Effects of Gamichunggantang on hyperlipidemia

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KEY WORDS hyperlipidemia; triglycerides; cholesterol; *O*-acyltransferase

ABSTRACT

AIM: To evaluate the therapeutic effects of Gamichunggantang (GCT) on hyperlipidemia through high cholesterol diet model. GCT is an Oriental herbal medication, which has been used for the treatment of fatty liver, hyperlipidemia or alcoholic liver disease in Daejeon University Oriental Hospital, Korea since 1999. **METHODS:** Rats were fed with high cholesterol diet for 4 weeks and GCT was administrated for 2 weeks from 2 weeks later in experimental days. The levels of serum total cholesterol, HDL-cholesterol, and triglyceride were analyzed every week. Absolute and relative liver weight to body, and histophathological changes were determined at last day. And, lipid metabolism-related gene expressions (ACAT and DGAT) in liver tissue were analyzed by using RT-PCR. **RESULTS:** In GCT group, TG levels were reduced at 3 and 4 weeks after GCT administration (39.4 %, $P<0.05$ and 36.3 %, $P<0.01$ respectively). Total cholesterol levels also were reduced at 3 weeks (20.5 %, $P<0.05$) and 4 weeks (35.86 %, $P<0.01$) after GCT administration, but HDL-cholesterol levels were increased significantly ($P<0.05$) at 3 weeks (14.7 %) and 4 weeks (25.5 %) compared with hyperlipidemia-induced group without GCT. In the GCT treated group, liver weight was lower and lipid accumulation was decreased in histological finding. ACAT gene expression was suppressed compared with hyperlipidemia-induced group but not DGAT. **CONCLUSION:** GCT possesses preventive or therapeutic effects on diet-induced hyperlipidemia by inhibiting the intestinal absorption and storage of exogenous and endogenous cholesterol.

INTRODUCTION

Coronary artery disease and brain stroke continue to be the leading causes of death in industrialized nations. The lowering of plasma cholesterol is beneficial for reducing the risk of developing cardiovascular disease[1].

Because cholesterol is available to body through two basic mechanisms-endogenous biosynthesis and absorption from the gastrointestinal tract, much of the research has been established in these pathways for lipid management[2-4]. The clinical complications of atherosclerosis could be diminished and life be prolonged when plasma lipid level was lowered by hypocholesterolemic agents[5]. But many of promising agents developed have serious side effects, especially on adrenal function[6]. From this, much of growing interest in oriental medicine that may have less toxicity has led to an increasing number of non-pharmacological therapies for lipid management[7]. Many kinds of hypocholesterolemic activi-

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ties from a lot of plant materials were confirmed till now[7-9]. Gamichunggantang (GCT) has been used for patients with fatty liver or hyperlipidemia in Daejeon Oriental Medicine Hospital, Korea since 1999. We evaluated its clinical effects from the patients suffering from these diseases. But the scientific basis of the effect on hyperlipidemia has not been examined in laboratory yet. Therefore, this present study is aimed to elucidate the effects of GCT on hyperlipidemia by determination of gene expressions related with lipid metabolism as well as serum triglyceride, total and HDL-cholesterol level, and histological changes.

MATERIALS AND METHODS

Materials The composition of Gamichunggantang (GCT) was listed in Tab 1. Total 162 g of GCT were purchased from Daejeon Oriental Medical Hospital as dried ones. Having been washed and dried again, mixture was added in 1.5 L of distilled water and left for 1 h at room temperature followed by boiling twice for 1 h each time and lyophilization. The CGT extract was 11.8 g as 7.3 % (w/w) of yield and this extract was suspended in distilled water to be given orally to rats once a day for 2 weeks. Only hyperlipidemia induced group was orally administrated with distilled water instead of CGT. Cholesterol, cholic acid, and olive oil were purchased from Sigma Inc (ST Louis, USA). DNA *Taq* polymerase was obtained from Bioneer (Cheongwon, Korea), M-MLV reverse transcriptase was obtained from Promega (Madison, USA). TRIzol reagent was obtained from Gibco (Maryland, USA). Rest reagents were purchased from Sigma Inc (St Louis, USA).

Tab 1. Prescription of Gamichunggantang (GCT).

General name	Part used	Voucher specimen number	Relative Amount (g)
Artemisiae capillaris	Herba	01104	10
Trionyx sinensis	Carapax	01939	8
Raphanus sativus var *hortensis* for *acanthiformis*	Semen	01785	8
Atractylodis macrocephalae	Rhizoma	01127	6
Poria cocos		01747	6
Atractylodis japonica	Rhizoma	01129	6
Polyporus umbellatus		01744	4
Amomum villosum	Fructus	01057	4
Glycyrrhizae uralensis	Radix	01449	2
Total amount			54

Experimental animals Five-week old male Sprague-Dawley rats were purchased from commercial animal breeder (Daehan BioLink, Korea). The rats were housed in an environmentally controlled room at (22±2) °C, relative humidity at (55±10) % and 12 h light/dark and fed with commercial pellets (Samyang Feed Ltd, Korea) and tap water *ad libitum*.

After 1-week acclimation, eighteen rats were divided into 3 groups of 6 animals each; naive group fed with normal diet, induced group fed with high cholesterol diet without GCT, and GCT group fed with high cholesterol diet with GCT. The two groups were fed with home made cholesterol diet containing 1 % cholesterol, 0.25 % cholic acid, and 2.5 % olive oil for 4 weeks *ad libitum.* Two weeks after giving high cholesterol diet, the rats in GCT group were administrated orally with GCT (200 mg/kg), induced group were administrated with distilled water on the other hand. Every week of the experimental period, the animals were fasted 4 h and blood was collected from the orbital vein under ether anesthesia. On the last day, the animals were fasted 12 h and whole blood was collected from abdominal aorta, and organs were weighted and fixed in phosphate buffered formalin.

Serum biochemical analysis After letting the bloods at room temperature for 1 h, serum was separated by centrifugation at 1500×*g* for 15 min. The levels of serum total cholesterol, HDL-cholesterol, and triglyceride were determined using Olympus Optical Reply (Olympus Ltd, Japan).

Histopathologic findings For the histomorphological evaluation, a portion of liver tissue was removed and fixed in 10 % phosphate buffered formalin. Tissues were embedded with paraplast and sectioned 4 μm in thickness. Liver sections were stained with hematoxylin & eosin for histopathological examination.

DGAT and ACAT gene analysis with RT-PCR Just after sacrificing rats at the last day, total RNA was extracted from by the pieces of six livers per group with homogenization in TRIzol reagent (Gibco, Maryland, USA) according to the manufacturer's instructions. Reverse transcription and cDNA-PCR process were done according to general RT-PCR method. PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95 °C for 10 min; followed 35 cycles of 95 °C for 1 min, 60 °C for 40 s and 72 °C for 40 s; and final cycle of 72 °C for

10 min. The PCR products were run on 1 % agarose gel in 0.5X TBE buffer and measured as height value by using Windows ID main program (AAB, USA). Sequences of the deoxyribonucleotide for PCR are as Tab 2.

Statistical analysis The results obtained were expressed as mean±SD for the number of experiments. Student's *t*-test was used to make a statistical comparison between the groups. Results with $P<0.05$ were considered statistically significant.

RESULTS

Change of body weight Every group showed increased body weight throughout 4 weeks. But GCT group was lower than naive or induced group, and there was significant difference ($P<0.05$) between GCT and hyperlipidemia-induced group at the 4th week, especially (Fig 1).

Change of organ weight At the autopsy, GCT and induced groups together show some hypertrophy of liver being yellowish (Fig 2). Absolute and relative liver weight of hyperlipidemia-induced group increased significantly ($P<0.01$) compared with naive group. On the other hand, absolute and relative liver weights of GCT group were significantly lower than those of induced group ($P<0.01$, Fig 3).

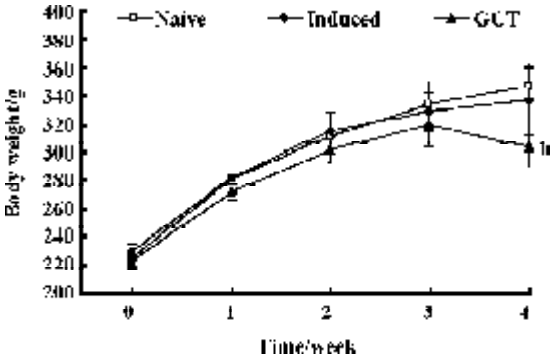

Fig 1. Changes in the mean body weight of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. *n*=6. Mean±SD. $^bP< 0.05$ *vs* hyperlipidemia-induced group.

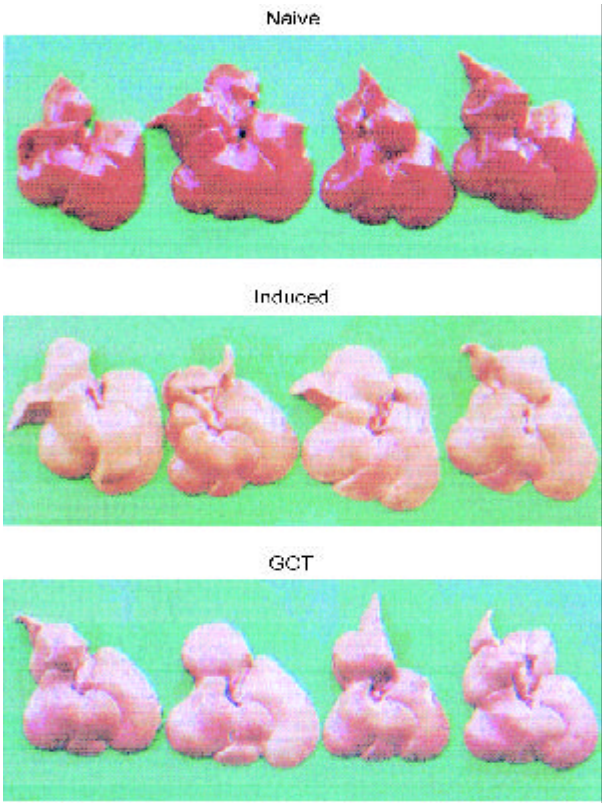

Fig 2. Gross finding in liver of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet.

Histopathological observations As shown in Fig 4, macrovascuolar cytoplasmic alterations of hepatocytes were detected around the periportal area of both induced and GCT groups. But these alterations were more severe in induced group than GCT group, which

Tab 2. Oligonucleotide sequences of primers.

Gene	Primer	Sequence	Product size/bp
β-Actin	Sense	5'- GTG GGG CGC CCC AGG CAC CA -3'	539
	Antisense	5'- CTC CTT AAT GTC ACG CAC GAT TTC -3'	
DGAT	Sense	5'-GAA TAT CCC CGT GCA CAA GT-3'	255
	Antisense	5'-CAC AGC TGC ATT GCC ATA GT-3'	
ACAT	Sense	5'-CCT CCC GGT TCA TTC TGA TA-3'	370
	Antisense	5'-ACA CCT GGC AAG ATG GAG TT-3'	

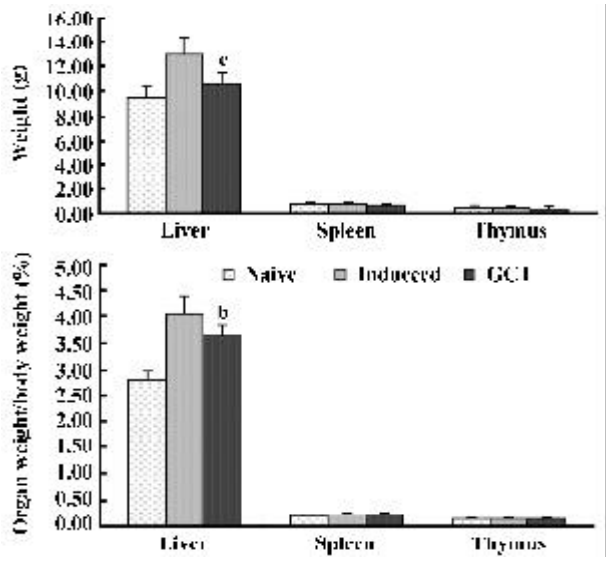

Fig 3. Absolute and relative organ weight of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. n=6. Mean±SD. ^bP<0.05, ^cP< 0.01 *vs* hyperlipidemia-induced group.

showed microvascuolar one. There were many apoptotic cell lysises in induced group especially.

Serum biochemical analysis Serum triglyceride levels were highest in induced group and lowest in GCT group especially at the 3rd week and the 4th week (39.4 %, P<0.05 and 36.3 %, P<0.01, respectively) (Fig 5). Total cholesterol levels were increased according to the period of high cholesterol diet administration in induced group, but the levels were reduced at 3 week

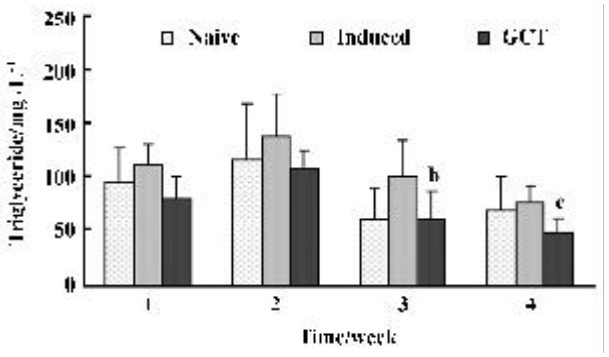

Fig 5. Serum triglyceride levels of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. n=6. Mean±SD. ^bP< 0.05, ^cP< 0.01 *vs* hyperlipidemia-induced group.

(20.5 %, P<0.05) and 4 week (35.86 %, P<0.01) after administration of GCT (Fig 6). On the contrary, HDL-cholesterol levels were increased significantly (P<0.05) at 3 weeks (14.7 %) and 4 weeks (25.5 %) in GCT group (Fig 7).

Gene expression of DGAT and ACAT ACAT gene expression was decreased by 21.3 % in GCT group compared with hyperlipidemia-induced group (Fig 8). DGAT gene expression was increased in both groups given high cholesterol diet and in GCT group more increased than in only given high cholesterol diet group.

DISCUSSION

Although our major metabolic energy is originated

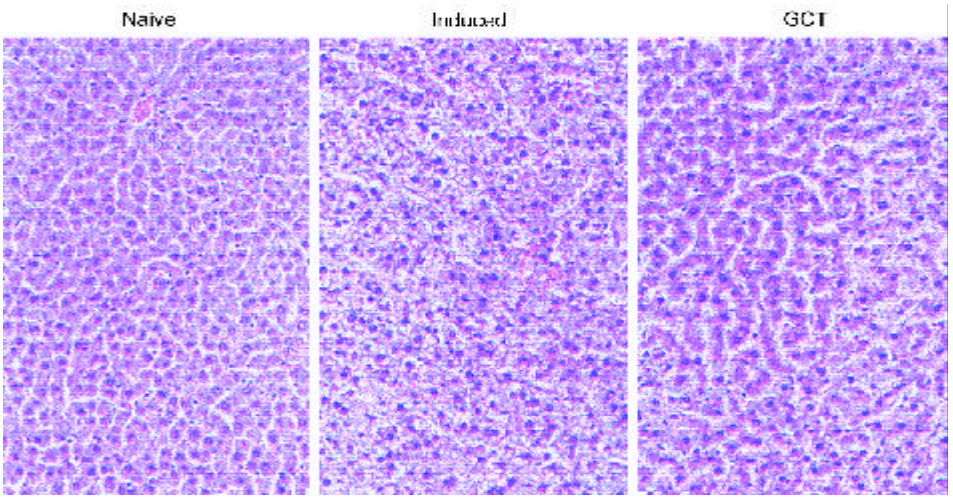

Fig 4. Histopathological findings in liver of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. H&E stain, ×200.

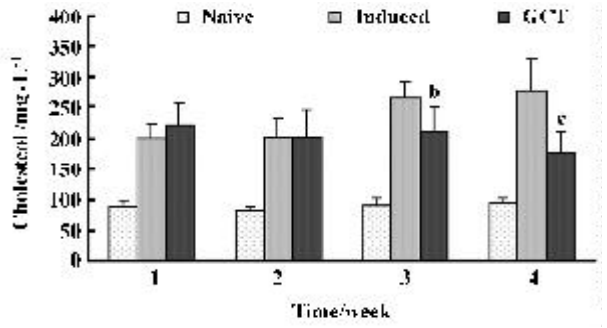

Fig 6. Serum cholesterol levels of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. *n*=6. Mean±SD. $^bP< 0.05$, $^cP< 0.01$ *vs* hyperlipidemia-induced group.

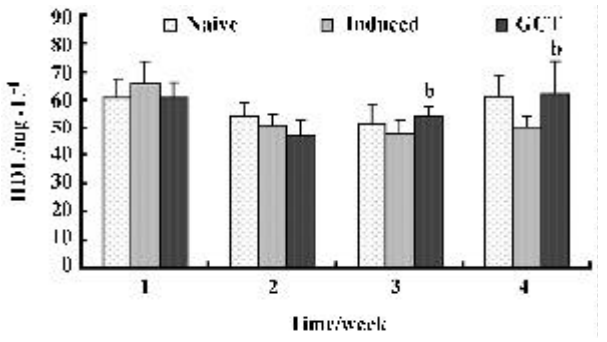

Fig 7. Serum high density lipoprotein (HDL) levels of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. *n*=6. Mean±SD. $^bP < 0.05$ *vs* hyperlipidemia-induced group.

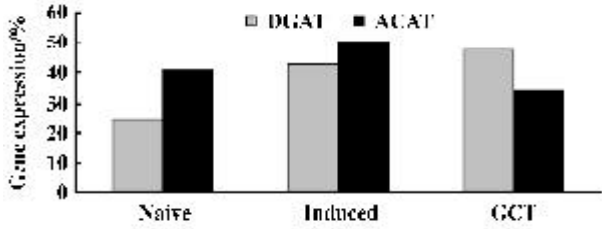

Fig 8. DGAT, ACAT gene expression in liver of rats fed with high cholesterol diet. Naive, given commercial diet; Induced, only given high cholesterol diet; GCT, 200 mg/kg GCT with high cholesterol diet. Relative gene expression of DGAT and ACAT was expressed as the ratio of DGAT/ACAT gene expression to b-actin gene expression.

from oxidation of fatty acid, excessive consumption of fatty acid might come to hyperlipidemia causing complication of vascular disease[10]. Recently, vascular disease demands a dreadful toll and, recent mass research has showed that coronary events in patients with symptomatic vascular disease could be reduced with cholesterol lowering agents[11]. Because chemical therapeutics have serious organ toxicity including adrenocortical degeneration, herbal remedies in many countries over the world have become potential candidates for the same purpose with less toxicity[7].

The body's supply of lipid energy comes from the diet in the small intestine or through endogenous fatty acid synthesis, primarily in the liver. Dietary fatty acids are esterified to form triglyceride (TG)[12] and cholesterol, which is stored by fat cells in lipid droplets[11]. Cholesterol, included in steroid lineage, is mainly synthesized in liver by internalizing lipoprotein and concentrated primarily in brain and spinal cord[13]. Upon demand, intracellular triglyceride and cholesterol are hydrolyzed by the action of hormone sensitive lipase to release free fatty acids in the forms of lipoprotein particle and oxidized to generate energy, which is mainly controlled by liver. Lipoproteins, known to transport cholesterol and triglycerol, solubilizes hydrophobic lipid and have searching signals to find target cells. These are as follows: chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL)[14-16]. Related with complication of atherosclerotic vascular disease in hypercholesterolemia, the crucial step is the oxidation of LDL, mainly composed of endogenous cholesterol ester, in the arterial wall. On the contrary, HDL may be protective by reversing cholesterol transport, inhibiting the oxidation of LDL, and by neutralizing the arterogenic effects of oxidized LDL. But in hypertriglyceridemic condition produced by several pathologies, mature HDL levels are reduced, from which hyperglycemia can be also considered as a vascular risk factor. In addition, many of enzymes are related to the absorption and storage of metabolic fatty acid. Diacylglycerol acyltransferase (DGAT)[17,18] catalyzes the final acylation of TG pathway, which is unique to TG synthesis. Acyl-CoA cholesterol acyltransferase (ACAT)[19], responsible for the esterification of cholesterol, is the primary enzyme in the intestinal mucosal cholesterol absorption and synthesizes the cholesterol esters both to flow into very low-density lipoproteins (VLDL) and to store in fatty cells. Because the inhibitors of these enzymes can lower plasma cholesterol and triglyceride levels by inhibiting absorption and storage of metabolic fatty acid, subsequently reduced VLDL production in liver could directly block atheriosclerotic lesion formation reducing the possibility of vascular attacks.

GCT was composed with nine herbal plants which have been well known as clinical and experimental effects on many kind of problems of liver, which play main role to control quantity and quality of blood in oriental medical theory. In the last study, GCT showed liver protective effects in rats administered alcohol[20]. *Artemisiae capillaries,* a main gradient of GCT, has a liver protective effect through inhibition of TGF-β induced apoptosis[21]. In Korea, many oriental medicines to protect and treat liver diseases contain *Artemisiae capillaries* and they also showed positive effects on liver[22,23]. *Polyporus umbellatus* and *Poria cocos* were studied about effects on hepatitis[24,25]. The present studies demonstrate that GCT can reduce the rise in plasma cholesterol and TG levels induced by a high cholesterol diet and also reversed pre-established hypercholesterolemia and hypertriglycemia. Even being decreasing of total cholesterol level, HDL-cholesterol was increased in GCT group, which shows the decrease of other lipoprotein fractions including LDL and IDL (not shown). In the analysis of absolute and relative liver weight, GCT significantly inhibited the weight gain induced by high cholesterol diet compared with induced group. We could suggest that GCT blocks the accumulation of fatty acid in the hepatocyte, then, lipid droplet and apoptotic changes in the GCT group were less serious compared with induced group in the histological observations. In the serum biochemical analysis, TG and total cholesterol levels were lower in GCT group than induced group, on the contrary, HDL-cholesterol levels were higher in GCT group compared with control group. It is obviously valuable in clinical purpose to reduce total cholesterol but to increase HDL-cholesterol by GCT. In the gene expression related with TG and cholesterol metabolism, GCT suppressed ACAT gene expression but not DGAT. By above results, GCT might have some effects to inhibit cholesterol absorption and synthesis related with ACAT, and that GCT's effect on reducing triglyceride resulted from not DGAT but other mechanism.

From this study, we can suggest that GCT extracts possess hypolipidemic effects by lowering serum total cholesterol and TG levels, but enhancing HDL levels. The possible mechanism of GCT effects on therapeutics or prevention for hyperlipidemia may be related to lowering ACAT gene expression, and decreasing intestinal absorption, synthesis or storage of exogenous and endogenous cholesterol. Then Gamichunggantang (GCT) could be used for patients with hyperlipidemia and need to be developed for more specific therapeutics.

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