

Effects of garlic on brachial endothelial function and capacity of plasma to mediate cholesterol efflux in patients with coronary artery disease

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ABSTRACT

Objective: This study investigated the effects of garlic on brachial endothelial function and THP-1 macrophage cholesterol efflux (CE) and examined whether garlic modulates ATP-binding cassette (ABC) A1 and ABCG1 mRNA expressions in peripheral blood mononuclear cells (PBMCs) isolated from patients with coronary artery disease (CAD).

Methods: In this randomized, placebo-controlled trial, patients with CAD were randomly divided into two groups: those receiving garlic powder or placebo tablets twice daily for 3 months. Brachial flow-mediated dilation (FMD) was assessed using ultrasound. Fasting blood samples were collected before and after period and PBMC and plasma were isolated. Human THP-1 monocytes were differentiated into macrophages, labeled with 3H-cholesterol, and incubated with plasma samples, and CE was assessed. ABCA1 and ABCG1 mRNA expressions were determined in PBMCs.

Results: After 3 months, brachial FMD values significantly improved (50.7%) in the garlic group compared with those in the placebo group ($p=0.016$). High-sensitive C-reactive protein (hs-CRP) levels significantly decreased in the garlic group, but the difference between the two groups was not statistically significant. No significant difference was observed with regard to CE and ABCA1 and ABCG1 mRNA expressions in PBMCs. CE was negatively correlated with hs-CRP levels.

Conclusion: Short-term treatment with garlic may improve the endothelial function and may affect hs-CRP levels; however, it could neither significantly improve THP-1 macrophage CE nor affect ABCA1 or ABCG1 expressions in PBMCs. (*Anatol J Cardiol* 2017; 18: 116-21)

Keywords: garlic powder tablet, cholesterol efflux, ATP-binding cassette transporter A1, hs-CRP, flow-mediated dilation

Introduction

Reverse cholesterol transport (RCT) is a physiological process by which cholesterol in peripheral tissues is transported by high-density lipoprotein (HDL) to the liver for excretion in the bile and feces (1). The first step is cholesterol efflux (CE) from cell membranes to circulating cholesterol acceptors. A cohort study revealed that CE capacity was inversely associated with cardiovascular diseases (2). Studies have shown that impaired CE is a factor that leads to cholesterol accumulation in macrophages and potentially fatal atheroma development in arteries. Therefore, increasing the CE capacity from macrophages may be an effective strategy for primary and secondary prevention of atherosclerosis (3–5).

ATP-binding cassette (ABC) A1 and ABCG1 are two major cellular transmembrane proteins that mediate CE. Animal studies

have demonstrated that ABCG1 and ABCA1 are key mediators of CE and play key roles in facilitating CE and RCT (6). Endothelial dysfunction precedes the manifestation of clinical cardiovascular problems and may have a critical role in its pathogenesis. Risk factors for coronary atherosclerosis, causing endothelial dysfunction, characterized by disorder in vasodilation, increase in adhesion molecule expression, and increase the risk for thrombosis. Studies have shown that brachial flow-mediated dilation (FMD), measured using ultrasound, is correlated with endothelial function in coronary arteries, and impaired brachial FMD is related to the prevalence of atherosclerosis in coronary arteries and predicts cardiovascular problems (7). Garlic has been used as food and medicine for many years; however, there is little scientific evidence regarding its therapeutic properties. Some sulfur-containing compounds such as allicin, ajoene, S-allylcysteine, S methylcysteine, diallyl disulfide, and sulfoxides may be

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responsible for the antiatherosclerotic activity of garlic (8–11). Aged garlic extracts increase the expressions of CE mediators or inhibit CD36 expressions and oxidized low-density lipoprotein uptakes in the human monocyte/macrophage cell line (THP-1) (12, 13). Similar to intact garlic cloves, dried garlic powder contains alliin, which is the inactive precursor of the biologically highly potent and powerful smelling allicin. The conversion of alliin to allicin depends on the enzyme alliinase, which is present in fresh garlic and in dried garlic powder tablets. Alliinase is not released from dried garlic tablets until the tablets are dissolved in the gastrointestinal tract (14). This study aimed to examine the effects of garlic tablet treatments on the ability of plasma to promote CE from macrophages and also the effects on brachial endothelial function in patients with severe CAD after angioplasty.

Methods

Study design and intervention

This study was a randomized, placebo-controlled, clinical trial. Male and female patients aged 25–75 years with severe coronary artery disease (CAD) who underwent angioplasty were recruited from the Rajaei Cardiovascular, Medical and Research Center, a university-affiliated medical center, from August 2013 to April 2014. Exclusion criteria included recent acute coronary syndrome (<6 months), smokers, diabetes, chronic kidney disease, body mass index (BMI) of >30 kg/m², consistent garlic use in the last month, and use of antioxidant supplements such as vitamin C supplements, vitamin E supplements, and selenium supplements. All patients were from urban regions. They gave written and informed consent, and the study was approved by the National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences Ethics Committee. The clinical trial has been registered in the public trials registry; the registration number is NCT01948453 (<https://register.clinicaltrials.gov>). Following baseline assessment, patients were randomized to receive either garlic powder or placebo tablets twice daily for 3 months, in adjunct to their prescribed medications. Garlic tablets were commercially manufactured (Amin Pharmaceutical Company, Isfahan, Iran) and contained alliin, ajoene, diallyl disulfide, and diallyl trisulfide, in addition to 1200 mg allicin per tablet. Placebo tablets were visually identical to the garlic tablets and contained corn starch. Simple randomization was conducted using blocks of 10 sealed opaque envelopes, assigning five patients to receive garlic tablets and five to receive placebo tablets. Treatment was initiated within 3 days following angioplasty. Blood pressure was measured by a trained research nurse, using a single calibrated mercury sphygmomanometer and appropriate sized cuffs, with their arm supported at the heart level and after 5-min rest. BMI was calculated as weight (in kilogram) divided by height squared (in m²). The patients in the two groups received dietary advice from a trained dietitian. The recommended diet was based on the therapeutic lifestyle change, and the weight maintenance energy calorie of

diets was estimated. All the patients completed a take home 3-day food recall (two weekdays and one weekend) at baseline and after completing the intervention, and these records were verified by a nutritionist. The Nutritionist IV software (First Databank, Hearst Corp, San Bruno, CA, USA) was used to compute dietary intakes of macronutrients and energy. Fasting plasma samples were collected for measuring lipids, lipoprotein a [Lp (a)], apolipoprotein A1 (Apo A1), high-sensitive C-reactive protein (hs-CRP), and CE. Plasma Apo A1, hs-CRP, and Lp (a) levels were determined by an immunoturbidimetric method, and HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) levels were determined using colorimetric enzymatic methods; all were determined using commercial kits (Pars Azmoon Co) with the Selectra E instrument.

CE from THP-1 macrophages to plasma in study patients

Human THP-1 monocytes (National cell bank, Pasteur Institute) were maintained in RPMI 1640 (Gibco, Maryland, USA) containing 10% fetal bovine serum (FBS). CE was assayed as previously described (14). The cells were plated on 24-well plates and treated with 100 ng/mL phorbol 12-myristate 13-acetate (Santa Cruz, CA, USA) in the growth medium. After 72 h, the medium was replaced with fresh medium containing 5% FBS, and macrophages were loaded with 3H-cholesterol (specific radioactivity, 53 Ci/mmoL; final radioactivity, 1 µCi/mL; Perkin Elmer, Waltham, MA, USA) for 48 h. After labeling, cells were washed with PBS; incubated overnight in a serum-free medium in the presence of the liver X receptor (LXR) agonist TO-901317 (Sigma, St. Louis, MI), with a final concentration of 1 µmol/L; and then incubated for further 2 h at 37°C with serum-free medium containing 2% plasma obtained from the study patients. The medium was removed and centrifuged. Total cell radioactivity was determined by adding 0.5 M NaOH to the cells. Medium and cell-associated radioactivity in the media was assayed by liquid scintillation counting using a Beckman counter (Biochemistry Department, Pasteur Institute). CE was determined as the radioactivity in the medium divided by that in the medium plus cells, after subtracting the background efflux in control incubations without added plasma and expressed as a percentage.

Real-time PCR

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll density gradient centrifugation, washed, and stored at –80°C until RNA extraction. Total RNA of PBMCs was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Maxima H Minus First Strand cDNA Synthesis kits (Thermo Scientific, Wilmington, DE, USA) was used for cDNA synthesis. Real-time PCR was performed using the BioRad MiniOpticon device and Maxima SYBR-green PCR Master Mix (Thermo Scientific). Primers were designed using the oligo primer analysis software and were either located in different exons or across exon–exon boundaries. In this study, the primer sequences used were as follows: ABCA1 (108 base pairs) forward:

5'-TCTGTAATGCCAACAACCCTG-3', reverse: 5'-ATGCCTTCATGCTGGTGTCT-3'; ABCG1 (113 base pairs) forward: 5'-GTCGCTC-CATCATTTGCACCA-3', reverse: 5'-ATTGCAGACTTTTCCCGG-GTA-3'. Peptidylpropyl isomerase B was amplified as the house keeping gene (forward 5'-GTGGATAATTTGTGGCCTTAGC-3' and reverse: 5'-GCCCTGGATCATGAAGTCCT-3'). Thermocycler conditions included holds for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and 30 s at 72°C. A melting curve analysis was performed for each reaction with 55°C–95°C ramp. Normalized Ct or delta Ct ($\Delta\text{Ct} = \text{Ct gene} - \text{Ct housekeeping}$) value was calculated, and the ΔCt data were statistically analyzed.

FMD

Brachial FMD was assessed by performing ultrasound, a non-invasive method, of the endothelial-dependent flow-mediated vasodilation of the brachial artery by a single radiologist blinded to the patients' data. All the patients were examined in the supine position, and measurements were conducted in a quiet environment. After approximately 10-min rest in a temperature-controlled environment (21°C), the endothelial function was assessed. To assess brachial FMD, the right brachial artery diameter was measured both at rest and during reactive hyperemia. Reactive hyperemia was induced by inflating a blood pressure cuff placed around the forearm to a pressure of 250 mmHg for 5 min. The brachial artery was imaged above the antecubital fossa in the longitudinal plane. Measurements of the arterial diameter were performed at a fixed distance from an anatomic marker at rest and at 30, 60, and 90 s after releasing the cuff. The diameter of the vessel in scans after reactive hyperemia was expressed as the percentage relative to the resting scan. The greatest value between 30 and 90 s was used to report the maximum FMD.

Statistical analysis

The minimum sample size was determined on the basis of a priori power analysis, considering FMD as the primary efficacy variable. The power analysis was conducted using the G* Power version 3.1.9.2 (Buchner, Erdfelder, Faul and Lang, 2014) on the basis of a two-sided t-test with $\alpha=0.05$, $\text{power}=0.80$, and an estimated medium effect size of $d=0.64$. Data for calculating the effect size were obtained from a previous similar study of patients with CAD (15). These calculations suggested the need for a minimum sample size of 40 patients (20 in each group). To determine the normality of data distribution, Kolmogorov–Smirnov test was performed. Plasma CE after 3 months was normally distributed. Therefore, log transformation was used in statistical tests. Paired t-test was used to compare before and after differences of CE, ΔCt mRNA expressions, endothelial function, and plasma variables in each group. Differences in mean values between the garlic and placebo treatments were estimated using independent t-test (for normally distributed data) or Mann–Whitney U test (for non-normally distributed parameters). Correlation data that assessed the association between plasma values and CE were analyzed using Pearson correlation analysis. A p value

Table 1. Baseline characteristics of the patients

Variable	Garlic group (n=21)	Placebo group (n=21)	P
Age, year	56.89±10.09	63.04±9.68	0.06
Sex, % male	68.40	73.90	0.69
BMI, kg/m ²	25.80±3.35	25.40±2.91	0.68
Systolic BP, mm Hg	121.89±15.68	120.91±13.54	0.82
Diastolic BP, mm Hg,	77.21±10.98	78.26±09.51	0.74
Ejection fraction, %	45.78±06.72	43.69±08.28	0.38

Data represent mean±standard deviation or percentages of patients for dichotomous measures. Patients were recruited from August 2013 to April 2014

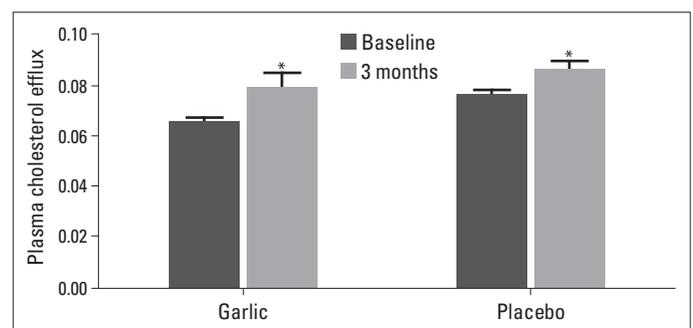


Figure 1. Effects of garlic or placebo tablets on cholesterol efflux from THP-1 macrophages to 25 plasma

Data are mean±standard error. *Significant difference compared to baseline ($p<0.05$)

of <0.05 were considered to be statistically significant. All statistical analyses were conducted using the SPSS software package (SPSS, Chicago, IL, USA).

Results

Of the 57 patients screened, only 42 plasma samples were available for measuring all parameters such as lipids, CE, and mRNA expressions (21 patients in each group). The clinical characteristics of the patients are shown in Table 1. Clopidogrel, aspirin, ACE inhibitor/ARB, and statins (atorvastatin) were prescribed to all the patients. There were no differences between and within both the groups with respect to total daily calorie and macronutrient intake at the study baseline. In addition, both the groups consumed the recommended diet (data not shown). Baseline plasma CE was different between the two groups ($p=0.001$). However, after 3 months of treatment, no difference was observed between the two groups using the analysis of covariance as the statistical test and considering baseline values as covariates (Fig. 1).

ABCA1 mRNA expressions decreased in both the groups, whereas ABCG1 mRNA expressions did not change after 3 months (Fig. 2). However, no differences were observed between the garlic and placebo treatment values. No significant correlations were observed between plasma CE and PBMC mRNA expressions of ABCA1 or ABCG1. Baseline brachial FMD values were similar in both the groups, with no statistically significant

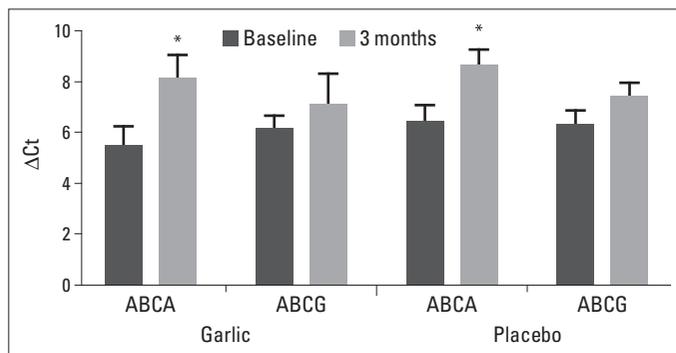


Figure 2. mRNA expression levels of ABCA1 and ABCG1 in PBMCs. Δ Ct was calculated using PPIB as the house gene (Δ Ct=Ct target gene- Ct PPIB). Higher values of Δ Ct indicate lower gene expression. Data are mean \pm standard error. *Significant difference compared to baseline ($p<0.05$)

Table 2. Endothelial function and plasma lipid and hs-CRP levels in the garlic and placebo groups

Variable		Baseline	3 months of treatment
BAD, mm	Garlic group	5.04 \pm 0.19	5.05 \pm 0.19
	Placebo group	4.74 \pm 0.17	4.75 \pm 0.17
	p	0.26	0.25
FMD, %	Garlic group	4.95 \pm 0.78	7.47 \pm 0.93**
	Placebo group	4.63 \pm 0.80	4.24 \pm 0.75
	p	0.48	0.016
hs-CRP, mg/L	Garlic group	2.43 \pm 1.90	1.72 \pm 1.82 *
	Placebo group	2.62 \pm 1.51	2.22 \pm 1.68
	p	0.72	0.36
Lp (a), mg/dL	Garlic group	28.28 \pm 17.08	28.45 \pm 18.87
	Placebo group	32.77 \pm 17.44	32.18 \pm 12.80
	p	0.34	0.45
Apo A1, mg/dL	Garlic group	101.05 \pm 21.03	119.94 \pm 30.86 **
	Placebo group	104.17 \pm 18.74	126.63 \pm 29.93 **
	p	0.63	0.46
HDL-C, mg/dL	Garlic group	33.26 \pm 10.60	37.05 \pm 10.12 **
	Placebo group	37.17 \pm 07.79	40.59 \pm 09.43 *
	p	0.17	0.25
LDL-C, mg/dL	Garlic group	52.31 \pm 15.51	60.0 \pm 3.2
	Placebo group	59.17 \pm 21.58	66.45 \pm 35.63
	p	0.25	0.23
Cholesterol, mg/dL	Garlic group	108.9 \pm 4.7	120.7 \pm 5.0
	Placebo group	111.8 \pm 5.4	128.1 \pm 10.1
	p	0.35	0.33

Apo A1 - apolipoprotein A1; BAD - baseline artery diameter; FMD - flow-mediated dilation; hs-CRP - high-sensitive C-reactive protein; HDL - high-density lipoprotein; Lp (a) - lipoprotein a; LDL - low-density lipoprotein. Data are mean \pm standard deviation. * $P<0.05$ compared with baseline; ** $P<0.01$ compared with baseline

differences between the groups. In our study, 65.4% of patients had FMD values $<5.3\%$. After 3 months of treatment, FMD values

(50.7%) significantly improved from the baseline value in the garlic group ($p=0.001$), whereas they did not in the placebo group ($p=0.92$). Final FMD values were significantly higher in the garlic group than in the placebo group (Table 2). Plasma HDL-C and Apo A1 significantly increased in both the groups after 3 months of treatment. However, no significant differences were found between the two groups with regard to HDL-C or Apo A1 levels (Table 2). Plasma LDL-C and Lp (a) levels did not change after 3 months of treatment, and they did not differ between the two groups. Plasma CE was not significantly correlated with plasma lipid or Apo A1 levels. After 3 months of treatment, hs-CRP levels significantly decreased in the garlic group ($p<0.05$), whereas no changes were observed in the placebo group. However, hs-CRP levels did not differ between the two groups. At baseline, plasma CE was negatively correlated with hs-CRP levels ($r=-0.334$, $p<0.05$), whereas after 3 months of treatment, no significant correlation was observed ($r=-0.175$). However, when baseline and posttreatment data were collectively analyzed, a significant correlation was observed ($r=-0.247$, $p<0.05$) (Fig. 3).

Discussion

Our study results suggest that a daily intake of 800 mg dry garlic powder tablet improved brachial endothelial function but did not improve CE from THP-1 macrophages and did not affect ABCA1 or ABCG1 mRNA expressions in PBMCs. Based on the study by Koyoshi et al. (16), the cut-off level of FMD, which had the greatest sensitivity and specificity for CAD, was 5.3%. In our study, 65.4% of patients had FMD values $<5.3\%$.

To study macrophage RCT, we used THP-1 macrophages that were well characterized and previously used (17, 18). Improvement in endothelial function in the garlic group in our study was consistent with that reported by Williams et al. (15), who demonstrated that a 2-week treatment with aged garlic extract significantly increased FMD values (44%, $p=0.04$) in patients with CAD. In our study, the effect of garlic tablet on endothelial function could be because of a stimulation of endothelial nitric oxide synthesis or preservation of nitric oxide by the garlic components (15). Allicin, ajoene, and antioxidants in the garlic inhibit inducible nitric oxide synthase in macrophages and reduce nitrite accumulation in atherosclerotic plaques (19). All the patients in our study were treated with clopidogrel, aspirin, ACE inhibitor/ARB, and statins (atorvastatin). Therefore, drugs did not affect the final FMD values. Based on recent studies, although CE from macrophages is only a small part of the overall flux through the RCT pathway, it is probably the component that is most associated to protection from atherosclerosis (5). Khera et al. (5) found that CE from macrophages was inversely associated with subclinical atherosclerosis and cardiovascular problems, which are associated to stenosis. These associations persisted after adjusting for common cardiovascular risk factors. Studies have shown that CE protect macrophages from LDL-induced apoptosis and increase endothelial function (20,

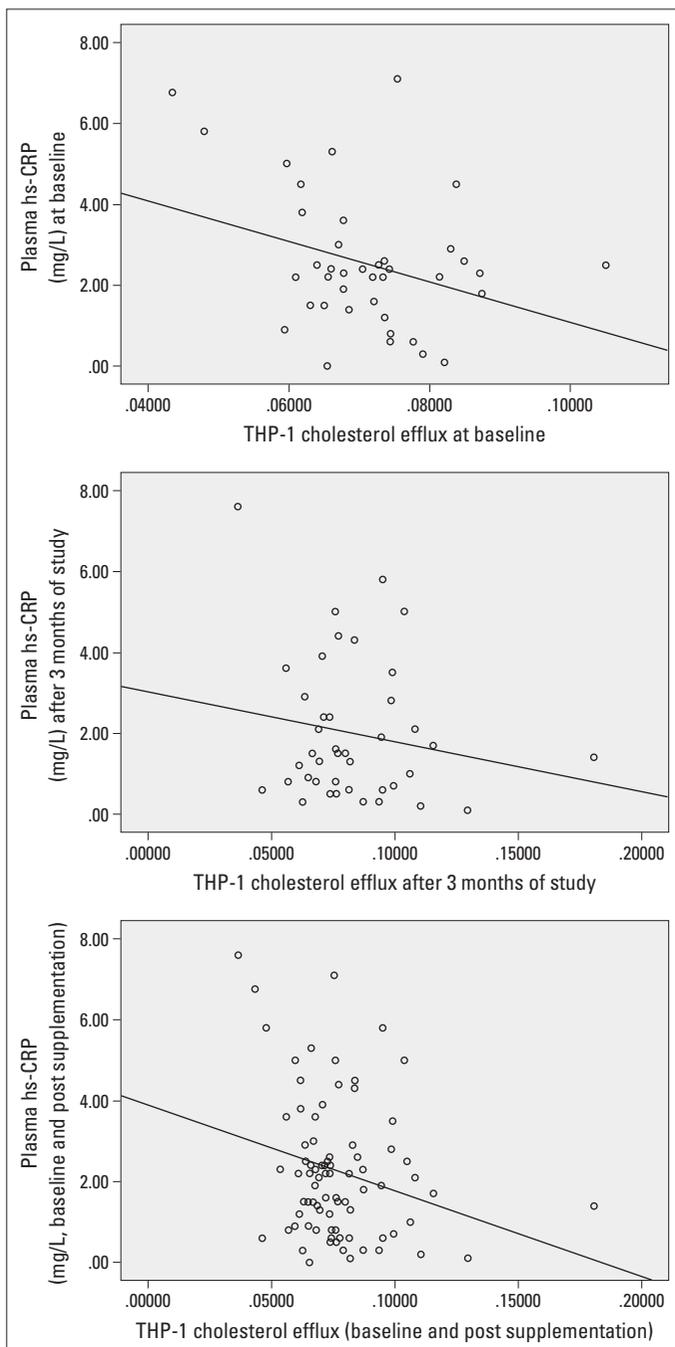


Figure 3. Scatter plot of CRP levels versus cholesterol efflux from THP-1 at baseline and after 3 months of treatment

21). Because ABCA1 is expressed in macrophages, these cells could serve as a suitable *in vitro* model to study the function of CE. Treating human THP-1 macrophages with S-allylcysteine, the most abundant organosulfur compound in aged garlic extracts, increased ABCA1 mRNA and protein expressions compared with the control, suggesting that S-allylcysteine is beneficial in promoting reverse CE (12). Mohammadi et al. (22) demonstrated that garlic extracts markedly increased LXR mRNA and protein expressions in the intestine of mice, and the activation of LXR leads to significantly increased ABCA1 mRNA

expression levels. In contrast, garlic regulates ABC genes by activating LXRs, and through this pathway, it may have a role in RCT (22). In this study, compared with baseline, plasma CE from THP-1 macrophage increased after 3 months of treatment in both the groups, whereas ABCA1 and ABCG1 mRNA expressions in PBMCs decreased in both the groups. This discrepancy that despite the decrease in ABCA1 and ABCG1 mRNA expressions, CE increased could be explained by the fact that ABCA1 and ABCG1 facilitate only a part of the total cholesterol removal from cells, and other routes such as the scavenger receptor class B also influence CE (23).

Both clinical and basic science studies have shown that CRP may increase the progression of atherosclerosis. Studies demonstrated a significant association between CRP or hs-CRP and *in vitro* CE (24, 25). Wang et al. (25) revealed that CRP inhibits CE from human macrophage-derived foam cells and decreases ABCA1 and ABCG1 mRNA expression levels. In this study, garlic treatment significantly altered hs-CRP levels. Few studies have reported the effect of garlic on hs-CRP level, an inflammation marker, in subjects treated with garlic. In a randomized, placebo-controlled, cross-over design with 2-week treatment and wash-out periods, aged garlic extract supplementation in 15 men with angiographically proven CAD patients treated with aspirin and a statin did not change markers of systemic inflammation (plasma CRP and interleukin-6) (18). In addition, a 12-week treatment with a high dose (2.1 g daily), chemically well-characterized garlic powder had no antiinflammatory effects in normolipidemic overweight smokers (26). However, in obese patients with type 2 diabetes who were treated with metformin, daily supplementation of garlic capsules (500 mg) for 12 weeks decreased CRP levels (27). In our study, plasma CE was negatively correlated with hs-CRP levels. The interaction of CRP with cholesterol-loaded macrophages can decrease CE, and the downregulation of ABCA1 and ABCG1 in these cells may be responsible for this effect (25). In patients with pronounced inflammation such as those with rheumatoid arthritis, treatments that are aimed at reducing inflammation are associated with an overall reduction in cardiovascular risk, despite increases in LDL-C levels. In a longitudinal cohort of rheumatoid arthritis, hs-CRP levels decreased and HDL CE capacity increased, whereas LDL-C levels increased. Moreover, there was significant correlations between reductions in hs-CRP levels, with increases in the CE capacity (24). In our study, plasma cholesterol levels were within the optimal range, and this could be one of the main reasons that garlic treatment did not have any effect on the cholesterol levels. Based on the hospital management protocol of patients with CAD undergoing angioplasty, a high dose (80 mg/dL) of atorvastatin was prescribed for all patients for a period of 2–3 days before angioplasty, and a constant maintenance dose (20 mg/dL) was continued for the rest of the study period. However, because the drug therapy protocol was relatively similar for all patients, it appeared that the impact of the treatment in both the garlic and placebo groups was relatively equal.

Study limitations

In our study, the duration of garlic treatment was relatively short, which restricts firm conclusions. Long-term studies with both healthy and patients with CAD could help provide more definitive conclusions.

Conclusion

The study results suggest that in patients with CAD, 3-month treatment with garlic tablet improves brachial endothelial function and decreases hs-CRP levels, whereas it could improve neither CE from THP-1 macrophages nor affect ABCA1 or ABCG1 mRNA expressions in PBMCs. In addition, the garlic treatment could not further improve plasma lipid levels in patients who were optimally treated with medications.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – J.N., M.M.; Design – J.N., M.M., P.M.; Supervision – J.N., P.M.; Materials – P.M.; Data collection &/or processing – M.M., J.N.; Analysis &/or interpretation – M.M., J.N., M.A.; Literature search – M.M., J.N.; Writing – M.M., J.N.; Critical review – P.M., J.N.

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