대한한방내과학회지 제26권 4호(2005년 12월)
Korean J.Orient.Int. Med. 2005:26(4):820-827

Poloxamer-407로 유발시킨 고지혈증에 대한 청간탕의 효과

박종학, 조정효, 신장우, 이연월, 유화승, 이남헌, 윤담희, 조종관, 손창규*
대전대학교 부속 한방병원 내과학교실

Lipid-lowering Effect of Chunggantang in Poloxamer-407 induced Hyperlipidemia Model in Rat

Jong-hak Park, Jung-hyo Cho, Jang-woo Shin, Yeon-Weol Lee, Hwa-seung Yoo, Nam-heon Lee, Dam-hee Yun, Chong-kwan Cho, Chang-gue Son*
Department of Internal Medicine, College of Oriental Medicine, Daejeon University

연구목적: 본 연구는 Poloxamer-407로 유발시킨 고지혈증에 대한 청간탕의 효과를 알아보기 위해 수행되어졌다.
실험방법: Poloxamer-407로 쥐에 고지혈증을 유발시킨후 청간탕과 Lipidil을 경구 투여하여 혈청 cholesterol, 고밀도 지단백, 중성지질을 측정하였으며, 지질대사와 관련된 ACAT, DGAT, CYP7αH, LDL receptor의 gene expression을 RT-PCR를 통해 비교 분석하였다.
실험결과: 청간탕 투여군은 혈청 콜레스테롤을 각각 32% 와 65% ($p<0.05$)로, 혈청 중성지방을 각각 21% 와 51% ($p<0.05$)로 감소시켰다. 또한, 청간탕은 LDL 수용체와 CYP7αH 유전자 발현을 증가시켰다.
결론: 이상의 연구로부터 우리는 청간탕이 지질의 흡수, 저장을 억제하고 콜레스테롤의 분비를 촉진함으로써 고지혈증에 일정한 효과가 있음을 알 수 있었다.

Key Words: Chunggantang, Poloxamer-407, Hyperlipidemia, LDL receptor

Ⅰ. Introduction

Hyperlipidemia is a group of disorders characterized by an excess of fatty substances such as cholesterol, triglycerides and lipoproteins present in the blood. Lipid disorders are one of important risk factors in developing atherosclerosis and heart disease[1,2]. In the previous epidemiological research, it has been recognized that high levels of low-density lipoproteins(LDL) cholesterol increases the risk of coronary heart disease and atherosclerosis, just as high levels of high-density lipoproteins (HDL) cholesterol lower that same risk[3,4].

Many studies have focused on effect of reducing plasma concentrations of low-density-lipoprotein associated cholesterol(LDL-C). However, many of promising agents developed have serious side effects such as failure of adrenal function. As with any drug, cholesterol drugs carry the risk of side effects, which vary depending on the type of drug. For this reason, the concern with natural products which may have less toxicity in medical research for lipid management has been growing in recent years[5,6].

· 접수 : 2005. 8. 26. · 채택 : 2005. 10. 11.
· 교신저자 : Chang-gue Son
(Tel. 82-42-470-9422, Fax. 82-42-229-6968,
E-mail : ckson@dju.ac.kr)

Chunggantang(CGT) is a new herbal formula modified from In-Jin-O-Ryoung-San(Korean pronunciation for 茵蔯五苓散) which has been a standardized formula for liver injury related jaundice for several centuries. CGT has clinically been used in Oriental Hospital of Daejeon University for the treatment of patient with diverse liver diseases since 2000. It has been also pharmacologically therapeutic properties especially for hyperlipidemia, alcohol-induced liver damages and other chronic liver disease[7,8]. However, the effect of CGT on hyperlipidemia has not been examined in laboratory yet.

This present study is aimed to elucidate the effects of CGT on hyperlipidemia by determination of gene expressions related with lipid metabolism, serum triglyceride as well as total and HDL-cholesterol level.

II. Materials and Methods

Materials

Medicinal herbs were purchased from Daejeon Oriental Medical Hospital. The composition of Chunggantang(CGT) formation was mentioned in the Table 1. After drying, 1 day dosage(148g) of the formulation for a human adult was mixed with 2 L of distilled water and left for 1 h at room temperature and the whole mixture was then boiled twice for 1 h each time. The CGT extract was then filtered and freeze dried. The yield CGT extract was 12% (w/w) in terms of the dried medicinal herbs. The CGT extract was suspended in distilled water and given orally to mice once daily for five weeks. Rats in the control group were orally given distilled water(Table 1.).

*DNA taq*polymerase was obtained from Bioneer (Cheongwon, Korea), M-MLV reversetranscriptase was obtained from promega(Madison, USA). TRIzol$^{\circ R}$reagent was obtained from Gibco (Maryland, USA). Lest reagentswere purchased from Sigma (ST. Louis, USA).

Experimental animals

Seven-week old male Sprague-Dawley rats were purchased from commercial animal breeder(Daehan BioLink, Korea). After one week of acclimation, rats were used in this experiment. The rats were housed in an environmentally controlled room at 22

Table 1. Prescription of Chunggantang(CGT)

General Name	Part used	Relative Amount(g)
Artemisia capillaries	Herba	10
Pueraria thuunbergiana	Radix	12
Trionyx sinensis	Carapace	10
Raphanus sativus var	Semen	10
Atractylodes macrocephala	Rhizoma	6
Poria cocos		6
Atractylodes japonica	Rhizoma	6
Pueraria thuunbergiana	Flos	4
Polyporus umbrellatus		4
Amomum villosum Lorur	Fructus	4
Glycyrrhiza uralensis	Radix	2
Total amount		74

±2℃, relative humidity at 55±10% and 12h light/dark and fed with commercial pellets(Samyang Feed Co, Korea) and tap water *ad libitum*.

Thirty two Sprague-Dawley rats were divided into 4 groups of 8 animals each. Hyperlipidemia was induced by 30% poloxamer-407 injection.One mililiter of poloxamer-407 were injected intraperitoneally.

The Rats in treatment group were administrated orally with CGT (200mg, 600mg/kg), Lipidil(3.33 mg/kg) respectively. The Lipidil was used as a positive control. The rats in control group were given 10mL · kg^{-1} of distilled water. On day 6, animals were fasted 16h and whole blood was collected from abdominal aorta. After being clotted for 1 h, the blood was centrifuged at 3000 rpm for 15 min to separate serum. The liver was removed and stored -80℃ till RNA extraction.

Serum biochemical analysis

The levels of serum cholesterol(CHOL), high density lipoprotein(HDL), and triglycerides(TG) were determined using Olympus optical reply(Olympus, Japan).

RT/PCR for analysis gene expression

Total cellular RNA was isolated by the TRIzol^{OR} reagent(Gibco, maryland, USA) according to the manufacturer's instructions. The mRNA levels were fixed quantity at 260nm by spectrophotometer (Cary50, Varian, USA).

Total RNA was extracted from homogenized liver sample of rats. The RNA(1μg) was reverse-transcribed(RT) into first strand cDNA in a RT mixture containing 2μL10mM dNTPs mix, 1μL oligo-dT primer(20pmol/ μL), 2μL 100mM DTT, 4μL 5×RT buffer(250mM Tris-Cl, pH 8.3, 375mM KCl, 15mM $MgCl_2$, RNase inhibitor 20U), 1μL M-MLV RT(200U/μL; Promega, U.S.A) and 2μL DDW. The RT mixture was incubated at 42℃ for 60min, heated to 72℃ for 10min to inactivate the reverse transcriptase activity, and chilled to 4℃ for 5min. A portion of the RT product(1μL) was then subjected to the polymerase chain reaction (PCR) in a DNA thermal cycler(TaKaRa, Tokyo, Japan).

To determine the expression pattern of DGAT, ACAT, CYP7αH and LDLr mRNA in rat liver, 1 μL of cDNA was amplified by a thermal cycler using the primers(Table 2). The PCR mixture was made as followings; 1.5 units of *Taq* DNA polymerase(Bioneer, Korea), 3 μL of 10mM dNTPs, 3μL of 10× PCR buffer, 1μL of 10 pmol sense and antisense primers, and 3 μL of cDNA in 19.7 μL of ultra distilled water.

Table 2. Oligonucleotide sequences of primers

Gene	Primer	Sequence	Product size(bp)
β-Actin	Sense Antisense	5'-GTG GGG CGC CCC AGG CAC CA-3' 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'	539
DGAT	Sense Antisense	5'-GAA TAT CCC CGT GCA CAA GT-3' 5'-CAC AGC TGC ATT GCC ATA GT-3'	255
ACAT	Sense Antisense	5'-CCT CCC GGT TCA TTC TGA TA-3' 5'-ACA CCT GGC AAG ATG GAG TT-3'	370
CYP7αH	Sense Antisense	5'-GGG AGT GCC ATT TAC TTG GA-3' 5'-GAT CCG AAG GGC ATG TAG AA-3'	329
LDL r	Sense Antisense	5'-ACC GCC ATG AGG TAC GTA AG-3' 5'-GTC CCC CAA TCT GTC CAG TA-3'	397

PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95?for 10 min; then 35 cycles at 95℃ for 1 min (denaturing), at 60℃ for 40s(annealing), and at 7 2℃ for 10 min. The PCR products were run on a 1% agarose gel in 0.5× TBE buffer(Table 2.).

Statistical analysis

Results were expressed as the mean±SE. Statistical analysis of the data was carried out by Student's t-test. A difference from the respective control data at the levels of $p<0.05$ and $p<0.01$ were regarded as statistically significant.

III. Results

Serum biochemical analysis

On 6 day after Poloxamer-407 injection, serum cholesterol level was 227mg/dL in control group. But, in CGT 200mg and 600mg/kg treatment groups, serum cholesterol levels were decreased 150 and 77 mg/dL($p<0.05$) respectively(Fig. 1.). Lipidil treatment

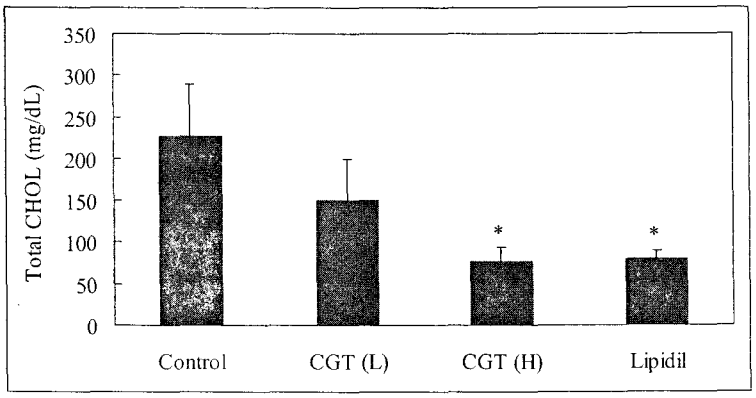

Fig. 1. Serum total cholesterol(CHOL) levels of rats injected poloxamer-407. Control, distilled water; CGT(L), 200mg/kg of CGT; CGT(H), 600mg/kg of CGT; Lipidil, 3.33mg/kg of Lipidil. The values were expressed as the mean±SE. *: p<0.05. significant differences compared with the group administrated distilled water only.

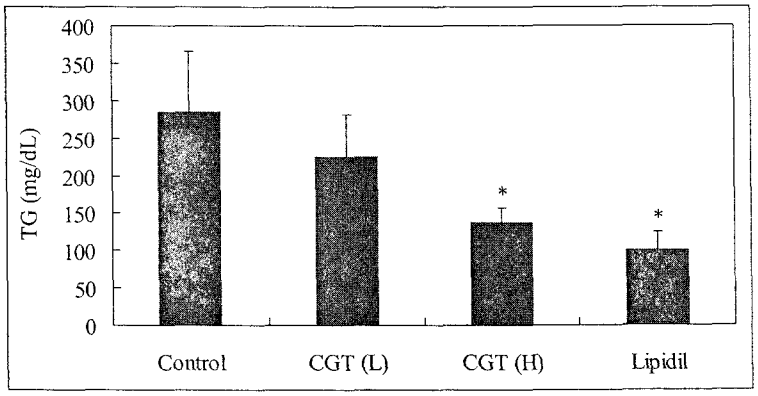

Fig. 2. Serum triglycerides(TG) levels of rats injected poloxamer-407. Control, distilled water; CGT (L), 200mg/kg of CGT; CGT(H), 600mg/kg of CGT; Lipidil, 3.33mg/kg of Lipidil. The values were expressed as the mean±SE. *: p<0.05. significant differences compared with the group administrated distilled water only.

also decreased serum cholesterol level significantly ($p<0.05$). In CGT treatment groups, TG levels were decreased 21% and 51% respectively(Fig. 2.). HDL levels in treatment groups were little decreased (Fig. 3.) but the ratio of HDL to LDL was higher than that in control group. Percentages of serum HDL to serum LDL were 55%, 121%($p<0.05$), 202%($p<0.05$) and 247%($p<0.01$) respectively in each group(Fig. 4.).

ACAT, DGAT, CYP7αH and LDL receptor gene expression

ACAT gene expression was little increased in the treatment groups. DGAT gene was upregulated only in Lipidil treatment group. CYP7αH gene expression was increased about 20% in both of CGT and Lipidil treatment groups and LDL receptor gene expression also increased about 33% in all treatment groups compared with thats of control(Fig 5.).

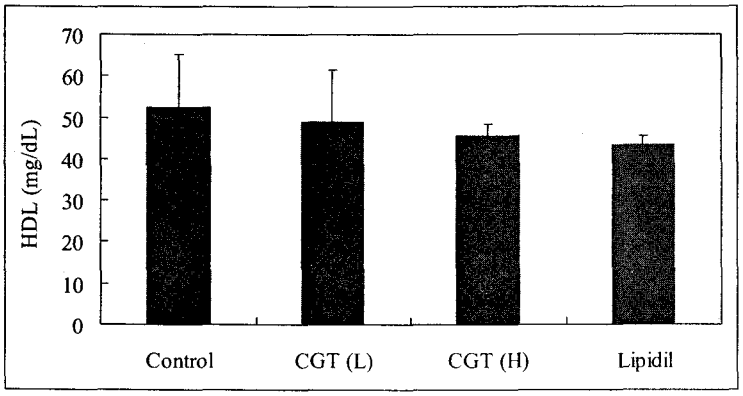

Fig. 3. Serum HDL levels of rats injected poloxamer-407. Control, distilled water; CGT(L), 200mg/kg of CGT; CGT(H), 600mg/kg of CGT; Lipidil, 3.33mg/kg of Lipidil. The values were expressed as the mean±S.E.. *: $p < 0.05$, : significant differences compared with the group administrated distilled water only.

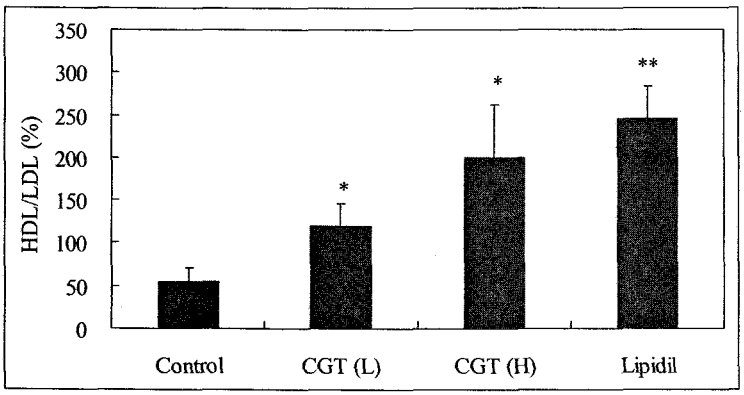

Fig. 4. Percentage of serum HDL to serum LDL in rats injected poloxamer-407. Control, distilled water; CGT(L), 200mg/kg of CGT; CGT(H), 600mg/kg of CGT; Lipidil, 3.33mg/kg of Lipidil. The values were expressed as the mean±SE. *: $p<0.05$, **: $p<0.01$. significant differences compared with the group administrated distilled water only.

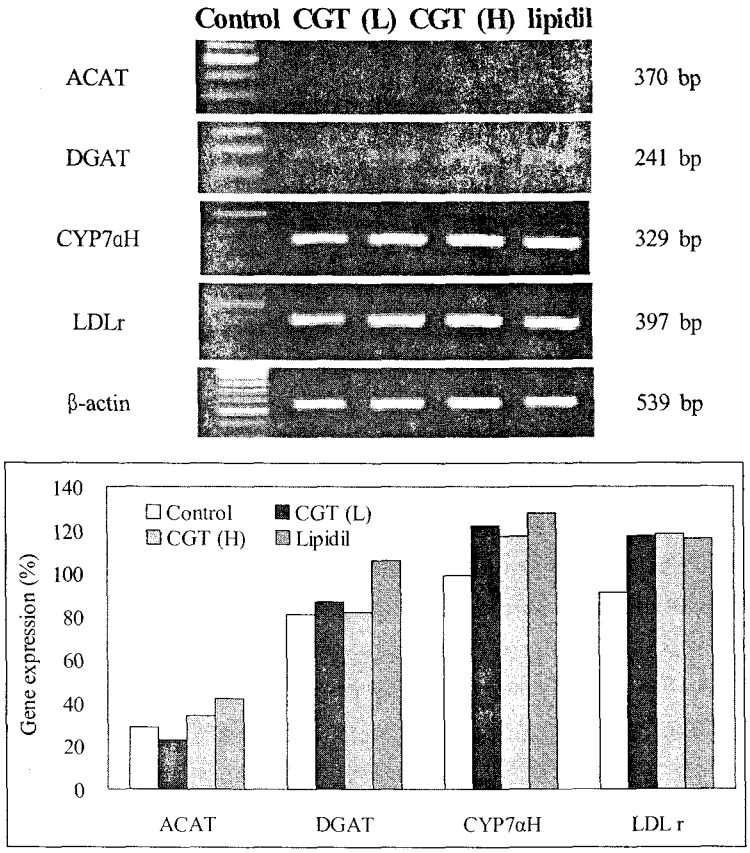

Fig. 5. ACAT, DGAT, CYP7αH and LDL receptor gene expressionin liver of rats injected poloxamer-407. Control, distilled water; CGT(L), 200mg/kg of CGT; CGT(H), 600mg/kg of CGT; Lipidil, 3.33mg/kg of Lipidil. Relative gene expression was expressed as the ratio of each gene expression to β-action expression.

Ⅳ. Discussion

The body's supply of lipid energy come 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 and cholesterol, which is stored by fat cells in lipid droplets[9,10]. Cholesterol, included in steroid lineage, is mainly synthesized in liver by internalizing lipoprotein and concentrated primarily in brain and spinal cord[11]. 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 oxidased 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)[12-14]. Related with complication of atherosclerotic vascular disease in hypercholesterolemia, the crucial step is known to oxidation of LDL, mainly composed of endogenous cholesterol ester, in the arterial wall.

Although our major metabolic energy is originated from oxidation of fatty acid, excessive consumption of fatty acid might come to hyperlipidemia, causing complication of vascular disease[15]. Recently, vascular disease demand an dreadful toll and recent research showed that coronary events in patients with symptomatic vascular disease, can be reduced with cholesterol lowering agents[10]. Therefore, the importance of cholesterol lowering in atherosclerotic vascular disease cannot be overemphasized. In our study, it was found from the result that CGT can reduce the rise in plasma cholesterol and TG levels induced by poloxamer-407. Moreover, the ratio of HDL to LDL in CGT treated group was higher than that in control group.

Cholesterol is normally eliminated into bile. CYP7αH catalyzes the biochemical reaction of cholesterol conversion to 7α-hydroxycholesterol and is considered to be rate controlling[16,17]. Poloxamer-407 was shown downregulation of CYP7αH in mice model[18]. CYP7αH is upregulated by depletion of the bile acid pool and feeding of bile acids inhibits CYP7αH and bile acid synthesis via the classic pathway. CYP7αH is important enzyme witch participates to the synthesis of bile acids from cholesterol and allows its beneficial excretion into gastro-intestinal tract[19]. In the present study, CGT increased CYP7αH gene expression in liver.

The LDL receptor is found on the surface of all cells and it recognizes apoB-100 and apoE[20]. The LDLr and its bound LDL taken an endosomal pathway initiated in clathrin-coated pits on the plasma membrane. In the lysosome, LDL-apoB is degraded to amino acids, while LDL-CE are hydrolyzed to free cholesterol and fatty acids. CGT increased LDL receptor gene expression in liver. LDL receptor upregulation lead to increase of LDL uptake and degradation.

DGAT is a key enzyme in triglyceride synthesis. DGAT catalyzes the final step in the glycerol pathway for triglyceride synthesis in cells. However it is unclear whether DGAT is rate-limiting for triglyceride synthesis[21]. Cholesterol acyltransferase (ACAT) 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[22]. Therefore, 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. In this study, ACAT gene expression was little increased in the treatment groups. But on the other hand, DGAT gene was upregulated only in Lipidil treatment group.

These results lead us to the conclusion that CGT possess hypolipidemic effect by lowering serum total cholesterol and TG levels. The possible mechanism for prevention of hyperlipidemia may be related with upregulation of LDL receptor and CYP7αH gene expression in poloxamer-407 model. Thus, CGT could be used for patients with hyperlipidemia and need to be developed for more specific therapeutics.

REFERENCES

1. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. J Clin Invest. 1973 Jul;52(7):1544-68.
2. Keys A. Coronary heart disease, the global

picture. Atherosclerosis 1975;22:149-92.
3. Price MJ, Shah PK. New strategies in managing and preventing atherosclerosis: focus on HDL. Rev. Cardiovasc. Med. 2002;3:129-37.
4. Rader DJ. High-density lipoproteins and atherosclerosis. Am. J. Cardiol. 2002;90:62-70.
5. Witztum JL. Drugs used in the treatment of hyperlipoproteinemias. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW. The Pharmacological Basis of Therapeutics, 9th. MCGTraw-Hill, New York. 1996;875-97.
6. Deepak B. Lipid-lowering drugs in the management of hyperlipidemia. Pharmacol. Ther. 1998; 79:205-30.
7. Son CG, Choi BL, Shin JW, Choi WJ, Cho JH, Cho CK. Effects of GamiChunggantang(GCT) on Alcoholic Metabolism and Alcoholic Liver Disease. Korean Journal of Oriental Medicine. 2001;2(1):89-98.
8. Cho JH, Lee YY, Seo SH, Yoo HS, Choi WJ, Lee YW, Son CG, Cho CK. A Clinical Report about 57 Patients with Chronic Liver Disease. Korean Journal of Oriental Medicine. 2000;21 (4):112-21.
9. Fernando DB, Carla DB, Marcelo C, Mario JY, Jean-Charles F, Graciela R, Castro R. VLDL compositional changes and plasma levels of triglycerides and high density lipoprotein. Clinica Chemica Acta. 1998;269:107-24.
10. Allan DS, Katherine C. Lipid and vascular disease: what do and do not know. Clinica Chemica Acta.. 1999;286:7-22.
11. Laura L, Natalie JM. Intracellular transport. Biochemica et Biophysica Acta. 1999;19-37.
12. She M, Wang Z. The protective role of high-density lipoproteins in atherosclerosis/experimental. Gerontology. 1999;34:539-48.
13. Hessler JR, Robertson ALJ, Chisolm GMI. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endithelial cells in culture. Atheriosclerosis. 1979; 32:213-29.
14. Mackness MI, Durrinngton PN. HDL, its enzymes, and its potential to influence lipid peroxidation. Atheriosclerosis. 1995;115:243-53.
15. Jumana S, Allan DS, Katherine C. Regulation of plasma fatty acid metabolism. Clinica Chemica Acta. 1999;286:163-80.
16. Shefer S, Hauser S, Berkersky I, Mosbach EH. Biochemical site of regulation of bile acid synthesis in the rat. J. Lipid Res. 1970;11: 404-11.
17. Myant NB, Mitopoulos KA. cholesterol 7α -hydroxylase. J. Lipid Res. 1997;18:135-53.
18. Johnston TP, Nguyen LB, Chu WA, Shefer S. Potency of select statin drugs in a new mouse model of hyperlipidemia and atherosclerosis. International J. Phamaceutics. 2001;229:75-86.
19. Chiang JYL, Miller WF, Lin GM. Regulation of cholesterol 7 alpha-hydroxylase in the liver. J. Biol. Chem. 1990;265:3889-97.
20. Schneider WJ, Bbeisiegel U, Goldstein JL, Brown MS. Purification of the low density lipoprotein recptor, an acidic glycoprotein of 164,000 molecular weight. J. Biol. Chem. 1982;269:2664-73.
21. Lehner R, Kuksis A. Biosynthesis of triacylglycerols. Prog Lipid. Res. 1996;35:169-201.
22. Keisuke K, Toshiya T, Rina A, Kouki T, Toshinobu M, Notio N, Takao O. Structure of the human acyl-CoA:cholesterol acyltransferase-2 (ACAT-2) gene and its relation to dyslipidemia Biochimina et Biophysica Acta. 2001;1531:230-40.