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Effect of garlic extract on some serum biochemical parameters and expression of *npc1l1*, *abca1*, *abcg5* and *abcg8* genes in the intestine of hypercholesterolemic mice

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Some compounds in the garlic inhibit cholesterol synthesis, resulting in lowering of serum cholesterol and triglycerides and increase in HDL level. However, the mechanism of this specific effect is not fully understood. In the small intestine, ATP-binding cassette transporters G5, G8 and A1 (ABCG5, ABCG8 and ABCA1), as well as Niemann-Pick C1 like 1 (NPC1L1) protein have important roles in cholesterol metabolism. In this study, we evaluated the beneficial effect of aqueous extract of garlic on lipid profile and also expression of *npc1l1*, *abca1*, *abcg5* and *abcg8* genes in the intestine of N-Marry mice fed a high cholesterol diet as a possible mechanism of garlic effect. Twenty-four mice were randomly divided into three groups: Group 1: hypercholesterolmic (received chow + 2% cholesterol + 0.5% cholic acid); Group 2: garlic (received chow + 4% (w/w) garlic extract + 2% cholesterol + 0.5% cholic acid); and Group 3: received chow only. After one month, mice were anesthetized and blood was collected from their heart. The jejunum was removed, washed with PBS and entrocytes were scraped and used for the experiments. Serum lipids were measured enzymatically and expression of mRNA levels for the above-mentioned proteins was determined by semi-quantitative RT-PCR. Garlic extract significantly reduced serum lipids ($p<0.05$), compared with the hypercholesterolemic group. Expression of the intestinal *npc1l1* was significantly decreased ($p<0.01$) in the garlic group, compared with the chow group, while *abcg5* ($p<0.01$), *abcg8* ($p<0.01$) and *abca1* ($p<0.05$) expressions were significantly increased. In conclusion, this study reveals a possible mechanism for the beneficial effects of the garlic in lowering serum lipids by decreasing the intestinal lipid absorption and increasing excretion of cholesterol back into the intestinal lumen.

Keywords: Garlic, Lipid profile, *npc1l1, abca1, abcg5, abcg8,* Hypercholesterolemic, Gene expression

Earlier studies have confirmed that lowering plasma total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and increasing high density lipoprotein cholesterol (HDL-C) are very useful in preventing the risk of cardiovascular diseases (CVD)[1-3].

Lipid lowering drugs, such as HMG-CoA reductase inhibitors and bile acid sequestrates have been successfully used for treating high-risk people. All of these drugs have adverse effects and some have been associated with possible carcinogenicity. For these reasons, many people tend to use natural substances for their ailments. Different forms of garlic extracts represent a common example of such natural substances that have shown beneficial effects in the management of variety of aspects of cardiovascular diseases[4]. Garlic has also shown antioxidant, anti-lipemic, hemostatic, hemodynamic and anti-platelet-aggregation activities[5].

Intestinal absorption is a good candidate process for control of hypercholesterolemia. In the small intestine, a number of proteins, such as scavenger receptor class B member 1 (SR-B1), aminopeptidase N and Niemann-Pick C1 like 1 (NPC1L1) have been proposed as cholesterol transporters. These transporters have a critical role in cholesterol uptake by enterocytes. Furthermore, studies on the ATP-binding cassette (ABC) transporters G5 and G8 (ABCG5 and ABCG8) proteins, known as intestinal cholesterol efflux transporters have provided definite evidence that cholesterol absorption is a protein-mediated, selective and active process[6]. Another intestinal protein, the ATP-binding cassette (ABC) transporter 1 (ABCA1), also has a crucial role in HDL formation. Mutations in

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Abbreviations: ABCA1, ATP-binding cassette transporter protein A1; ABCG5, ATP-binding cassette transporter protein G5; ABCG8, ATP-binding cassette transporter proteins G8; CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, Low-density lipoprotein cholesterol; LXR, liver X receptor; NPC1L1, Niemann-Pick C1 like 1; TG, triglyceride.

the ABCA1 cause the Tangier disease. This disease is characterized by almost complete absence of plasma HDL, abnormal build-up of cholesteryl esters in many tissues and early occurrence of atherosclerosis[7].

In this study, we have investigated the beneficial effects of consuming aqueous extract of garlic on lipid profile and expression of *npc1l1, abca1, abcg5* and *abcg8,* which are involved in cholesterol absorption and transport, in the intestine of mice (N-marry) fed a high-cholesterol diet.

Materials and Methods

Animals and treatment

Twenty-four N-marry mice (3-4 months old) weighing 28-32 g were acclimatized for 2 weeks prior to the experiments[8]. Animals were maintained at room temperature with a photoperiod of 12 h light/12 h dark and temperature of 24 ± 1ºC. The study was approved by the Ethics Committee of Kerman University of Medical Sciences, Iran.

Animals were randomly divided into three groups (n = 8): Group 1: the hypercholesterolmic group (received chow + 2% cholesterol + 0.5% cholic acid): Group 2: the garlic group (received chow + 4% (w/w) garlic extract powder + 2% cholesterol + 0.5% cholic acid), and Group 3: received the chow only. The dose of garlic extract was selected on the basis of previous experiment[3]. After one month, animals were starved overnight for 12 h, anaesthetized with ether and blood was collected from their heart. Jejunum was removed and washed with phosphate buffered saline (PBS) and entrocytes were scraped and used for the experiments. For serum preparation, blood samples were centrifuged at 1000 × *g* for 15 min[9].

Water extract of garlic

Cloves of garlic obtained from the local market were washed with distilled water, crushed and dried at room temperature. The shadow-dried garlic (20 g) was pulverized and macerated in 200 ml of distilled water for 24 h. After filtration, the extract was dried at 40°C in an incubator. Dried extracts were powdered and kept in dark vials at -20°C[10]. Powdered extract was mixed with animal's diet in a 4% w/w ratio.

Biochemical determinations

Triglycerides (TG) and total cholesterol were measured by an enzymatic method[11-13]. LDL-C and VLDL-C were calculated using the Friedwald equation: [LDL-C (mg/dl) = Total cholesterol (mg/dl) - (HDL-C (mg/dl) + TG/5) (mg/dl)][12,13].

Semi-quantitative RT-PCR

Total RNA was isolated from the enterocytes using AccuZolTM Total RNA Extraction Reagent (Bioneer, Korea) according to the manufacturers' protocol. cDNA was synthesized according to the manufacturer's instructions (Fermentas, Lithuania). Thirty-five cycles of PCR amplification were performed with denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. All reactions were finished with a single extension cycle at 72°C for 5 min. Products were then electrophoresed on 1% agarose (Sigma) gel and band densities were quantified by densitometry using Lab Works analyzing software (UVP, UK)[14,15].

The following primers were used in the study: Mouse β-actin — forward: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', reverse: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; *abcg5* — forward: 5'-TGC CCT TTC TGA GTC CAG AG-3', reverse: 5'-GTG CTC TTT CAA TGT TCT CCA G-3'; *abcg8* — forward: 5'-ATG AGC TGG AAG ACG GGC TG-3', reverse: 5'-GCC AGT GAG AGC AAG GCT GA-3'; *abca1* — forward: 5'-TCT CTG CTA TCT CCA ACC TCA TC-3', reverse: 5'-ACG TCT TCA CCA GGT AAT CTG AA-3'; and *npc1l1*— forward: 5'-GCT TCT TCC GCA AGA TAT ACA CTC CC-3 and reverse: 5'-GAG GAT GCA GCA ATA GCC ACA TAA GAC-3'.

Statistical analysis

Data were analyzed using one-way with ANOVA, followed by Tukey test and expressed as mean ± SEM. P-value less than 0.05 was considered as statistically significant[16].

Results

Lipid profiles

After four weeks of feeding animals with cholesterol supplemented diet, significant changes were found in the serum lipid profiles in the hypercholesterolemic group, compared with those in normal diet group. In the hypercholesterolemic group, LDL-C, VLDL-C and TG were significantly increased (Table 1). No significant changes were observed in the serum HDL in the garlic group, compared with the hypercholesterolemic group. The mean serum TC, LDL-C, VLDL-C and TG were significantly decreased in the garlic group, compared with the hypercholesterolemic group ($P<0.05$) (Table 1).

Table 1—Serum lipid profile in different groups of mice after four weeks of treatment

[Data represent mean ± SEM (n = 8)]

Biochemical factors	Hypercholestrolemic	Garlic	Chow
Body weight (g)	37.5 ± 1.5[f]	35.7 ± 0.8[e]	30.7 ± 0.7
Fasting blood glucose (mg/dl)	160.1 ± 5.6	152.7 ± 4.6	138.1 ± 9.7
TC (mg/dl)	230.1 ± 4.5[f]	191.4 ± 5.4[a]	129 ± 13.4
TG (mg/dl)	160.5 ± 7.5[e]	137.4 ± 4.5[a]	133.5 ± 4.0
VLDL-C (mg/dl)	32.1 ± 1.5[e]	27.5 ± 0.9[a]	26.7 ± 0.8
HDL-C (mg/dl)	110.2 ± 8.1[e]	106.2 ± 3.8[e]	87.4 ± 9.7
LDL-C (mg/dl)	92.8 ± 9.8[f]	57.6 ± 7.4[a]	24.9 ± 5.5
HDL-C/LDL-C ratio	1.195 ± 0.5[d]	1.859 ± 0.4 [b]	3.625 ± 0.7
Non-HDL-C	120 ± 6.3[e]	85 ± 5.5[b]	42 ± 6.1

Group I; Hypercholestrolemic, Group II; Garlic, Group III; Chow, TC; total cholesterol, TG; triglyceride, LDL-C; low-density lipoprotein cholesterol, HDL-C; high-density lipoprotein cholesterol, VLDL-C; very low-density lipoprotein cholesterol.

[a]$p<0.05$ as compared to hypercholestrolemic group; [b]$p<0.01$ as compared to hypercholestrolemic group; [c]$p<0.001$ as compared to hypercholestrolemic group; [d]$p<0.05$ as compared to chow group; [e]$p<0.01$ as compared to chow group; [f]$p<0.001$ as compared to chow group.

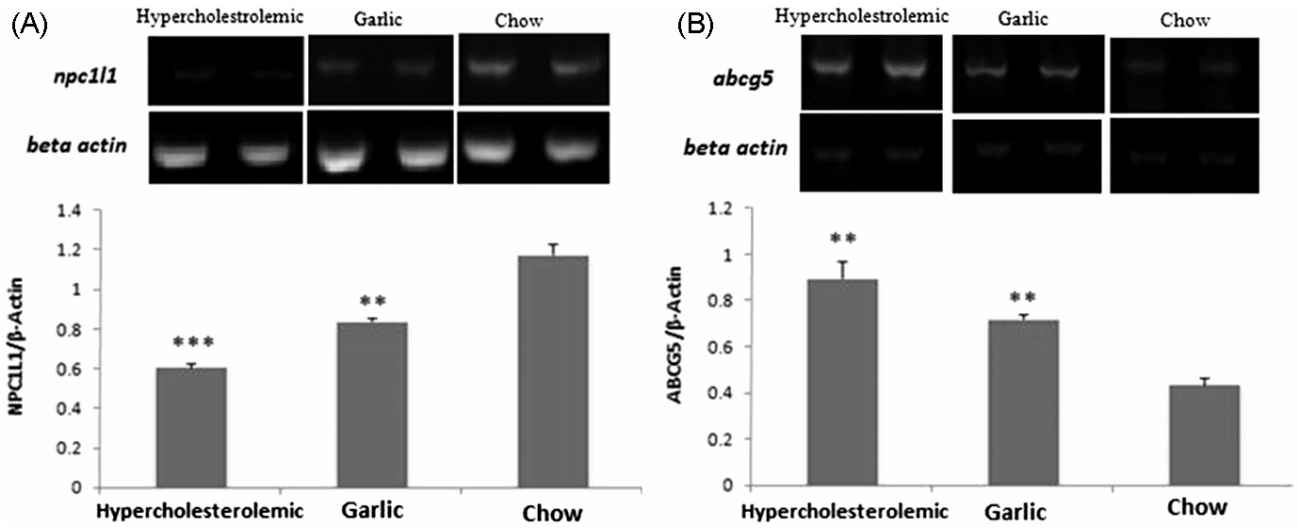

Fig. 1—(**A**) Expression of npc1l1 mRNA in intestine of hypercholesterolemic, garlic and chow groups (n = 8) [npc1l1 mRNA expression was significantly reduced in hypercholesterolemic and garlic groups. ***P<0.001 and **P<0.01 as compared to chow group. Data presented as mean ± SEM]; (**B**) Expression of abcg5 mRNA in intestine of hypercholesterolemic, garlic and chow groups (n = 8) [Abcg5 mRNA expression was significantly increased in hypercholesterolemic and garlic groups. **P<0.01 as compared to chow group. Data presented as mean ± SEM]

Gene expression

We studied the effect of garlic extract on the regulation of *npc1l1, abca1, abcg5* and *abcg8.* Dietary supplementation with 2% cholesterol and 0.5% cholate is usually used to provoke atherosclerosis in mice. The feeding of cholesterol or cholate alone has fewer prominent effects[17]. Semi-quantitative RT-PCR analysis of intestine membrane revealed a marked down-regulation of *npc1l1* in response to the dietary ($P<0.01$) garlic and hypercholestrolemic diet (2% cholesterol and 0.5% cholate) ($P<0.001$). Also, *abcg5* and *abcg8* mRNA significantly increased in the garlic and hypercholestrolemic groups ($P<0.001$). The increase of *abcg5* and *abcg8* mRNA in the hypercholestrolemic group was higher than the garlic group. Expression of *abca1* significantly increased ($P<0.05$) in the garlic extract and hypercholestrolemic groups (Fig. 1).

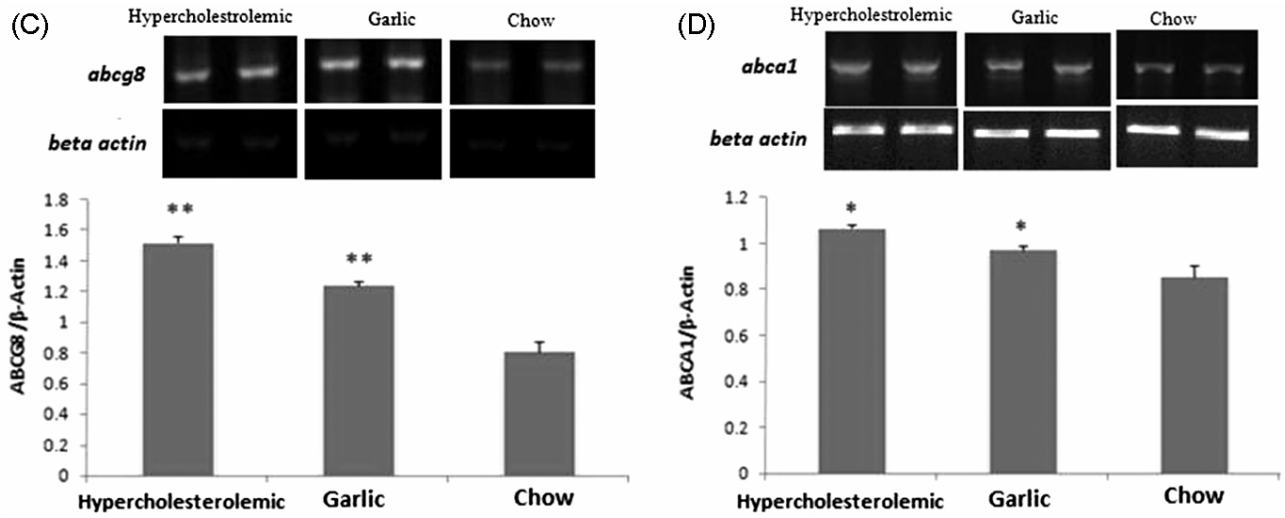

Fig. 1—(**C**) Expression of *abcg8* mRNA in intestine of hypercholesterolemic, garlic and chow group (n = 8) [*abcg8* mRNA expression was significantly increased in hypercholesterolemic and garlic groups. **P<0.01 as compared to chow group. Data presented as mean ± SEM]; and (**D**) Expression of *abca1* mRNA in intestine of hypercholesterolemic, garlic and chow group (n = 8) [*npc1l1* mRNA expression significantly increased in hypercholesterolemic and garlic groups. *P<0.05 as compared to chow group. Data presented as mean ± SEM]

Discussion

Dietary supplementation with 2% cholesterol and 0.5% cholate is usually used to provoke atherosclerosis in mice. The feeding of cholesterol or cholate alone has fewer prominent effects[17]. Some studies have demonstrated the cholesterol lowering effect of garlic in hypercholesterolemic animals[5,17], while others have shown that garlic has no beneficial effect on the lipid profile[18-20]. This study showed that treatment with aqueous extract of garlic for four weeks significantly reduced TC, LDL-C, VLDL-C and TG in the hypercholesterolemic rats, suggesting the garlic having an effective lipid lowering activity. Garlic contains allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate), which is thought to be the principal bioactive compound present in aqueous extract or raw garlic homogenate, It also contains S-allyl cystein (SAC) and di-allyl-disulfide (DADS), which are potent inhibitors of cholesterol synthesis[5,21]. The cholesterol lowering effect observed with garlic extract might be due to the inhibition of HMG-CoA reductase and cholesterol biosynthesis[22]. Garlic extract is also shown to decrease plasma TG level in the mice fed on garlic containing diets; the effect has been attributed to the inhibition of hepatic fatty acid synthesis[15].

Differences in HDL levels were not significant between the garlic extract and hypercholesterolemic groups. The garlic extract markedly increased HDL levels in comparison with chow diet. This result was contrary to an earlier study, which has shown that aqueous extract of garlic has no effect on HDL levels[24].

ABCG5 and ABCG8 are expressed mainly in the hepatocytes and enterocytes. ABCG5 and ABCG8 deficiency, which occurs in sitosterolemia is associated with increasing cholesterol absorption and a decrease in biliary sterols excretion[25]. As evident from this study, these proteins increased in the hypercholesterolemic and garlic extract treated groups, compared with the chow group. This result was similar to the results obtained in an earlier study, which has shown that consumption of high-cholesterol diet leads to a significant increase in the expression of both genes[26]. It is also shown that increased expression of ABCG5 and ABCG8 in intestine and liver leads to a significant rise in biliary cholesterol secretion and hepatic cholesterol synthesis, as well as reduction of cholesterol absorption[27].

Intestinal ABCA1 is known as a sterols transporter and lack of this protein leads to Tangier disease (severe HDL deficiency). Inactivation of *abca1* increases dietary cholesterol absorption. Diet supplemented with cholesterol up-regulates ABCA1 expression[28]. In agreement with an earlier study[29], results in our study showed that *abca1* expression was increased significantly in both hypercholesterolemic and garlic extract treated groups, compared with chow control. Increase in ABCA1, ABCG5 and ABCG8 in cases of high intracellular-free-cholesterol

or administration of liver X receptor (LXR) agonists suggests that ABCG5 and ABCG8 transport intracellular sterols out of the cells, probably in assistance with ABCA1[29].

NPC1L1 has been recognized as a critical protein in the intestinal cholesterol absorption. It has been shown that NPC1L1 is highly expressed in the jejunum, the middle section of the small intestine. Ezetimibe, a cholesterol lowering drug, through the inhibition of NPC1L1 reduces cholesterol absorption, as well as plasma cholesterol and subsequently inhibits the atherosclerosis development and progression in mice[30].

In this experiment, NPC1L1 expression was significantly reduced in the hypercholestrolemic and garlic groups, compared with the chow group. The difference in the expression of NPC1L1 between the garlic extract and hypercholestrolemic groups was not significant; however, the reduction in the expression of NPC1L1 in the hypercholestrolemic group was higher than the garlic group. Earlier, it has been shown that feeding mice with a diet containing 1% cholesterol and 0.5% cholate leads to 75% decrease in intestinal *npc1l1* expression in wild-type mice, compared with chow-fed mice. The *npc1l1* is localized in the jejunal enterocytes and has a critical role in the intestinal cholesterol and phytosterol absorption[31,32].

In conclusion**,** this study shows beneficial effects of garlic extract consumption on the cardiovascular risk factors in a short period of time. Garlic might inhibit intestinal cholesterol absorption by reducing expression of *npc1l1* and increasing expression of *abcg5,* and *abcg8.* The increased expression of *abca1* leads to rise of HDL levels. These findings reveal a possible mechanism for the lipid lowering effect of the garlic. However, further studies are needed to assay NPC1L1, ABCA1, ABCG5 and ABCG8 protein levels.

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