Regulatory Effects of Cheunggansoyo-san on Pathophysiological Changes Induced by Hyperlipidemic Diets in the Mice

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Hyperlipidemia is caused by high dietary intake of cholesterol and saturated fats, and is known as a major risk factor for atherosclerosis. In the oriental medicine, Cheunggansoyo-san (CGSYS) has been used for supplementing hematopoietic function and for treating cardiovascular disorders. In the present study, CGSYS was administered into hyperlipidemic mice. Increases in body weight and cholesterol levels induced by hyperlipidemic diets for 6 weeks were significantly inhibited by CGSYS administration. Serum levels of glucose, triglyceride, sGOT, and sGPT values were all decreased by CGSYS treatment compared with hyperlipidemic dietary mice. Moreover, CGSYS decreased LDL-cholesterol, but increased HDL-cholesterol levels in hyperlipidemic mice. Thus, the present results suggest that CGSYS appears to be effective for down-regulating risk factors of hyperlipidemia.

Key words : Cheunggansoyo-san(CGSYS), hyperlipidemic dietary mice, cholesterol, glucose, triglyceride level

Introduction

Hypertension is a major cardiovascular disease that is directly or indirectly associated with myocardial hypertrophy and myocardial infarction, cerebral infarction, stroke, and kidney diseases. A pathologic state caused by high dietary intake of cholesterol and saturated fatty acid is referred to hyperlipidemia[7,9,13,19]. Pathological significance of hyperlipidemia is evident when the blood cholesterol levels are unusually high, and thus recognized as hypercholesterolemia. Numerous studies suggest that the hypercholesterolemia is one of the critical factors for atherosclerosis because accumulation of cholesterol transported into the atheroma contributes to the development of atherosclerosis[16,18,27]. In addition to dietary influences, genetic factors such as defects in LDL receptor gene mutation can also cause familial hypercholesterolemia[2,25,26].

Use of experimental animals has accelerated to identify molecular factors related to hyperlipidemia[23,29,31]. Regular feeding of high cholesterol and saturated fats accelerates increases in body weight and also raises the plasma cholesterol levels as well as fat molecules[38,40,41]. Since all these changes are similarly occurring in humans, experimental animal model is valuable for investigating related factors in human diseases and further examining drugs.

There have been growing reports that oriental medicinal drugs are effective for the treatment of cardiovascular diseases including hypertension[21,22]. In the present study, we investigated possible regulatory effects of CGSYS on hyperlipidemia. Several herbal drug components of CGSYS are known to promote hematopoietic function, increase blood circulation, and promote proliferation of leukocytes including lymphocytes. Moreover, some components (e.g., Baikbokryong; Poria cocos) are known to promote diuretic activity thus lowering blood pressure. Together, informations on CGSYS based on the oriental medicine suggest possible regulatory function of hyperlipidemic constituents in the blood. Here, we generated hyperlipidemic mice by daily feeding of high fat food for 6 weeks. Our data show that CGSYS administration on hyperlipidemic mice can down-regulate the risk factors of hyperlipidemia.

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Materials and Methods

1. Materials

1) Animals and feeding protocol

C57BL/6 mice were obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB) and given water and chow for basal diet (Samyang Co, Korea) or hyperlipidemic diet (Bio-serve, USA) ad libitum. The animals were housed in standard plastic cages at 22±2℃ at least for one week and then used for the experiment. The compositions for basal diet and hyperlipidemic diet are shown in Table 1.

Table 1. Dietary composition of animal food

(a) Composition of basal diet	
Crud proteins	22.1%
Crude fats	8.0%
Crude fibers	5.0%
Crude minerals	8.0%
Calcium	0.6%
Phosphorus	0.4%
(b) Composition of Hyperlipidemic Diet*	
Total High Fat	17%
Cholesterol	1.25%
Cholic acid	0.5%
Normal diet	81.25%

* Telklad Premier Lab. Diet, No. TD 90221 ; paigen high Fat Diet

2) Herbal drugs

The Cheunggansoyo-san (CGSYS) was purchased from Daejeon University Oriental Medicinal Hospital and the composition of the seal is as follows.

Table 2. The Compositions of Cheunggansoyo-san(CGSYS)

Common name	Scientific name	amount (g)
Hyangbuja (향부자)	*Cyperi rhizoma*	10
Baikjakyak (백작약)	*Paeoniae radix alba*	6
Baikchul (백 출)	*Atractylodis macrocephalae rhizoma*	6
Chungpi (청 피)	*Citri reticulatae viride pericarpium*	4
Siho (시 호)	*Bupleuri radix*	4
Maikmundong (맥문동)	*Liriopis tuber*	4
Danggui (당 귀)	*Angelicae gigantis radix*	4
Baikbokryong (백복령)	*Poria cocos*	4
Sanchija (산치자)	*Gardeniae fructus*	2
Bakha (박 하)	*Menthae herba*	2
Gamcho (감 초)	*Glycyrrhizae radix*	2
Dansam (단 삼)	*Salviae miltiorrhizae radix*	6
Seokchangpo (석창포)	*Acori graminei rhizoma*	6
Total amount		60

3) Reagents and Instruments

FBS, trypsin, EDTA, trichloroacetic acid, Dulbecco's phosphate buffered saline, and 3.8% sodium citrate were purchased from Sigma (U.S.A), Normal saline was purchased from Jungoi Pharmaceutical Co (Korea), and the strips for the measurements of blood cholesterol and glucose levels were from Roche (Germany). Other chemicals were the highest quality commercially available.

The instrument and the manufacturers are listed below. Accutrend GC (Roche, Germany), blood glucose meter (LifeScan, USA), ice-maker (Vision, Korea), serum separator (Green Cross, Korea), Minos-ST (Cobas Co., France), centrifuge (Beckman Co., USA.), rotary vacuum evaporator (Büchi 461, Switzerland), deep freezer (Sanyo Co., Japan), freeze dryer (Eyela Co., Japan), autoclave (Hirayama, Japan), ultrasonic cleaner (Branson Ultrasonics Corp., USA.), roller mixer (Gowon scientific technology Co., Korea), herbal drug extractor (S-15000, Saeil Chemical, Korea).

2. Methods

1) CGSYS preparation

Two seals of CGSYS (120 g) were suspended in 3 liters of distilled water (Pulmuwon) in an autoclave designed for the purpose of herbal drug exaction (S-15000, Saeil Medical) and boiled at 100℃ and 0.5 kgf/cm^2 of pressure for the first 30 min and at 121℃ and 1.5 kgf/cm^2 for 2.5 hr. CGSYS extract was further concentrated by processing with a rotary vacuum evaporator (Büchi 461, EYELA) and freeze-dried for 24 hr using the freeze-drier (EYELA, FDU-540, Japan). The yield was 21.9 grams of GYSYS powder from two seals (120 g) and the product was kept at -75℃ of deep freezer, and resuspended in distilled water immediately before use.

2) Human fibroblast cells (hFC) culture

The human skin tissues were dissected, washed three times with cold PBS and cut into small pieces. The tissues in 15 ml conical tube were centrifuged at 1400 rpm for 5 min. After adding DMEM containing collagenase A (5 ㎎/㎖, BM, USA), DNase type I (0.15 ㎎/㎖, Sigma, USA) and antibiotics (penicillin 104 U/㎖, streptomycin 10 ㎎/㎖, amphotericin B 25 ㎍/㎖), the dissociated tissue was incubated at 37℃ for 2 hr, and further incubated for 30 min in the presence of 0.5% trypsin-0.2% EDTA. Then the tissue was washed twice with cold PBS, centrifuged at 1500 rpm to remove the supernatant, resuspended in DMEM-10% FBS, and cultured for 7 days. The cells were detached from the plate by 0.5% trypsin-0.2% EDTA treatment, and plated on 96 well plate with cell concentration of 10^5 cells/㎖ in DMEM-5% FBS culture medium.

3) Cytotoxicity assay

The cytotoxicity was measured by modified SRB method. After culture at 5% CO_2 incubator at 37℃ for 1 hr, cells were dissociated by trypsin-EDTA solution. The culture medium was removed and cells (2.0 x 10^4 well in 96 well plate) were grown at 37℃, 5% CO_2. incubator. Cells were treated with 1000 ㎍/㎖. 500 ㎍/㎖. 250 ㎍/㎖. 125 ㎍/㎖, 62.5 ㎍/㎖, 32 ㎍/㎖, 16 ㎍/㎖, 1.6 ㎍/㎖ of CGSYS for 48 hr. Cells were then washed with PBS twice, treated with 50 ㎕ of 50% trichloroacetic acid for 1 hr at 4℃, washed with distilled water five times, and air dried. Cells were stained with SRB solution (0.4%/1% acetic acid; 100 ㎕/well) for 30 min at room temperature, and washed with 0.1% acetic acid for 4-5 times. Cells were air dried and solublized in 10 mM Tris base (100 ㎕/well). Cells in the plate were resuspended in the culture medium by using a plate shaker (Lab-Line, USA) for 5 min and used for the measurement of optical density at 540 nm using ELISA reader (Molecular Devices, USA).

4) Dietary and reagent treatments

The animals were divided into normal untreated group supplied with basal diet, control group with hyperlipidemic diet, and experimental group having CGSYS administration. Animals in each group were fed for 6 weeks. In the experimental group, CGSYS (150 mg/dL) was orally administerd on a daily basis for 4 weeks along with hyperlipidemic diets.

5) Body weight measurement and serum analyses

The body weight was measured at 10 A.M. at every seventh day. Blood (0.01 ml each time) was collected from tail veins once every week for 6 weeks and cholesterol levels were determined by using Accutrend strip. Blood glucose levels were analyzed by blood glucose meter (LifeScan, USA). Activities of triglyceride, HDL-cholesterol, LDL-cholesterol, sGOT (aspartate aminotransferase), and sGPT(alanine aminotransferase) were measured by using biochemical analytical instruments following the principles of JSCC UV methods.

6) Statistical analysis

All number data were represented in mean ± standard error of mean (SEM). The statistical comparison was made by Student's t-test and accepted as being significant at a criterion of $p < 0.05$.

Results

1. Cytotoxicity of CGSYS in human fibroblast cells

Toxicity of CGSYS in cultured hFLS was determined by treating cells with different doses of CGSYS. As shown in Table 3, there were some decreases in cell viability by increasing CGSYS concentrations. The cell viability at ㎍/㎖ of CGSYS was decreased to 82% compared with no CGSYS treated cells. The cell viability was increased linearly as increases in CGSYS doses.

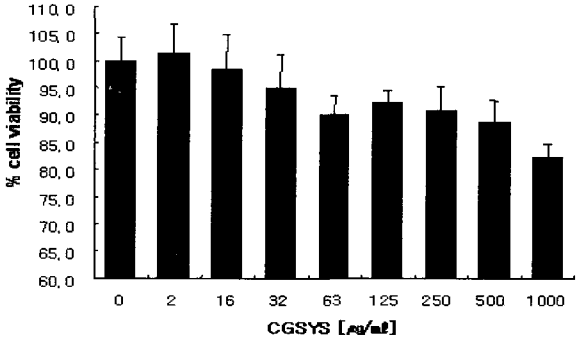

Fig. 1. Dose-dependent increases in viability of human fibroblast. Cells were treated with various concentrations of CGSYS and % cell viability relative to untreated control was plotted. Error bars denote SEM.

2. Effects of hyperlipidemia on body weight changes

To examine the effects of hyperlipidemic diet, changes in body weight were traced for 6 week period in normal, control, and CGSYS-treated animal groups. Hyperlipidemia mice showed rapid increases in body weight at 4-6 weeks compared with normal animal group. Administration of CGSYS 2 weeks after initial hyperlipidemia diet caused retarded increase in body weight compared with the group with hyperlipidemia diet (Fig 2).

The rate of body weight increase for 6 week period showed that the control group with hyperlipidemia diet showed 13.6% of increased body weight gain compared with normal diet group. In CGSYS-treated experimental group, the body weight increase was lowered to 6.3% compared with the hyperlipidemia control group, suggesting that CGSYS administration had an inhibitory effect on body weight increase by hyperlipidemia diet.

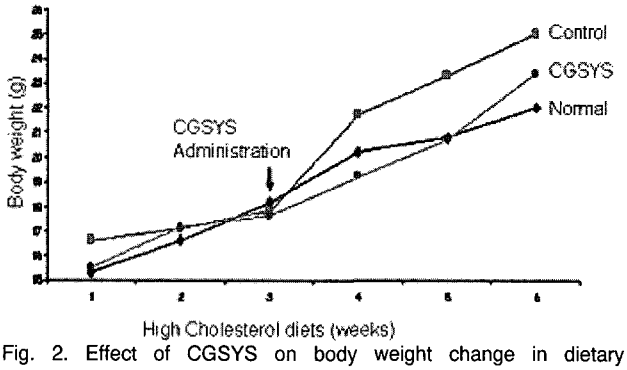

Fig. 2. Effect of CGSYS on body weight change in dietary hyperlipidemic rats. Normal: normal diet Control: hyperlipidemic diet CGSYS: hyperlipidemic diet plus CGSYS(150 mg/dL) administration

3. Time-dependent changes in cholesterol levels after

hyperlipidemia diet

Cholesterol levels were measured on a weekly basis in three animal groups. As shown in Fig. 3, serum cholesterol was not altered in normal animal group maintaining a basal level. In a group with hyperlipidemic diet, cholesterol levels were rapidly increased at 1-3 weeks after beginning hyperlipidemia diet and remained at high levels with about 200 mg/dL. Administration of CGSYS showed similar increases in cholesterol for the first 3 weeks, and then, cholesterol levels were declined by 6 weeks, resulting in much lower levels compared with hyperlipidemia diet group.

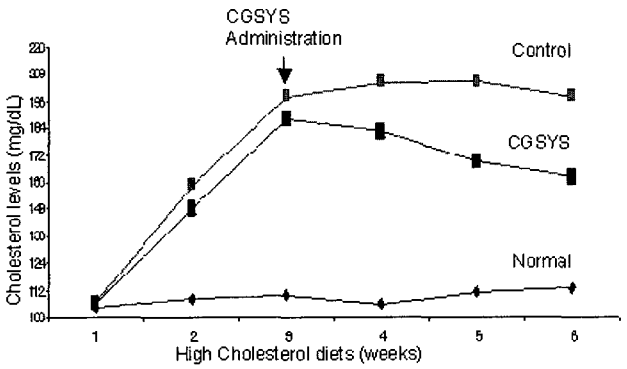

Fig. 3. Effect of CGSYS on cholesterol change in dietary hyperlipidemic rats. Normal: normal diet Control: hyperlipidemic diet CGSYS: hyperlipidemic diet plus CGSYS(150 mg/dL) administration

4. Effects of CGSYS on total cholesterol, glucose, and triglyceride levels in the serum

Levels of total cholesterol in the serum of hyperlipidemic control group were significantly increased compared with normal group. In CGSYS group, cholesterol levels were also higher than normal group, but significantly lower compared with the control group. Serum glucose levels were highly increased to more than three folds compared with untreated control. There was also a large increase in CGSYS group, but the comparison between control and CGSYS groups revealed significant decreases in CGSYS group. A similar pattern of inhibition effects of CGSYS was observed in serum triglyceride levels. Changes in cholesterol, glucose, and triglyceride are summarized in Table 3.

Table 3. Effects of CGSYS on serum cholesterol, glucose, and triglyceride levels

Group		Parameter of serum level (mg/dL)		
		Tot. Cholesterol	Glucose	Triglyceride
Normal		89.8 ± 1.9	78.0 ± 6.3	65.0 ± 4.2
HL diet	Control	123.6 ± 4.3^{++}	271.4 ± 9.4^{+++}	179.4 ± 9.4^{+++}
	CGSYS	103.4 ± 4.8*	200.6 ± 17.7**	124.0 ± 9.5*

+: Statical comparisons with normal animal group (+: p <0.05, ++: p <0.01, +++: p <0.001) *: Statical comparisons with control animal group (*: p <0.05, **: p <0.01, ***: p <0.001) HL: hyperlipidemic

5. Effects of CGSYS on serum sGOT, sGPT, and LDL/HDL cholesterol levels

Aspartate aminotransferase (sGOT) is one of the metabolic enzyme which is strongly upregulated by hyperlipidemic diet. sGOT levels was significantly increased by hyperlipidemic diet, then decreased by CGSYS treatment. Serum levels of a metabolic enzyme alanine aminotransferase (sGPT) also showed two fold increases by hyperlipidemic diets. Then, CGSYS administration decreased sGPT values similar to those of normal animal group(Table 4).

HDL cholesterol is known to be inversely related to hyperlipidemic diet. In the current experiment, HDL cholesterol levels were 94.5 ± 4.5 (mg/dL) in the normal-diet animal group, and significantly decreased in the animal group with hyperlipidemic diet (70.0±2.0(mg/dL), $p<0.01$ compared with normal diet group). CGSYS administration in the hyperlipidemic diet animal significantly increased HDL (90.2 ± 4.7(mg/dL), $p<0.01$ in comparison with hyperlipidemic diet group). Thus, CGSYS diet increased HDL cholesterol in hyperlipidemic animal. LDL cholesterol levels in the serum was 14.6 ± 0.8(mg/dL) in normal diet animal group and then significantly elevated after hyperlipidemic diet (19.2 ± 1.2(mg/dL), $p<0.001$). Administration of CGSYS effectively deceased LDH levels to 19.2 ± 1.2(mg/dL) which were significantly lower than hyperlipidemic diet group ($p<0.001$) and similar to normal animal. Changes in number data for serum constituents are summarized in Fig. 5.

Table 4. Effects of CGSYS on serum sGOT, sGPT, and LDH/HDL cholesterol.

Group		Parameter of serum level (mg/dL)			
		sGOT	sGPT	HDL-Chol.	LDL-Chol.
Normal		76.8 ± 12.9	24.0 ± 3.3	94.5 ± 4.5	14.6 ± 0.8
HL diet	Control	133.8 ± 13^{++}	57.0 ± 2.2^{+++}	70.0 ± 2.0^{++}	42.8±1.3^{+++}
	CGSYS	117.4 ± 8.0	24.2±1.0***	70.0 ± 2.0^{++}	19.2 ± 1.2***

+: Statical comparisons with normal animal group (+: p <0.05, ++: p <0.01, +++: p <0.001) *: Statical comparisons with control animal group (*: p <0.05, **: p <0.01, ***: p <0.001) HL: hyperlipidemic

Discussion

CGSYS is a complex prescription composed of Soyo-san (SYS) with additional 5 more herbal drugs. Major physiological activity of SYS herbal drugs are associated with regulation of cardiovascular activities. Hematopoiesis and improved blood circulation by Baikjakyak, regulation of diuretic function and blood glucose levels by Baikbokryong, increased blood circulation by Siho, Bakha, and Danggui, regulation of blood pressure by Maikmundong are a few examples for

cardiovascular activity known to be controlled by CGSYS[20]. Even though CGSYS has thus been used for the treatment of related cardiovascular diseases, informations on the regulatory mechanism at cellular and molecular levels are not known. Therefore in the current study, CGSYS was investigated using hyperlipidemia-induced mice.

Hyperlipidemia model can be generated by dietary regulation, and previous studies indicated that pathological features in hyperlipidemic and hypercholesterolemic mice are, in general, confident for reliable application to human diseases[28,29,31]. Hyperlipidemia refers to a pathophysiological state of abnormally increased body fat levels, particularly high in cholesterol and triacylglyceride, and is recognized as a major risk factor for atherosclerosis[30,39].

In the current study, an experimental model of hypercholesterolemia was developed in the mice by supplying hyperlipidemic diet on a daily basis for 6 weeks. The animal showed typical hyperlipidemic symptoms such as gaining excess body weight, increased levels of serum cholesterol, LDL cholesterol, and triglyceride. HDL cholesterol levels was decreased in hyperlipidemic mice. HDL is known to mobilize cholesterol from developing and existing atheroma and transport it to the liver for excretion in the bile[5]. Thus, HDL participates in reverse transport of cholesterol, thereby called as the good cholesterol. In contrast, LDL-cholesterol is the major component of the total serum cholesterol associated with increased risk of atherosclerosis[12].

Our study further showed that daily administration of CGSYS for the last 4 weeks changed several physiological parameters related with hyperlipidemia. Increase rate of rat's body weight was largely retarded by CGSYS. 4 week administration of CGSYS inhibited increases in body weight similar to that of normal control mice. Time-dependent measurement of blood cholesterol also showed that inhibitory actions of CGSYS appeared to be effective, when considering that the difference of blood cholesterol levels between CGSYS and control group were consistently increased(Fig 3).

In the blood from the subjects with obesity or atherosclerosis, glucose or fat molecules such as triacylglyceride, unsaturated fatty acid, cholesterol levels are consistently elevated[9,11,15,32]. Excess amount of blood glucose can cause insulin resistance, a feature of type II diabetes[35,37,39]. Increased serum triacylglyceride can lead to hypertrophy of adipose tissue and fat accumulation in the body[1,14,39]. All these parameters that can be observed from hyperlipidemic mice could be correlated well with increased body weight.

Then, oral administration of CGSYS decreased the parameters indicating the physiological consequences of high fat diet. CGSYS was the most effective for returning values of sGPT and LDL-cholesterol almost to normal levels. sGOT (aspartate aminotransferase) and sGPT (alanine aminotransferase) are largely present in the liver cells, skeletal muscle and blood vessels, and released into the blood when the these cells are damaged or undergo necrosis[8,33,34]. Thus, serum level of these enzymes are used for a diagnosis of liver diseases, chronic hepatitis, fatty liver, cirrhosis, and liver cancer[10,36,37]. Several studies have reported that hyperlipidemia and increased body weight increases fatty liver[3,4]. Persistent fatty liver inhibits normal cell functions and thus increases the release of sGOT and sGPT. Other studies further suggest that hyperlipidemia are related with increased insulin resistance, which in turn, exacerbate liver cell damage.

Inhibitory effects of CGSYS on serum sGPT and sGOT levels could improve liver cell function, insulin regulation, cardiovascular activity and/or others. At this moment, possible cellular and molecular mechanism of the protective effects of CGSYS on hyperlipidemia-related pathological events are unknown. There have been several reports on the regulatory activity mediated by oriental medicinal drugs for the treatment of cardiovascular disorders related to hypertension and hyperlipidemia and hypercholesterolemia, but the mechanistic explanations remain to be explored[21,22,6,17]. As discussed above, there are more than ten different herbal constituents, and thus, their effects might be multi-functional. It is obvious that investigations of the protective effects of individual constituents on hyperlipidemia would be critical to determine active ingredients regulating hyperlipidemia and for the therapeutic application.

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