Original Article

Effect of lycopene on the vascular endothelial function and expression of inflammatory agents in hyperhomocysteinemic rats

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The aim of this study was to investigate the effect of lycopene on the vascular endothelial function and the expression of inflammatory agents in hyperhomocysteinemic rats. Fifty male Sprague-Dawley rats weighed 145-155g were on a commercial rat chow diet for seven days, and then were randomized into five groups: normal control group (NC) fed with AOAC diet and four hyperhomocysteinemic groups fed with AOAC diet plus 3% L-methionine. Four hyperhomocysteinemic groups were daily supplemented with 0 (HC), 10 mg/kg (HL1), 15 mg/kg (HL2), 20 mg/kg (HL3) lycopene dissolved in corn oil respectively by intragastric administration for 12 weeks. At the end of experiment, their blood and abdominal aortas were collected after etherization. Serum levels of Hcy were determined by HPLC, nitric oxide (NO) and nitric oxide synthase (NOS) by chromatometry, endothelin-1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) by ELISA. Hematoxylin and eosin staining and oil red staining were used to analyze abdominal aortas histologically. Moderate hyperhomocysteinemia was induced in hyperhomocysteinemic groups. Serum level of NO was lower and ET-1 was higher in HC rats than in NC, NL2 and NL3 rats (p<0.01). There was no difference of serum NOS activity among five groups. There were some foam cells and depositions of lipochondria in aortic tunica intima in HC and HL1 rats, which were not found in HL2 and HL3 rats. Serum levels of VCAM-1, MCP-1, IL-8 were higher in HC rats than in NC, NL1, NL2 and NL3 rats (p<0.01). The present study indicated that lycopene exerts an antiatherogenic effect by inhibiting the expression of inflammatory agents in hyperhomocysteninemic rats.

Key Words: lycopene, hyperhomocysteinemia, vascular endothelium, inflammatory agents, atherogenesis

Introduction

Numerous epidemiological studies have demonstrated that hyperhomocysteinemia (HHcy), which refers to total plasma homocysteine(Hcy) above 15μmol/L, is a strong and independent risk factor for atherosclerosis and thrombotic disease.[1-2] Several hypotheses have been proposed to explain the cellular mechanisms including oxidative stress, endoplasmic reticulum (ER) stress and the activation of pro-inflammatory factors.[3] A few recent in vitro studies have indicated that Hcy affects the expression of some inflammatory factors. Hcy increases the expression of monocyte chemoattractant protein-1(MCP-1) in both human aortic endothelial cells[4] and monocytes.[5] Chronic exposure to Hcy also increases the production of intracellular adhesion molecule-1 (ICAM-1) in endothelial cells.[6] These results suggested that the atherogenic effects of Hcy result at least in part from its inflammatory effects.

Lycopene, which is a naturally present carotenoid in tomatoes and tomato products, is the most potent singlet oxygen quencher among the natural carotenoids.[7] Recent epidemiological studies have shown an inverse relationship between the intake of tomatoes and lycopene and serum and adipose tissue lycopene levels and the incidence of coronary heart disease. An *in vitro* study indicated that among five carotenoids, lycopene appears to be the most effective in reducing both human aortic endothelial cells (HAEC) adhesion to monocytes and expression of adhesion molecules on the cell surface, suggesting an important role for lycopene in attenuating atherogenesis.[8] To date there is not any similar study *in vivo*. The aim of this study was to investigate the effect of lycopene on the vascular endothelial function and the expression of inflammatory agents in hyperhomocysteinemic rats.

Materials and methods

Animals

The experiment used an animal protocol approved by Public Health Animal Care and Use Committee, the Second Military Medical University. Male Sprague-Dawley rats (n=50; body wt 150.5±7.3g) were obtained from the Second Military Medical University Animal Center.

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The rats were housed individually in stainless cages in an air-conditioned room maintained at 24°C with a 12-h light–dark cycle. They were fed a nonpurified diet for a 1-wk acclimation period, then were randomly divided into 5 groups of 10: normal control group (NC) fed with AOAC(Association of Officia1 Analytical Chemists) diet[9] and four HHcy groups fed with AOAC diet plus 3% L-methionine. Four HHcy groups were daily supplemented with 0 (HC), 10 mg/kg (HL1), 15 mg/kg (HL2), 20 mg/kg (HL3) lycopene dissolved in corn oil respectively by intragastric administration for 12 weeks, the control group was supplemented with the same volume of corn oil. The rats consumed food and water ad libitum. Food intake was measured daily and body weight weekly during the 12-wk experimental period. At the end of the experiment, all rats were deprived of food overnight, anesthetized with diethyl ether, and killed. Serum was stored at-20°C for laboratory analysis. The abdominal aorta was harvested from each rat for histopathological analysis.

Chemicals

L-methionine was purchased from Xinjiang Westar Bioengineering Co.,Ltd, China. Lycopene, >85% pure, was purchased from North China Pharmaceutical Group Corporation,China. DL-Hcy was purchased from Sigma USA.

Serum Hcy

The concentration of total serum Hcy was analyzed by high performance liquid chromatography (HPLC).[10] Briefly, 100μL of serum was added to 10μL of 0.345 mol/L tri-*n*-butylphosphine (Sigma) in dimethyl formamide (Sigma). Then, 100μL of 0.5 mol/L perchloric acid containing 0.5mmol/L EDTA-Na_2 was added. The mixture was centrifuged at 4000×*g* for 10 min. To 30μL of the supernatant, a mixture consisting of 15μL of 0.5 mol/L NaOH and 30μL of 7-fluorbenzo-2-oxa-1,3-diazle-4-sulfonic acid (SBD-F; Sigma) [4.25 mmol/L in 0.1 mol/L borate buffer (pH 9.5) containing 2 mmol/L of EDTA] was added. A 10μL sample of the supernatant was analyzed by HPLC, using an HP 1050LC series liquid chromatograph and workstation and an F1046 fluorescence detector (excitation 385 nm, emission 515 nm; Hewlett-Packard). Separation was carried out using a reversed-phase column(C18 BDS, 150×4.6 mm; Hypersil). Analysis was performed under isocratic conditions (40 mmol/L ammonium formate and 30 mmol/L ammonium nitrate buffer with 5% acetonitrile, pH3.5) at a flow rate of 1 mL/min for 15 min. The concentration of serum Hcy was calculated using Hcy as an external standard. The retention time for Hcy was～3 min.

Serum nitric oxide (NO)

Serum nitrite concentration, as an indicator of NO production, was measured by the Griess reaction.[11] 100μL serum was mixed with the same volume of Griess reagent (58 mmol/L sulfanilamide in 0.5 mol/L phosphoric acid and 3.85 mmol/L naphthylethylenediamine dihydrochloride in water). The absorbance at 550 nm was assayed using sodium nitrite as the calibration standard. The assay was performed using a commercially available kit (NO determination kit, Jiancheng Biotechnology,China)

Serum nitric oxide synthase (NOS) activity

NOS can catalyze L-arginine and O_2 to produce NO, which can react with the nucleophilic compound to produce colored chemical compound. According to the OD value of the colored compound, which can be tested at 530nm, the activity of NOS can be calculated.[12] The assay was performed using a commercially available kit (NOS activity determination kit, Jiancheng Biotechnology,China).

Serum vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8) and endothelin-1 (ET-1)

Serum was analyzed using commercially available Rat detector kits (Rapidbio Lab. USA) according to the manual provided with the kits by ELISA respectively.[13] VCAM-1 as the example, diluted serum samples were added to a 96-well plate precoated with anti-VCAM-1 antibody. After incubating for 1 h at room temperature, the plate was washed 3 times. Biotinylated antibody reagent was added to each well, and the plate was again incubated for 1 h. Then streptavidin-horseradish peroxidase (HRP) solution was added to each well, and the plate was incubated for 30 min at room temperature. After washing, tetramethyl benzidine (TMB) substrate solution was added, and the enzymatic color reaction was developed in darkness at room temperature for 30 min without covering the plate. The reaction was terminated by adding the stop solution. Absorbance was detected at 450 nm, using a Titertek ELISA reader (Bio-TEK Instruments).

Histopathological analysis

The abdominal aorta was excised from each rat after sacrifice and dissected into 2 segments: one was fixed in 10% buffered formalin, embedded in paraffin, and sections stained with hematoxylin and eosin; another was frozened at-20°C, sections stained with oil red.

Statistical analysis

Results were presented as means±SD. Data were analyzed by one-way ANOVA and a post-hoc least significant difference (LSD)-*t* multiple comparison test using the SPSS 12.0 for Windows. Results were considered statistically significant at $p<0.05$.

Results

Serum Hcy concentration: Moderate HHcy was induced in rats fed with AOAC diet plus 3% L-methionine (Fig 1). There was no diference of serum Hcy concentrations among HHcy groups.

Effect of lycopene on serum levels of NO, ET-1 and NOS activity: Serum NO level was lower and ET-1 was higher in HC group than in NC group (p=0.0001). Serum NO levels were higher and ET-1 were lower in HL2 and HL3 groups than in HC group (p=0.0001). There was no difference of serum NOS activity among five groups (p=0.876) (Table 1).

Effect of lycopene on serum levels of VCAM-1, MCP-1 and IL-8: Serum levels of VCAM-1, MCP-1 and IL-8 were higher in HC group than in NC group (p=0.0001). Serum levels of VCAM-1, MCP-1 and IL-8 were lower in HL1, HL2 and HL3 groups than in HC group (p=0.0001).

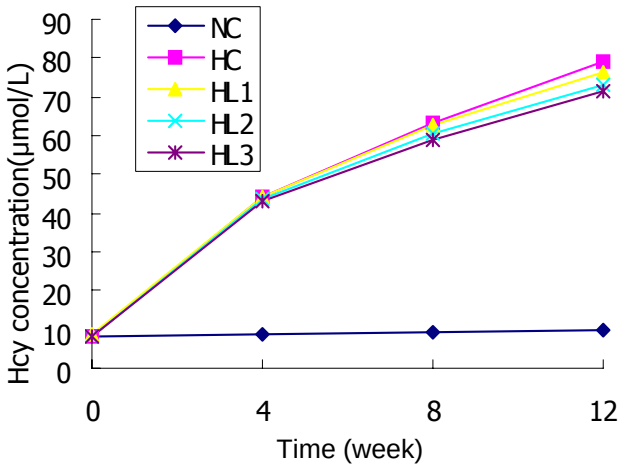


Figure 1. The serum levels of homocysteine of rats in each experimental group.

Serum levels of VCAM-1 and MCP-1 were also lower in HL3 group than in NC group (p=0.0001, p=0.014). A serum level of VCAM-1 was also lower in HL2 group than NC group (p=0.0001) (Table 2).

Histopathological analysis: There were some foam cells and depositions of lipochondria in aortic tunica intima in HC and HL1 groups, which were not found in HL2 and HL3 groups (Fig 2, 3).

Discussion

Homocysteine, a sulfur-containing amino acid, is an intermediate metabolite of the essential amino acid methionine. It is generally thought total plasma Hcy 5～15μmol/L is normal, 16～30μmol/L is mild HHcy, 31～100μmol/L is moderate HHcy, above 100μmol/L is severe HHcy. Numerous clinical and epidemiological studies have indicated that HHcy is an independent risk factor for atherothrombotic disease. HHcy is found in 30% of patients with premature atherosclerosis of carotid and peripheral arteries.[14] Up to 40% of patients diagnosed with premature coronary artery disease, peripheral vascular disease, or recurrent venous thrombosis present with HHcy.[1-2,15] This study showed that serum Hcy concentration of HC group fed with AOAC diet plus 3% L-methionine increased significantly, reached up to 78.86 μmol/L at 12-wk . Serum NO level was lower and ET-1 was higher in HC group than in NC group. Histopathological analysis showed that there were some foam cells and depositions of lipochondria in aortic tunica intima in HC group, which hinted premature atherosclerosis. This study indicates that moderate HHcy directly leads to endothelial dysfunction and premature atherogenesis.

In general, the development and progression of atherosclerosis is considered to be a form of chronic inflammation.[16-17] Endothelial dysfunction is the key process promoting inflammatory reactions. I*n vitro* studies have demonstrated that Hcy enhances the production of several pro-inflammatory cytokines. Expression of MCP-1 is increased in cultured human vascular endothelial cells[4], smooth muscle cells[18] and monocytes treated with homocysteine[5], leading to enhanced monocyte chemotaxis and adhesion to endothelial cells. Hcy has also been shown to increase expression of IL-8 in cultured endothelial cells.[4] *In vivo* study has also demonstrated that HHcy stimulates the expression of MCP-1, VCAM-1, and E-selectin in rats, leading to increased monocyte adhesion to the aortic endothelium.[19] Such an effect may contribute significantly to the development of atherosclerosis by facilitating monocyte/macrophage infiltration into the arterial wall. Our results also showed that serum levels of VCAM-1, MCP-1 and IL-8 were significantly higher in HC group than in NC group and there were some foam cells and depositions of lipochondria in aortic tunica intima in HC group.

Lycopene is the most potent singlet oxygen quencher among the natural carotenoids. Its quenching ability is twice as high as that of β-carotene, 100 times as that of α-tocopherol. [7] Lycopene is the most predominant carotenoid in human plasma. Recent epidemiological studies have shown an inverse relationship between the intake of tomatoes and lycopene, serum and adipose tissue

Table1. Effect of lycopene on serum levels of NO, ET-1 and NOS in HHcy rats

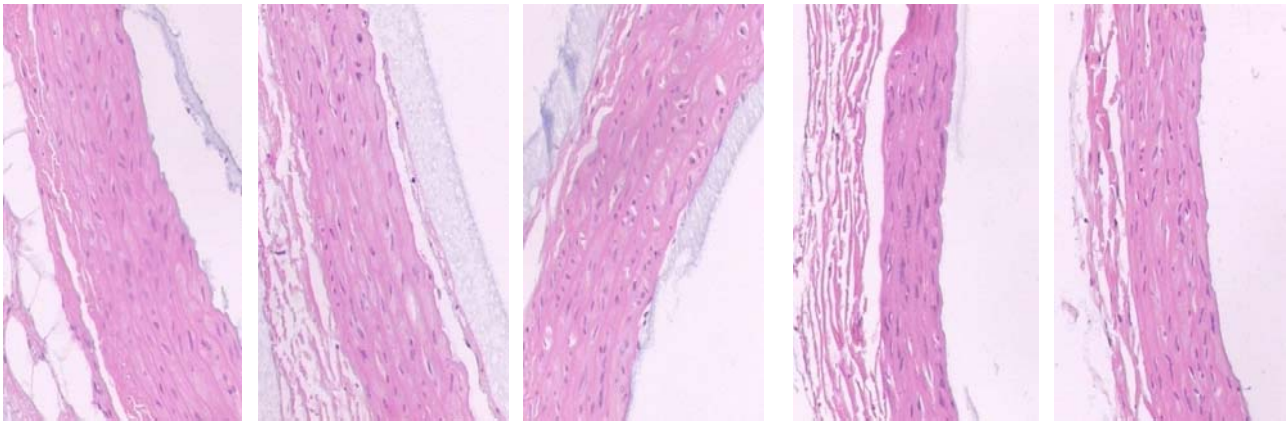
Group	NO(μmol/L)	ET-1(pg/mL)	NOS(U/mL)
NC (n=10)	58.0±10.6*	34.0±5.47*	34.5±1.86
HC (n=10)	37.9± 9.40	58.0±7.06	33.9±2.64
HL1(n=10)	47.8±15.4	36.5±4.89*	33.1±2.34
HL2(n=10)	58.59± 9.20*	37.73±7.41*	33.29±3.85
HL3(n=10)	61.06±14.36$^{*\#}$	37.89±5.99*	33.61±3.84

* p<0.01 vs HC, # p<0.05 vs HL1.

Table 2. Effect of lycopene on serum levels of VCAM-1, MCP-1 and IL-8 in HHcy rats

Group	VCAM-1(ng/mL)	MCP-1(pg/mL)	IL-8(pg/mL)
NC (n=10)	6.76±1.56	287±37.4	178±34.1
HC (n=10)	12.6±1.05**	686±92.9**	377±36.5**
HL1(n=10)	5.90±0.56$^{\#\#}$	279±54.1$^{\#\#}$	177±19.7$^{\#\#}$
HL2(n=10)	4.15±0.83$^{**\#\#\$\$}$	271±43.3$^{\#\#}$	168±28.4$^{\#\#}$
HL3(n=10)	2.97±0.53$^{**\#\#\$\$+}$	219±48.7$^{*\#\#\$\$}$	188±34.2$^{\#\#}$

* p<0.05 ** p<0.01 vs NC, ## p<0.01 vs HC, $ p<0.05 $$ p<0.01 vs LH1,+ p<0.05 vs LH2.



NC group HC group HL1 group HL2 group HL3 group

Figure 2. The histopathological change in vascu1ar structure (hematoxylin and eosin staining) ×200

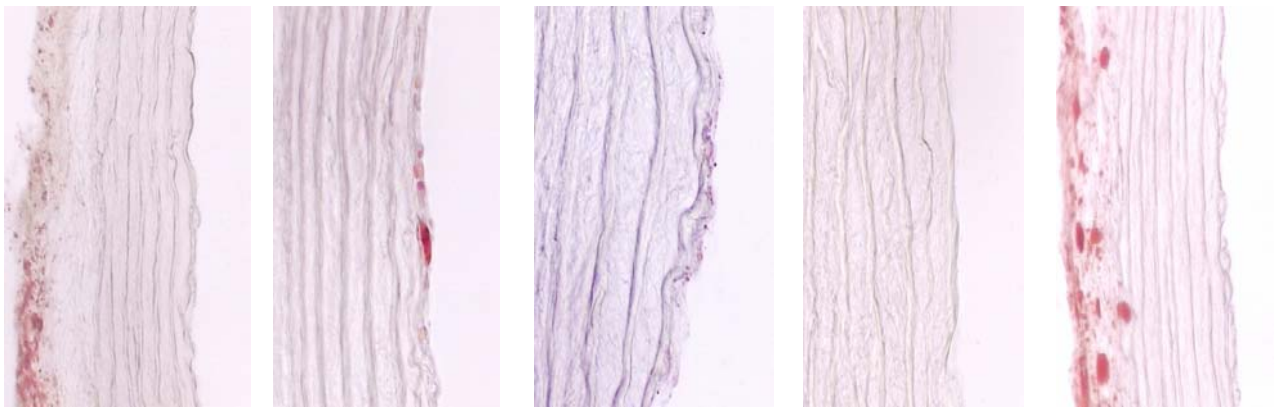


Figure 3. The histopathological change in vascu1ar structure (oil red staining) ×200

lycopene levels and the incidence of coronary heart disease. Some 725 middle-aged men, free of coronary heart disease and stroke, took part in the Kuopio Ischemic Heart Disease Risk Factor (KIHD) study. Men in the lowest quartile of serum levels of lycopene had a 3.3 fold increased risk of an acute coronary event or a stroke as compared to the other groups.[20] In another study, the association between plasma lycopene concentration and intima-media thickness of the common carotid artery wall (CCA-IMT) was examined. 520 asymptomatic men and women participated in. It was found that low plasma levels of lycopene were associated with an 18% increase in IMT in men compared with men whose plasma levels were higher than the median. In women, the difference did not remain significant after the adjustment. It indicated that lycopene maybe could prevent premature AS.[21] Our results showed that lycopene administration could increase serum NO levels, decrease serum ET-1 levels of HHcy rats, ameliorate endothelial dysfunction and prevent early AS induced by HHcy.

Van Herpen-Broekmans evaluated the association between serum antioxidants and markers of endothelial function and inflammation in 379 subjects sampled from the general population. They found inverse associations between lycopene and soluble ICAM-1.[22] Martin, Wu and Meydani examined *in vitro* the effect of the five most prevalent plasma carotenoids-α-carotene, β-carotene, lutein, zeaxanthin and lycopene on the expression of key adhesion molecules involved in the atherosclerosis process, and determined the subsequent binding of U937 monocytic cells when carotenoids were incubated with human aortic endothelial cells (HAEC). While other carotenoids were ineffective, lycopene attenuated interleukin-1β-stimulated and spontaneous HAEC adhesion to U937 monocytic cells by 20 and 25%, respectively. Thus, among all the carotenoids tested, lycopene appears to be the most effective in reducing both HAEC adhesion to monocytes and expression of adhesion molecules on the cell surface. These results suggest an important role for lycopene in attenuating atherogenesis.[8]This study showed lycopene administration decreased serum VCAM-1, MCP-1 and IL-8 levels of HHcy rats significantly, inhibited high expressions of inflammatory agents induced by HHcy.

In summary, our findings indicate that lycopene can inhibit high expressions of inflammatory agents induced by HHcy, ameliorate endothelial dysfunction and prevent atherogenesis.

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