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Anti-atherosclerotic effects of garlic preparation in freeze injury model of atherosclerosis in cholesterol-fed rabbits

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ABSTRACT

Background: Garlic (*Allium sativum L.*) is one of the most popular substances used to reduce various risks associated with cardiovascular disease. However, little is known on the direct effects of garlic on atherosclerosis.
Purpose: In the present study we have examined the effect of per oral administration of the time-released garlic herbal preparation on serum atherogenicity and formation of intimal thickening after freeze injury in cholesterol-fed rabbits.
Methods: Group 1 rabbits maintained on the standard cholesterol-rich diet served as the control. Group 2 rabbits were fed the cholesterol-rich diet and treated with garlic preparation containing 300 mg garlic powder.
Results: Local thickening of the aortic media (i.e., the neointima formation) in the freeze injury zone was observed in all the rabbits. Regular garlic preparation therapy prevented the neointima formation and the accumulation of free and esterified cholesterol, triglycerides, phospholipids and collagen in the neointima, the effects being statistically significant. Garlic preparation also decreased serum lipid content by 1.5-fold and lowered atherogenic activity of blood serum (ability to induce lipid accumulation in cultured cells) induced by cholesterol-rich diet.
Conclusion: The results obtained indicate that garlic preparation prevents the development of cholesterol-induced experimental atherosclerosis and possesses the direct anti-atherogenic activity.

Introduction

Garlic (*Allium sativum L.*) is one of the most popular substances used to reduce various risks associated with cardiovascular disease. However, little is known on the direct effects of garlic on atherosclerosis.

Atherosclerosis-related effects of garlic and garlic-derived preparations may be realized at the arterial level, preventing new and suppressing the existing atherosclerotic. The mechanisms of these effects have been shown to be associated with the effects of the garlic components on blood lipid profile. Recently, using an animal experimental model, Mohammadi and Oshaghi (2014) studied effects of garlic on lipid profile as well as LXRα expression in intestine and liver. The study found that garlic extract reduced LXRα expression in the liver and increased its expression in the intestine. The study of Mohammadi and Oshaghi (2014) demonstrated a role of garlic in reducing serum triglyceride and cholesterol. It seems that garlic could affect directly intimal vascular cells. Supporting this possibility is a study of Orekhov et al. (1996). Which demonstrated that water extract of garlic powder produces the direct anti-atherogenic effect on cultured smooth muscle cells isolated from grossly normal human aortic intima by preventing the intracellular accumulation of cholesterol and reducing cellular proliferation stimulated by atherogenic serum. Many studies reported anti-atherogenic effects of garlic preparations observed *in vivo* (Bordia et al., 1975; Jain, 1975; Kritchevsky, 1975; Jain and Konar, 1978; Mirhardi et al., 1991; Rafieian-Kopaei et al., 2013).

In the present study we have examined the effect of orally administered garlic powder time-released garlic preparation containing

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300 mg dried garlic powder from *Allium sativum L.* bulbs (Allicor, INAT-Pharma, Russia) on the atherogenic properties of blood serum and on the formation of intimal thickening in a freeze-injured aorta of cholesterol-fed rabbits. Long-acting garlic preparations nave not been examined previously for their anti-atherogenic effect in the animal model.

Material and methods

Aorta injury model

The experiment procedures carried out in the present study compiled with the "Animal Research: Reporting *In Vivo* Experiments" (ARRIVE) guidelines. The animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Institute of Experimental Cardiology, Russian Cardiology Research Center; Moscow, Russia. The study was approved by the Ethical committees of Institute of Atherosclerosis Research, Moscow, and by Russian Cardiology Research Center, Moscow.

Commercially available garlic preparation was used in this study (Allicor, INAT-Pharma, Russia; batch no. 13213, quality control protocol no. 172/13). The single herbal constituent of Allicor was dried garlic powder from bulbs of *Allium sativum L.* The plant name has been checked with http://www.theplantlist.org (*Allium sativum L.*; Status: accepted; Confidence level: ***; Source: WCSP; Date supplied: 2012-03-23). One tablet contained 300 mg dried garlic powder standardized by allicin content, the most recommended bioavailable marker compound to ensure a consistent quality and reproducible pharmacological activity (Borlinghaus et al., 2014). In brief, 150 mg powdered tablets were extracted with 2.5 ml water for 20 min., and further with 150 μl 40% NaOH for 3 min., and neutralized by adding 475 ± 25 μl 10% HCl and 500 μl phosphate buffer (pH 7.0). Then methanol was added to final volume of 10 ml, and the mixture was centrifuged to obtain clear supernatant. The content of active ingredient in the final product was determined by measuring free allicin by HPLC. Allicin content accounted for 0.7%, or 2.1 mg per tablet. The fingerprint of extract sample obtained by HPLC analysis is shown at Fig. 1. According to manufacturer's specification, the delayed disintegration of tablets and, thus, the prolonged biological effect of garlic preparation was provided by polymeric matrix containing dried garlic particles.

Experiments were performed on male Chinchilla rabbits, aged 12–15 weeks weighing 3.0–3.5 kg. The animals were maintained on the standard diet. Rabbits were anesthetized with Nembutal (40 mg/kg). Rabbit aorta was denuded by freeze injury (Malczak and Buck, 1977). A 12 mm aluminium bar, cooled down to the temperature of liquid nitrogen was placed on the exterior of the abdominal aorta (infrarenal segment) for 1 min.

Twelve weeks after the procedure, the rabbits were divided into two groups of 12 animals each. Group 1 rabbits (control) were given cholesterol (Fluca Chemie, AG, Buchs, Switzerland) perorally in dose of 2 g. Group 2 rabbits received cholesterol in the same dose and commercially available garlic preparation containing 300 mg dried garlic powder standardized by allicin content (0.7%) (Allicor, INAT-Pharma, Russia). Cholesterol and garlic preparation were mixed with vegetable oil and given to the rabbits once daily for 35 days.

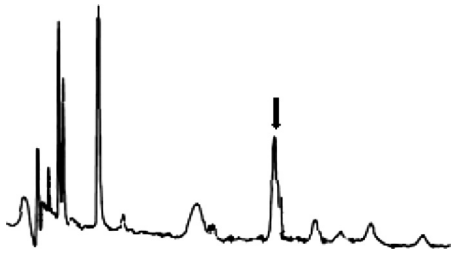

Fig. 1. The fingerprint of water extract of garlic preparation obtained by HPLC analysis. Precolumn Spherisorb ODS 2.10 μm × 10 mm, column Spherisorb ODS 2.5 μm × 120 mm, isocratic carrier phase methanol/water, 70:30 vol:vol, 0.5 ml/min., ultraviolet detection at 230 nm. Allicin peak is shown by arrow, retention time 10.30 min.

Blood was drawn before the aorta denudation procedure, 12 weeks after it, and on days 7 and 35 of the long-term treatment.

Light microscopy

Aortas were dissected in 17 weeks after freeze injury. The animals were sacrificed with Nembutal (80 mg/kg). For morphological studies, an aortic segment (5 mm wide) from the central part of the injury was fixed with 2.5% glutaraldehyde. The thickness of the media and intima was measured on semithin sections with an ocular micrometer (Opton-3 light microscope, Karl Zeiss, Jena, Germany). The remainder of the aorta was used in biochemical studies. Cell content in the neointima was determined by the ethanol-alkaline dissociation method as described previously (Orekhov et al., 1984).

Biochemical studies

The intima and the media were separated mechanically. Lipids were extracted with a chloroform:methanol mixture (2:1) and separated by thin-layer chromatography using the following systems: (1) benzene-diethyl ether-ethanol-acetic acid (50:40:2:0.2) and, (2) petroleum ether-diethyl ether-acetic acid (90:10:1). Phospholipids, total and esterified cholesterol and triglycerides were measured densitometrically (Orekhov et al., 1995). Collagen content was determined as described elsewhere (Orekhov et al., 1984).

Cell culture

Rabbit blood serum was added to cultured peritoneal macrophages of BALB/c mise obtained as described (Tertov et al., 1989). Cells were washed with medium 199 and incubated for 3 h in medium 199 containing glutamine, antibiotics (all reagents - "GIBCO Europe", Paisley, UK) and 10% of the serum tested. Cells were extensively washed and the total cholesterol content was determined as described previously (Orekhov et al., 1989).

Serum lipids measurements

The total cholesterol content in blood serum samples was determined colorimetrically using a Cholesterol Monotest kit (Boehringer Mannheim, GmbH, Mannheim, Germany). The triglyceride level was evaluated colorimetrically using a Peridochrome Triglyceride kit (Boehringer Mannheim, GmbH).

Statistics

The significance of the differences was evaluated by the dispersion analysis using a BMDP statistical software (Dixon and Brown, 1977).

Results

Effect of garlic preparation on serum lipid levels

Cholesterol-rich diet caused hyperlipidemia in all rabbits. The total serum cholesterol content significantly increased from 141 ± 10 to 1533 ± 94 mg/dl ($p < 0,05$) and the triglyceride content from 129 ± 9 to 270 ± 67 mg/dl ($p < 0,05$). Garlic preparation treatment prevented the rise of serum blood lipids, which was seen after 1 and 5 weeks on cholesterol-rich diet (Fig. 2). By the end of the study (5 weeks on cholesterol-rich diet) in garlic preparation-treated animals the cholesterol and triglyceride contents were 1.2- and 1.7-fold lower,