (GraphPad, USA). A P value of <0.05 was considered statistically significant.

Results

Figure 1 shows the effect of 0.3% AS or combined use of 0.3% AS and 0.45% FSE on liver weight in high-fat-fed rats. AS resulted in a significant reduction in liver size in rats fed a high-fat diet. Nonetheless, the combined use of 0.3% AS and 0.45% FSE did not significantly augment this effect on liver weight, compared with AS alone. There was no significant difference in total liver lipid levels between rats with 0.3% AS and rats with 0.3% AS + 0.45% FSE (data not shown). These results indicated that the combined use of FSE with AS exerted no further beneficial effect on diet-induced non-alcoholic fatty liver disease (NAFLD), compared with AS alone.

Although FSE appeared to exert no synergistic effect with AS on diet-induced NAFLD, FSE in combination with AS exerted significant protective effects on AS-induced liver toxicity by: (1) reducing liver enzyme (ALT and AST) levels, (2) improving

liver glutathione level, (3) reducing liver reactive oxidative species, and (4) a trend of reducing calcium-induced membrane permeability transition within the liver (data not shown).

To determine whether FSE would affect the plasma and liver concentrations of AS and its metabolites during their combined use, a preliminary pharmacokinetic study was performed. Figure 2 shows the effect of the combined use of FSE and AS on plasma AS and its metabolite concentrations. The combined use of 0.3% AS and 0.45% FSE induced a significant increase in plasma AS concentration (Fig 2a). The plasma concentrations of ortho-hydroxy AS and para-hydroxy AS, two of the metabolites from AS (Figs 2b and 2c), were also increased. There were no detectable levels of schizandrin in the plasma of any group.

Figure 3 shows the effect of the combined use of AS and FSE on the liver AS and its metabolites. The combined use of 0.3% AS and 0.45% FSE significantly reduced liver AS and its metabolites, compared with 0.3% AS alone. The combined use of 0.3% AS and 0.45% FSE significantly reduced the level of liver schizandrin, compared with 0.45% FSE alone.

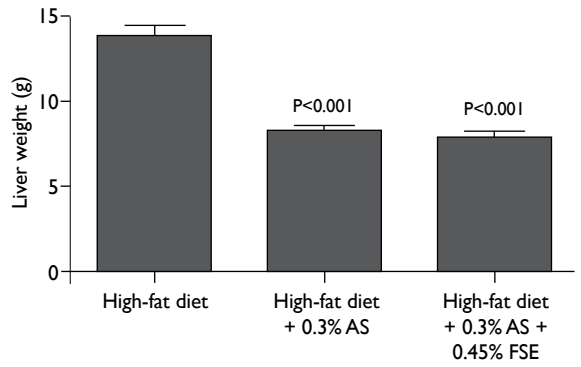

FIG 1. Effect of atorvastatin (AS) with or without Fructus Schisandrae aqueous extract (FSE) on liver size in high-fat-fed rats

Discussion

Combined use of FSE and AS exerted no further synergistic effect on diet-induced NAFLD in rats, compared with AS alone. Nonetheless, FSE could exert a potent protective effect on statin-induced toxicity. One possibility for the absence of a synergistic effect of FSE and AS on diet-induced NAFLD could be the very high dose use of AS. The use of AS alone may have optimal beneficial effects and its combined use with FSE may exert no further beneficial effect on diet-induced NAFLD. In addition, there are concerns about the adverse effects of statins on the liver. Clinically, a mild-to-moderate increase in liver transaminases is a common side effect that is regularly monitored in patients prescribed with statin. The ability of dietary FSE supplementation to exert a potent hepatoprotective effect on AS-induced liver toxicity offers great potential.

The preliminary pharmacokinetic studies of AS and FSE suggested that their combined use may enhance the concentrations of AS and its metabolites, ortho-hydroxy AS and para-hydroxy AS within the plasma. The concentrations of AS and its metabolites were reduced in the livers of rats given a combination of FSE and AS, compared with those given AS alone. AS requires CYP3A4 for metabolism. Our results indicated that FSE could enhance AS concentration within the plasma. Although the exact mechanism is unknown, it is possible that schizandrin, which requires CYP3A4 for metabolism, competes with AS

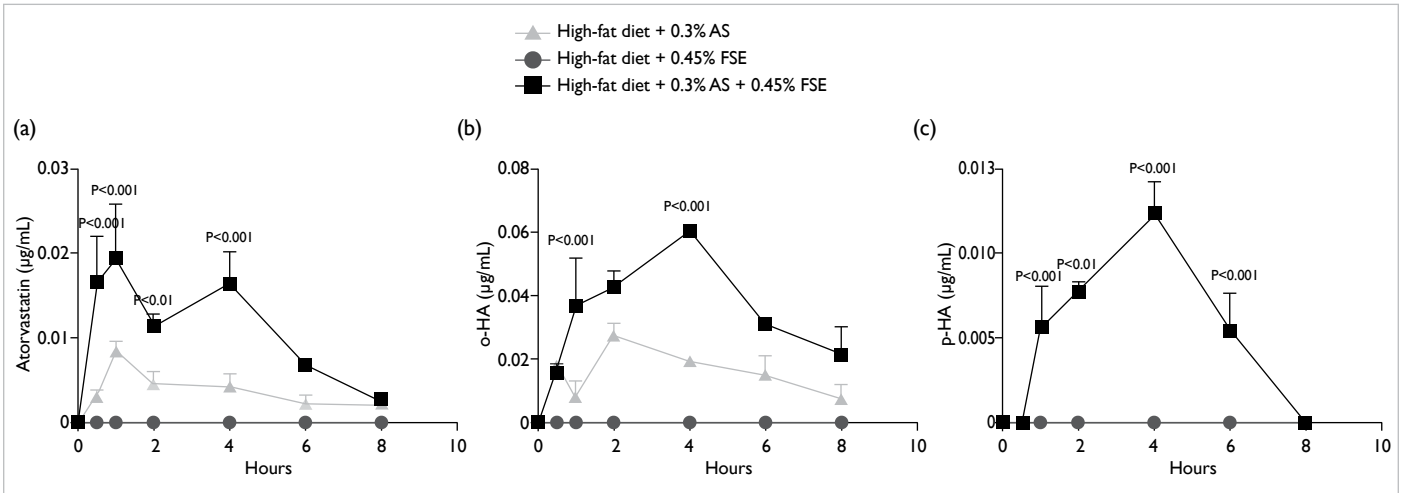

FIG 2. Effect of the combined use of atorvastatin (AS) and *Fructus Schisandrae* aqueous extract (FSE) in rats on plasma concentrations of (a) AS, (b) orthohydroxy AS (o-HA), and (c) para-hydroxy AS (p-HA). There was no detectable plasma level of schizandrin in any rat.

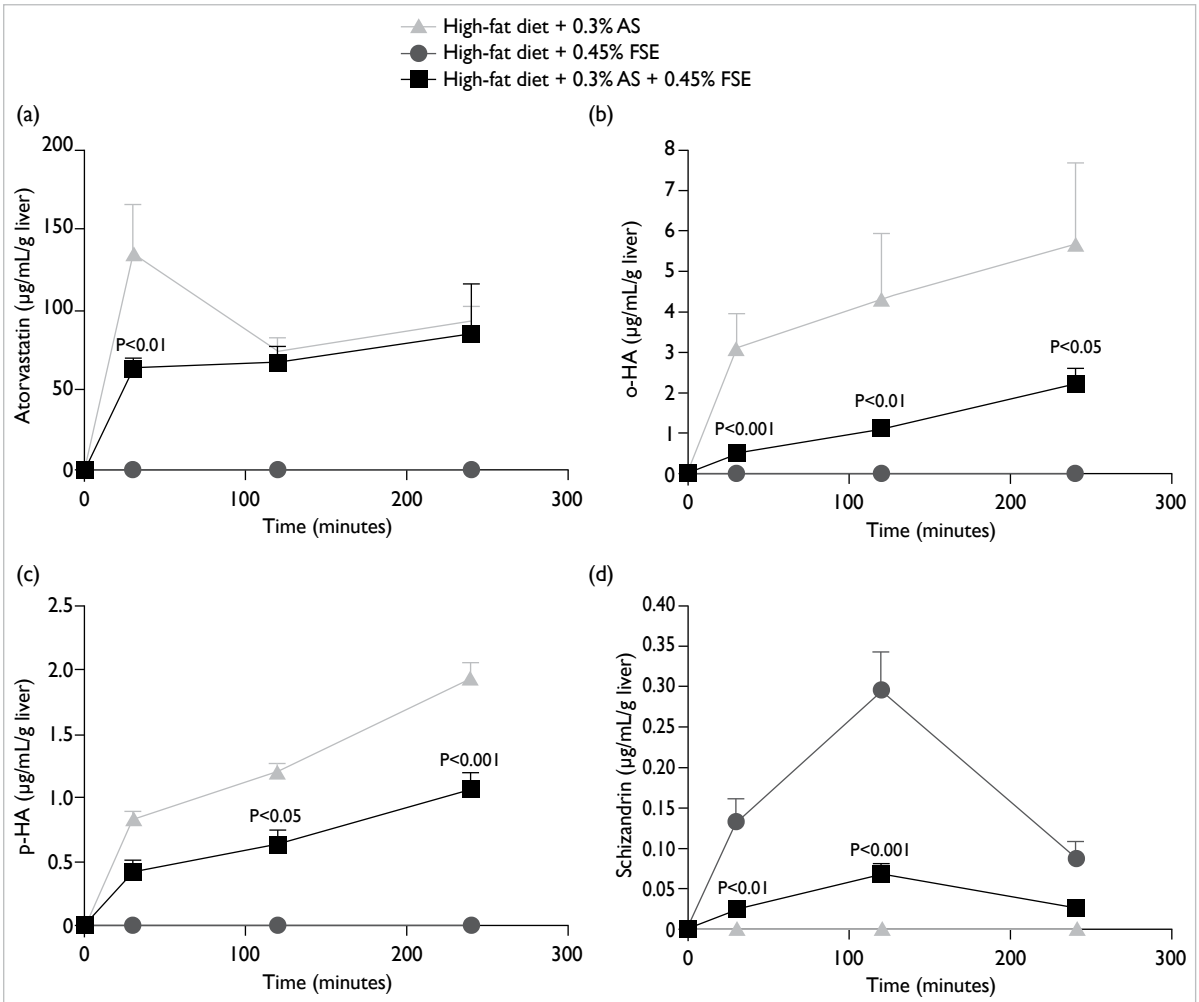

FIG 3. Effect of the combined use of atorvastatin (AS) and *Fructus Schisandrae* aqueous extract (FSE) on rat's liver concentrations of (a) AS, (b) orthohydroxy AS (o-HA), (c) para-hydroxy AS (p-HA), and (d) schizandrin.

for CYP3A4 metabolism and leads to accumulation of AS and its metabolites in the plasma. Nonetheless, schizandrin is only a weak inhibitor of CYP3A4,[5] as is its inhibitory effect on the metabolism of AS.

Our preliminary pharmacokinetic study could not explain the exact mechanism by which FSE interacts with AS to affect the plasma and liver concentrations of AS and/or its metabolites. Further studies are needed to determine whether the combined use of FSE and AS is safe in humans.

Conclusion

Combined use of FSE with AS exerts no further synergistic effect on diet-induced NAFLD in rats compared with AS alone. Nonetheless, the use of FSE can protect high-fat-fed rats from AS-induced liver toxicity. FSE may enhance plasma metabolites of AS but reduce the liver metabolites of AS. Further studies are warranted to determine the safety and underlying mechanism of the protective effects of FSE on AS-induced toxicity.

Acknowledgement

This study was supported by the Health and Health Services Research Fund, Food and Health Bureau, Hong Kong SAR Government (#09100241).

References

1. Golomb BA, Evans MA. Statin adverse effects: a review of the literature and evidence for a mitochondrial mechanism. Am J Cardiovasc Drugs 2008;8:373-418.
2. Pan SY, Dong H, Zhao XY, et al. Schisandrin B from Schisandra chinensis reduces hepatic lipid contents in hypercholesterolaemic mice. J Pharm Pharmacol 2008;60:399-403.
3. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.
4. Hermann M, Christensen H, Reubsaet JL. Determination of atorvastatin and metabolites in human plasma with solid-phase extraction followed by LC-tandem MS. Anal Bioanal Chem 2005;382:1242-9.
5. Iwata H, Tezuka Y, Kadota S, Hiratsuka A, Watabe T. Identification and characterization of potent CYP3A4 inhibitors in Schisandra fruit extract. Drug Metab Dispos 2004;32:1351-8.