2006. Vol. 27. No. 4. *96-104*
Korean Journal of Oriental Medicine

Original Article

Antihyperlipidemic and Antioxidant Effects of *Insamsansa-eum* (*Renshenshanzha-yin*) in Hypercholesterolemic Rats

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Insamsansa-eum (*Renshenshanzha-yin*, ISE) is a new medicine developed to prevent and treat atherosclerotic diseases. To explore antiatherosclerotic effects of ISE, we evaluated the effects of ISE on serum lipids of hypercholesterolemic rats in vivo, as well as its antioxidant activities *in vitro*. *In vitro*, ISE showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical scavenging activities, and inhibited hemolysis induced by 2,2' -azobis-2-amidinopropane dihydrochloride (AAPH) in a dose-dependent manner. *In vivo*, ISE significantly inhibited increase of total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and triglyceride (TG) values in both high cholesterol diet and Triton WR-1339 models. It also significantly inhibited decrease of high-density lipoprotein cholesterol (HDL) value in the high cholesterol diet model. Collectively, our data suggest that ISE has the potential to control the risk of atherosclerosis development.

Key Words : *Insamsansa-eum (Renshenshanzha-yin,* ISE), antioxidant, hyperlipidemia, atherosclerosis

Introduction

Hypercholesterolemia is a major cause of cardiovascular disturbance, such as atherosclerosis and coronary heart disease[1]. Although several factors, such as cigarette smoking, high-fat diet, high blood pressure, physical inactivity, age and heredity play a significant role in causing atherosclerosis, high blood cholesterol is mainly responsible[2,3].

In arteries in hypercholesterolemic animals and patients, vascular superoxide production and oxidative stress are increased[4]. Oxidation of low-density lipoprotein cholesterol (LDL) is considered an important step in the development of atherosclerosis. Oxidized LDL (oxLDL) is cytotoxic to a variety of vascular cells[5], induces the synthesis of monocyte chemotatic protein-1[6], recruits inflammatory cells[7], and stimulates the production of autoantibodies[8].

Antioxidants have the ability to prevent oxidation of LDL. For example, dietary antioxidants such as vitamin E protect LDL from oxidation[9]. In addition to inhibition of LDL oxidation, antioxidant therapy has been shown to produce beneficial effects on atherosclerosis and to prevent the progression of atherosclerosis in animal models by limiting vascular oxidative stress and superoxide production[10-13].

Received 12 October 2006;received in revised form 16 October 2006;accepted 28 October 2006
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Table 1. Composition of Insamsansa-eum

Constitute herbs	Scientific name	Mass (g/capsule)
Ginseng Radix	*Panax ginseng* C. A. MEYER	1
Crataegii Fructus	*Crataegus pinnatifida* BGE.	3
Total		4

Insamsansa-eum (*Renshenshanzha-yin*, ISE) is a combinatorial drug consisting of Ginseng Radix and Crataegii Fructus. Many reports from clinical and experimental studies suggested that ginseng and Hawthorn (*Crataegus*) fruit may have an antihyperlipidemic effect[14-18] and the ability to prevent oxidation of LDL[19-27]. In clinical study, ISE showed significant lipid-lowering effect[28]. In the present study we intended to explore the antiatherosclerotic effect of ISE by assessing the antihyperlipidemic potential *in vivo*, and the antioxidant action *in vitro*.

Materials and Methods

1. Materials

ISE is a capsulated water extract (300 mg per one capsule) of Ginseng Radix and Crataegii Fructus (Table 1). Each herbal medicine was extracted twice with boiling water for 2 h. These extracts were filtered and evaporated in a rotary vacuum evaporator and then finally lyophilized with a freezing dryer. To standardize the quality of ISE, ginsenoside Rg1 in Ginseng Radix was quantitatively assayed according to the previous methods[29].

2. Animals and diets

Sprague-Dawley male rats (200-250 g) were acclimatized for a week in colony cages. The animals were kept at a constant temperature (22-26°C) and humidity (50-55%), and were fed with commercial diet (Samyang, Korea). Water was allowed ad libitum.

3. DPPH radical scavenging activity

Different concentrations of the extract were measured for hydrogen donating or radical scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to Blois[30]. The reaction mixture containing 1 mL of a DPPH methanolic solution (1×10^{-4} M) plus 4 mL of the extract at different concentrations was incubated at room temperature for 30 min, and the absorbance was measured spectrophotometrically at 520 nm. The percent of DPPH discoloration of the sample was then calculated. The results were expressed as percent inhibition.

4. Superoxide anion scavenging activity

Superoxide anion scavenging activity of the ISE extract was determined according to Amakura et al. by measuring the superoxide radicals generated by the xanthine/xanthine oxidase system[31]. 0.02 mL of different concentrations of the extract, 3 mM xanthine, 3 mM EDTA, 0.75 mM nitroblue tetrazolium (NBT) and 0.15% bovine serum albumin (BSA) were added to 0.48 mL of 0.05 M Na_2CO_3 buffer and incubated for 10 min at room temperature. The reaction was initiated by the addition of 6 mU xanthine oxidase and carried out at 25°C for 20 min. After this period, the reaction was stopped by the addition of 6 mM CuCl, and absorbance was measured at 560 nm. The results were expressed as percent inhibition.

5. AAPH-induced hemolysis in erythrocytes

We determined the hemolysis of erythrocytes mediated by 2,2'-azobis-2-amidinopropane dihy-

drochloride (AAPH) using a modification of a method described elsewhere[32]. Blood samples were obtained by cardiac puncture in heparinized tubes and centrifuged (1500 rpm, 10 min). After removing the supernatant, the pellet was washed three times with 0.15 M NaCl. During the last washing, the erythrocytes were centrifuged (1500 rpm, 10 min). A 10% suspension of erythrocytes was prepared by adding PBS of pH 7.4. Test samples (0.2 mL) at different concentrations and 0.2 mL of 100 mM AAPH were added in succession to 0.2 mL of erythrocyte suspension. The reaction mixtures were incubated at 37°C for 3 h with gentle shaking. After incubation, an aliquot of the reaction mixture was diluted 20 times with PBS and centrifuged at 1000×g for 10 min. The absorbance of the supernatant (A) at 540 nm was read. Similarly, another aliquot of the reaction mixture was diluted with distilled water to yield complete hemolysis and the absorbance of the supernatant (B) after centrifugation was measured at 540 nm. Inhibition percentage of hemolysis exhibited by each sample was calculated by the equation (1-A/B) × 100%.

6. High cholesterol (HC)-diet model

Rats were given 1% cholesterol, 0.25% cholic acid and 2.5% olive oil with standard equilibrated diet (Samyang, Korea) for 2 weeks. Then twenty-four hypercholesterolemic rats were selected and randomly divided into four groups. The first group was given HC-diet and administered orally with ISE extract 220 mg/kg once a day for a week (study group 1). The second group was administered with ISE extract 440 mg/kg using the same method (study group 2). The third group was administered with lovastatin 50 mg/kg using the same method (positive control group). The fourth group was administered with only normal saline (untreated control group). The fifth group was given only standard equilibrated diet during whole study period and administered with normal saline for a week (normal group). After a week of medication, animals were kept for overnight fasting. Then they were anaesthetized with ether and blood samples were obtained by cardiac puncture.

7. Triton WR-1339 model

Thirty rats were randomly assigned to five groups. After a week of acclimation, all groups except the fifth were injected i.p. with a 10% aqueous solution of Triton WR-1339 (Sigma, USA) at 200 mg/kg. Food was withdrawn 16 h prior to Triton WR-1339 injection. To the first group, ISE extract 220 mg/kg was administered orally once a day for three days; the last administration was 1 hour before Triton WR-1339 injection (study group 1). Using the same method, ISE extract 440 mg/kg was administered to the second group (study group 2) and lovastatin 50 mg/kg to the third group (positive control group). The fourth group (untreated control group) was only injected with Triton WR-1339. The fifth group was injected with only normal saline (normal group). Blood samples were obtained by cardiac puncture 18 h after the injection of Triton WR-1339.

8. Determination of serum lipopoproteins

Blood samples were allowed to clot for 30-40 min. Serum was separated after centrifugation (3000 rpm, 30 min) and used for biochemical analysis. Analysis of blood serum for triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL) was performed

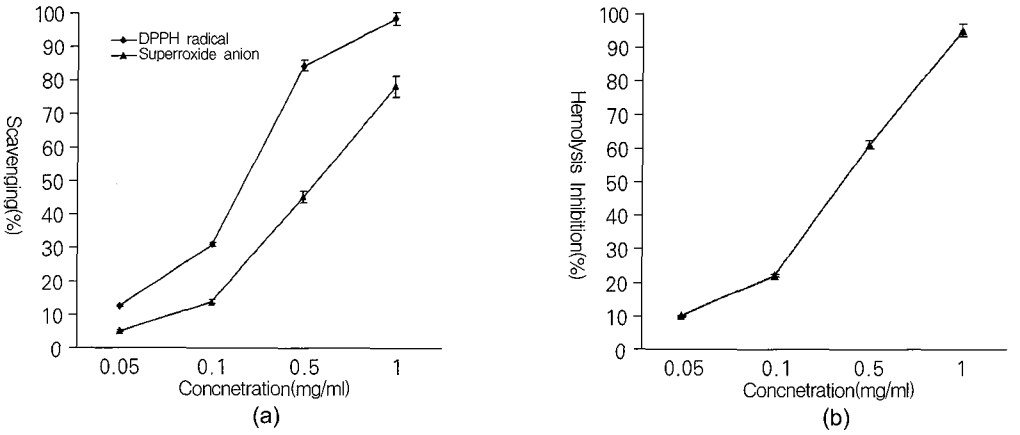

Fig. 1. Antioxidant activities of *Insamsansa-eum* (ISE) *in vitro*. (A) Concentration-response curves for the scavenging of free radicals by ISE. Data are expressed as percentage of scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), and superoxide radicals generated by the xanthine/xanthine oxidase system. (B) Concentration- response curve for the inhibition of hemolysis by ISE. Data are expressed as percentage of inhibition of 2,2′-azobis-2-amidinopropane dihydrochloride (AAPH)-induced hemolysis in erythrocytes. Values are means ± SD of three different assays.

using standard enzymatic assay kits (Asan Pharmacy Ltd., Korea). Serum low-density lipoprotein cholesterol (LDL) was determined using LDL-cholesterol kit (bioMerieux Inc., France).

9. Statistical analysis

All results are expressed as mean ± SD unless otherwise stated. Data were analyzed by independent samples t-test. A value of $P<0.05$ was considered

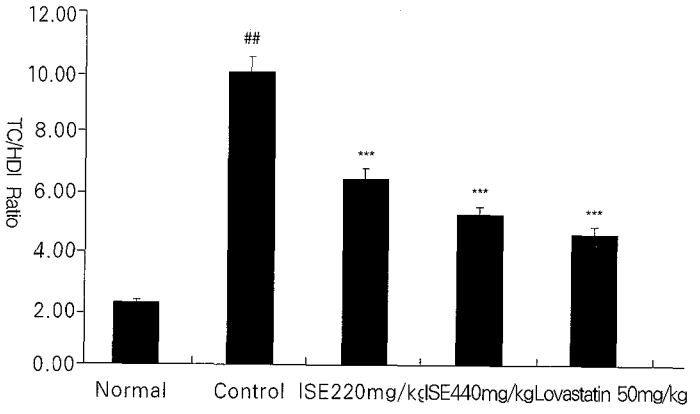

Fig. 2. Effects of *Insamsansa-eum* (ISE) on TC/HDL Ratio in High Cholesterol Diet-Induced Hyperlipidemic Rats
TC and HDL are total cholesterol and high-density lipoprotein cholesterol, respectively.
is $P<0.001$ by independent samples t-test vs. normal group.
*** are $P<0.001$ by independent samples t-test vs. control group

Table 2. Effects of Insamsansa-eum (ISE) on Serum Lipid Levels in High Cholesterol Diet-Induced Hyperlipidemic Rats

Group	Number	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)
Normal	6	82.0±2.81	21.2±1.59	36.5±1.97	75.0±3.71
Control	6	241.0±9.36$^{\#\#}$	67.3±3.39$^{\#\#}$	23.8±2.82$^{\#}$	135.5±5.90$^{\#\#}$
ISE 220mg/kg	6	193.5±6.05***	60.7±3.13	29.8±2.46	117.2±8.53
ISE 440mg/kg	6	166.5±5.19***	41.2±3.35***	31.7±2.75*	94.5±5.62***
Lovastatin 50mg/kg	6	150.0±6.37***	36.8±2.99***	32.5±0.92**	88.7±7.88***

TC, TG, HDL, and LDL are total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol, respectively.
is P<0.01 by independent samples t-test vs. normal group.
is P<0.001 by independent samples t-test vs. normal group.
* is P<0.05 by independent samples t-test vs. control group.
** is P<0.01 by independent samples t-test vs. control group.
*** are P<0.001 by independent samples t-test vs. control group.

significant. All calculation was performed by SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL).

Results

1. Antioxidant activity of ISE in vitro

To explore antioxidant activity of ISE, we determined inhibitory effects of ISE on DPPH and superoxide radical generation and AAPH-induced hemolysis. As shown in Fig.1A, ISE showed DPPH and superoxide radicals scavenging activities in a dose-dependent manner. ISE also inhibited hemolysis induced by AAPH in a dose-dependent manner (Fig.1B).

2. Hypolipidemic activity of ISE in vivo (high cholesterol-diet model)

The TC, LDL and TG values of the HC-diet group (control group) were significantly higher than that of the normal diet group (P<0.001). ISE (440 mg/kg) and lovastatin (50 mg/kg) significantly inhibited increase of TC, LDL and TG values (P<0.001). The HDL value was significantly lower in the control group than that in the normal diet group (P<0.01). ISE (440 mg/kg) and lovastatin (50 mg/kg) significantly inhibited decrease of HDL value (P<0.05 and P<0.01 respectively) (Table 2). TC/HDL ratio was significantly higher in the control group than that in the normal diet group. ISE (220 mg/kg), ISE (440 mg/kg) and lovastatin (50 mg/kg) significantly inhibited increase of TC/HDL ratio (Fig. 2).

3. Hypolipidemic activity of ISE in vivo (Triton WR-1339 model)

The TC, LDL and TG values in the Triton WR-1339 administered group (control group) were significantly higher than that in the normal

Table 3. Effects of Insamsansa-eum (ISE) on Serum Lipid Levels in Triton WR-1339 Induced Hyperlipidemic Rats

Group	Number	TC (mg/dL)	LDL (mg/dL)	TG (mg/dL)
Normal	6	78.7±5.92	11.7±1.43	102.5±7.61
Control	6	249.2±22.6$^{\#\#}$	81.2±5.16$^{\#\#}$	323.7±7.61$^{\#\#}$
ISE 220mg/kg	6	231.0±18.2	75.2±7.74	296.5±19.2
ISE 440mg/kg	6	182.0±13.0*	56.7±5.45*	277.7±12.8*
Lovastatin 50mg/kg	6	170.3±7.46**	52.3±2.63**	268.2±16.1**

TC, TG and LDL are total cholesterol, triglyceride and low-density lipoprotein cholesterol, respectively.
is P<0.001 by independent samples t-test vs. normal group.
* is P<0.05 by independent samples t-test vs. control group.
** is P<0.01 by independent samples t-test vs. control group.

diet group ($P<0.001$). ISE (440 mg/kg) and lovastatin (50 mg/kg) significantly inhibited increase of TC, LDL and TG values ($P<0.05$ and $P<0.01$ respectively) (Table 3).

Discussion

It is well established that elevated blood lipids levels constitute the major risk factor for atherosclerosis.[33] Lipids are very susceptible to attack by free radicals, and oxidized LDL species appear to contribute to the atherosclerosis pathobiology within the artery wall. *In vitro* studies have demonstrated that oxidized LDL (ox-LDL) activates endothelial cells to increase the expression of chemoattractant molecules leading to stimulate transendothelial migration of monocytes and formation of foam cells. Ox-LDL also increases production of growth factors including platelet-derived growth factor that stimulates migration and proliferation of smooth muscle cells. Furthermore, ox-LDL impairs endothelium-derived nitric oxide (NO) production[34,35].

ISE is a new medicine developed to prevent and treat atherosclerotic diseases. To explore antiatherosclerotic effects of ISE, we evaluated the effects of ISE on serum lipids of hypercholesterolemic rats, as well as its antioxidant activities. The present in vitro study shows that ISE has anti-oxidative activities as demonstrated, that this drug has a radical scavenging activity and is capable of inhibiting AAPH induced hemolysis.

Many studies reported that ginseng might have anti-oxidant activities. It was reported to scavenge DPPH and superoxide radicals, inhibit AAPH-induced hemolysis and metal ion-induced lipid peroxidation, and increase superoxide dismutase (SOD) activity and nitric oxide synthesis [22,25,36-41]. Hawthorn (*Crataegus*) fruit was also reported to scavenge hydrogen peroxidase and superoxidase, and inhibit Cu^{2+}- mediated lipid peroxidation[26,27]. Therefore, ISE is thought to work by way of integrating the actions of both herbs, as most traditional herbal formulations do.

In addition to the antioxidant activity, ISE showed a significant hypolipidemic activity, decreasing serum total cholesterol, triglycerides and LDL cholesterol in both hypercholesterolemic models. The TC/HDL ratio is a better indicator of coronary heart disease risk than individual lipoprotein concentration[42,43]. In this study, the TC/HDL ratios of ISE-treated groups were significantly lower than that of the control group in HC diet model.

The mechanism by which ISE decreases serum cholesterols remains unclear. Serum cholesterol can be lowered at several metabolic points including decreased synthesis, activation of LDL receptors, inhibition on absorption of dietary cholesterol, and conversion of cholesterol to bile acids. It was previously reported that ginseng decreases blood cholesterol levels by increasing cholesterol excretion through bile acid formation[44,45], and may increase LDL receptors by promoting the synthesis of LDL receptors in rats[46]. Muwalla and Abuirmmeileh reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was significantly lowered by ginseng, which shows the mechanism of the hypocholesterolemic action of ginseng involves the suppression of cholesterol biosynthesis [14]. Hawthorn (*Crataegus*) fruit was reported to increase LDL-receptor activity of hepatic membrane in rats[47]. In addition to the activation of LDL receptors, it was also reported to increase excretion of bile acids through up-regulation of hepatic cholesterol 7α -hydroxylase (CH) activity,

and inhibit cholesterol absorption through down-regulation of intestinal acyl CoA:cholesterol acyl-transferase (ACAT) activity[16]. Based on the studies reported, it was thought that those action mechanisms of Ginseng Radix and

Crataegii Fructus on lipid metabolism might be involved in the present study.

To summarize, ISE was able to reduce total cholesterol, triglycerides, LDL cholesterol and to increase HDL cholesterol in serum. It also showed anti-oxidant activity through radical scavenging activity and inhibition of AAPH induced hemolysis. Therefore, these data suggest that ISE has the potential to control the risk of atherosclerosis development

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