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A comparative study of the antithrombotic effect through activated endothelium of garlic powder and tomato extracts using a rodent model of collagen and epinephrine induced thrombosis

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Abstract In this study, garlic powder, tomato extract and a mixture of both were analyzed for anti-thrombotic effects using a collagen and epinephrine induced thrombosis model. Rats were randomly assigned to control, thrombosis induced control (COL/EP), garlic powder (G), tomato extract (T) and mixture of garlic powder and tomato extract (GT) groups. Test materials were administered for 7 days and thrombosis was induced by collagen and epinephrine injection. The results showed that G, T, and GT delayed activated partial thromboplastin time and reduced the expression of intracellular adhesion molecule-1 mRNA. Histological analysis of aorta and lung revealed that thrombosis was partially improved by G, T, and GT. Although there was no synergistic effect in GT compared to G and T treatment, this study showed that G, T, and GT have anti-thrombotic effect.

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Introduction

Atherothrombotic disease is recognized as a major cause of disease morbidity and mortality worldwide, and has become a concern in western society where obesity, diabetes and metabolic syndrome are increasing (Ajjan and Grant, 2006). Vascular endothelial cells may inhibit thrombosis and inflammation by suppressing blood vessel diameter, and platelet activation to release nitrogen monoxide (nitric oxide) (Palmer et al., 1988). However, excessive lifestyle changes and deterioration of vascular endothelial function due to aging decrease the release of nitrogen monoxide and activate platelets, thereby increasing the risk of thrombosis and atherosclerosis (Cines et al., 1998).

Garlic is a functional spice containing a large amount of sulfur compounds and flavonoids. It has been used extensively as a food material in Europe as well as in Korea. In vitro studies have shown that treatment of endothelial cells with garlic has the effect of preserving nitric oxide bioavailability, and decreasing adhesion molecule expression (Lau et al., 2013; Son et al., 2006; Weiss et al., 2013). Garlic powder improves fibrinolytic activity and pulse wave velocity (Kiesewetter et al., 1990; Turner et al., 2005). Treatment with lycopene isolated from tomato or garlic, has been shown to help maintain endothelial function in blood vessels in some studies (Suganuma and Inakuma, 1999; Vilahur et al., 2015; Xaplanteris et al., 2012). Garlic and tomatoes are foods that are consumed at the same time, especially in Europe. In animal models of

gastric cancer, combined treatment of S-allylcysteine and lycopene has an additive or synergistic effect on antioxidant production (Kumaraguruparan et al., 2005; Sengupta et al., 2003; 2004). Thus, garlic and tomato extracts could have similar functionalities or show further synergistic effects, even though they have different chemical composition. In addition, intravenous injection of collagen and epinephrine were used to induce platelet activation and thromboembolism (Westrick et al., 2007) and this model was further validated from previous studies (Jeong et al., 2017; Kang et al., 1999; Lim et al., 2016). Therefore, in this study, garlic powder, tomato extract and the mixture were prepared and compared for their anti-thrombotic effects using a rodent model of collagen and epinephrine induced thrombosis.

Materials and methods

Preparation of garlic powder and the mixture of garlic and tomato extract

To exclude the unpleasant taste and odor of garlic, microencapsulated garlic powder was prepared by coating with whey protein isolates (WPI) using a fluidized bed dryer (Retsch, Haan, Germany). Lyc-O-Mato (6% of lycopene) was used as tomato extract (Lycored Ltd. Be'er Sheva, Israel). Tomato extract and the mixture of garlic powder and tomato extract were homogenized to make them soluble. Lycopene and alliin were then analyzed using a high-performance liquid chromatography system equipped with a UV–Vis detector (Ultimate-3000, Dionex Corp., CA, USA) and a reverse phased C18 column (250 × 4.6 mm, 5 μm; Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of acetonitrile, methanol, methylene chloride, and hexane (4:4:1:1). The lycopene concentration was 10.8 mg/g in the mixture of garlic powder and tomato extract and 11.6 mg/g in tomato extract. The alliin concentration was 9.6 mg/g in garlic powder and 10.5 mg/g in and the mixture.

Animals and treatments

Five-week male Sprague–Dawley rats were obtained from Jung-Ang Lab Animal, Inc. (Seoul, Korea). After a week of acclimatization, the rats were randomly assigned into the following experimental groups (n = 8 per group): saline + saline (CON), saline + collagen and epinephrine (COL/EP), microencapsulated garlic powder (500 mg/kg BW) + collagen and epinephrine (G), mixture of microencapsulated garlic powder and tomato extract (500 mg and 5 mg as lycopene/kg BW) + collagen/epinephrine (GT), tomato extract (5 mg as lycopene/kg BW) + collagen/epinephrine (T). Test materials were administered orally to the rats once daily for 7 days by gavage. Following the last pre-treatment, a mixture of 150 μg of collagen and 3 μg of epinephrine or saline was injected to each rat via the caudal vein to induce platelet activation. After 1 h, the rats were euthanized by carbon dioxide inhalation, and the blood was collected in ethylenediaminetetraacetic acid tubes by cardiac puncture. The aortas were carefully excised, snap-frozen in liquid nitrogen, and stored at − 80 °C until analysis. The protocol was approved by the Institutional Animal Care and Use Committee of Ewha Womans University (Seoul, Korea). Animal care and handling was conducted in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Measuring anticoagulation activity

Blood was collected in citrate tubes with 3.2% buffered sodium citrate solution. Platelet-rich plasma (PRP) was prepared by centrifugation at 1500 × *g* for 15 min. Prothrombin time (PT) and activated partial thromboplastin time (APTT) (Thermo Fisher Scientific Inc, Waltham, MA, USA) were measured using automated coagulation analyzer (Behnk Elektronik, Norderstedt, Germany) with PRP. PT was determined by incubating 100 μL PRP solution for 3 min at 37 °C, followed by addition of 200 μL thromboplastin agent. PT results were expressed as international normalized ratios (INR). APTT was determined by incubating 100 μL PRP solution with 100 μL APTT-activating agent for 3 min at 37 °C, followed by the addition of 100 μL $CaCl_2$ (0.02 M). Saline was used as a control. The clotting time was then recorded for all samples.

Quantitative RT-PCR analysis

Total RNA was isolated from the aorta using TRIzol reagent (Invitrogen; San Diego, CA, USA). The RNA concentration and quality were measured using a BioSpec-nano (Shimadzu Corp; Tokyo, Japan). Total RNA was reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, CA, USA). The TaqMan method was used to quantify the expression of intracellular adhesion molecule-1 (ICAM-1, Icam; Rn00564227_m1), vascular cell adhesion molecule-1 (VCAM-1, vcam; Rn00563627_m1) and β-actin (Actb; Rn00667869_m1) in the aorta. The relative amounts of these mRNAs were normalized to the amount of β-actin, and the relative amounts of the RNAs were calculated using the comparative cycle threshold (CT) method.

Hematoxylin and eosin (H&E) staining of aorta and lung

Formalin-fixed aortas and lungs were dehydrated in various concentrations of ethyl alcohol (70–100% ethyl alcohol). Alcohol was removed with xylene, embedded in paraffin, and cut into 3-μm sections. For H&E staining, aortas and lungs were deparaffinized in xylene, rehydrated in different concentrations of ethyl alcohol (100–70% ethyl alcohol) in reverse order, and then stained with H&E. Slides were dehydrated, the alcohol was removed, and samples were mounted using Permount, and assessed for inflammation and tissue damage using an Olympus microscope (Olympus optical Co., Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). All results are expressed as the means ± standard errors (SEs). Analysis were performed using one way of ANOVA followed by post hoc Duncan's multiple comparison analysis. Statistical significance was determined as $p < 0.05$.

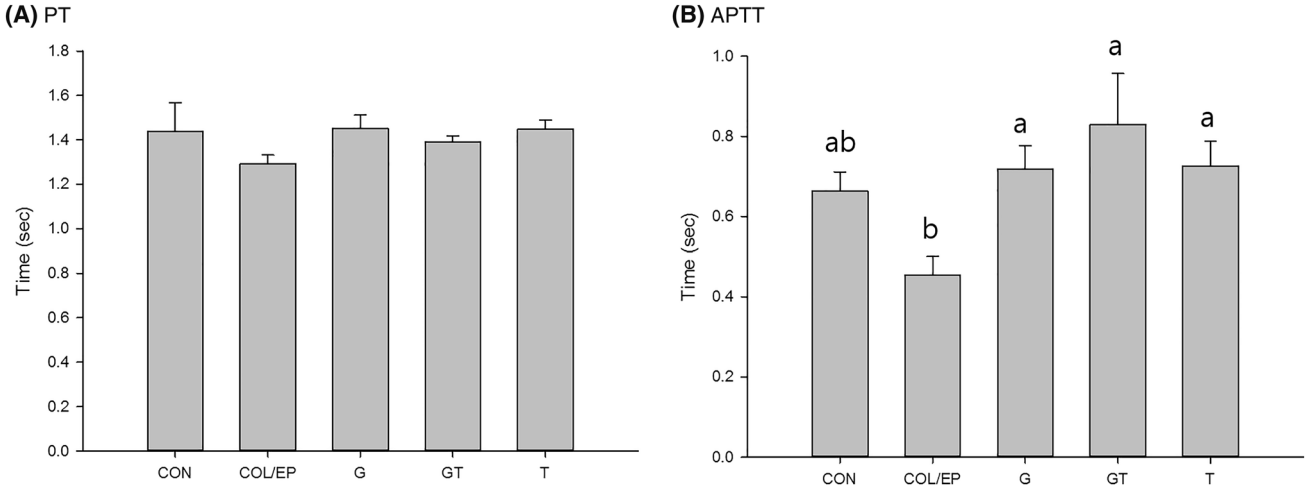

Fig. 1 Change of (**A**) PT and (**B**) APTT when thrombosis-induced rats were administered G, T, and GT. Values are presented as mean ± SE ($n = 8$). Significance was analyzed by one-way ANOVA followed by Duncan's multiple range test. Values having different superscript alphabets differ significantly at $p < 0.05$. PT, prothrombin time; APTT, activated partial thromboplastin time; G, garlic powder; T, tomato extract; GT, the mixture of garlic powder and tomato extract

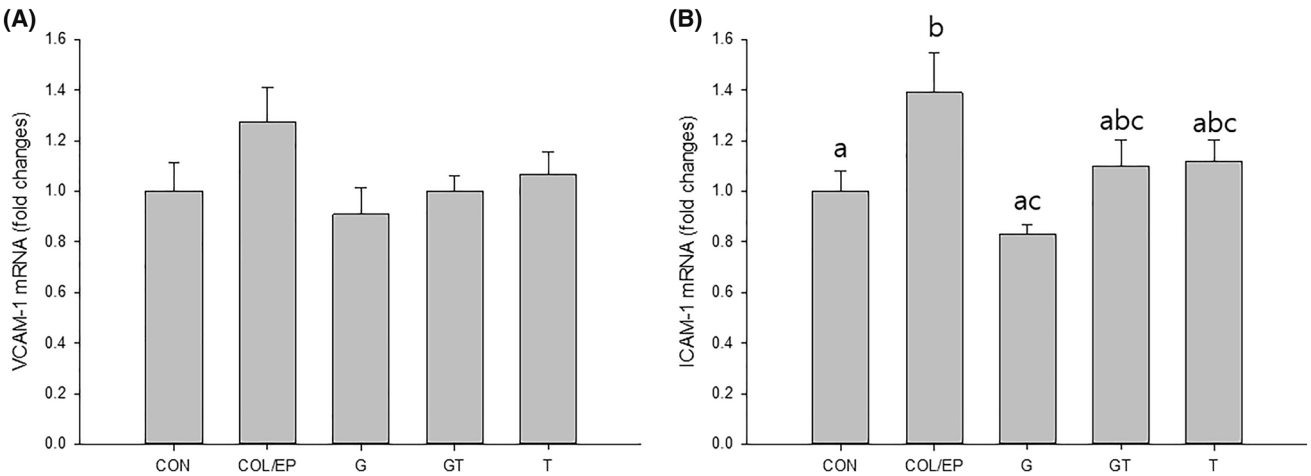

Fig. 2 Change in (**A**) VCAM-1 and (**B**) ICAM-1 mRNA expression levels when thrombosis-induced rats were administered G, T, and GT. Values are presented as mean ± SE ($n = 8$). Significance was analyzed by one-way ANOVA followed by Duncan's multiple range test. Values having different superscript alphabets differ significantly at $p < 0.05$. G, garlic powder; T, tomato extract; GT, the mixture of garlic powder and tomato extract

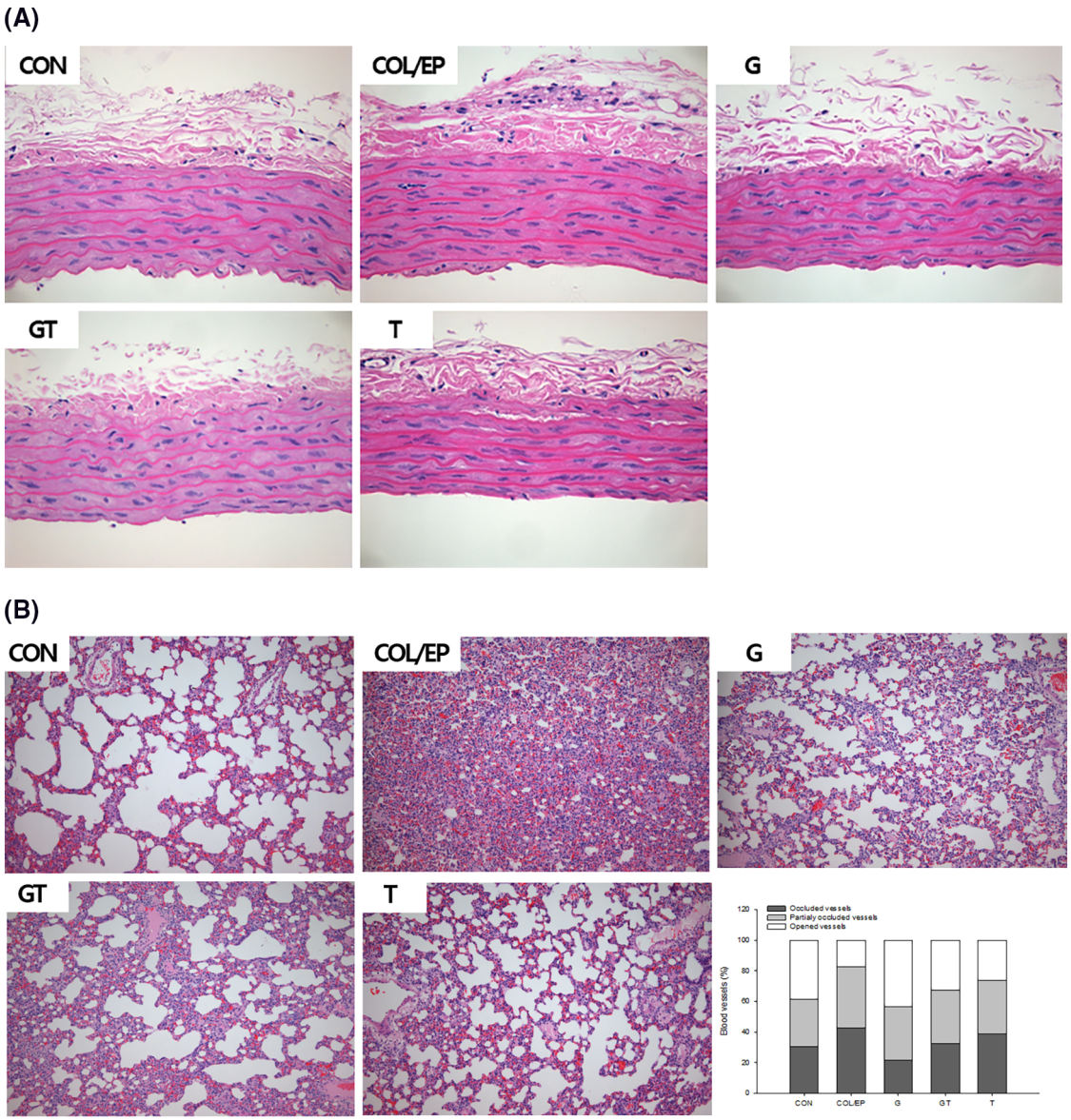

Fig. 3 Representative histopathological changes in the (**A**) aorta and (**B**) lung tissues (magnification × 400 for aorta and × 200 for lung). G, garlic powder; T, tomato extract; GT, the mixture of garlic powder and tomato extract

Results and discussion

To monitor prolonged blood coagulation time, PT and APTT were measured using PRP after collagen and epinephrine were injected (Fig. 1). PT was observed to be 1.44, 1.29, 1.45, 1.39 and 1.45 s in CON, COL/EP, G, GT, and T, respectively. Although limited statistical significance, injection of collagen and epinephrine reduced PT and APTT. All treated groups including G, T, and GT prolonged PT, however, there were no statistically significant differences. APTT was found to be 0.66, 0.46, 0.72, 0.83 and 0.73 s for CON, COL/EP, G, GT, and T, respectively. In spite of between-treatment differences, all treated groups including G, T, and GT, had significantly prolonged APTT compared to COL/EP group. These suggest that garlic powder and tomato extract might have the potent and preferential inhibitory activity toward the intrinsic and/or common pathway of coagulation, not the extrinsic pathway. Thrombus formation in the early stage is accompanied with platelet adhesion, activation and aggregations results from injuries of vascular endothelium (Furie and Furie, 2008). Activated endothelium express chemokines and adhesion molecules which involve the attachment of platelets (Mestas and Ley, 2008). Adhesion molecules such as VCAM-1 and ICAM-1 promote platelet adhesion and leukocyte recruitment and play an important role in atherogenesis (Lalor et al., 2007; Lin et al., 2014). In this study, mRNA expression levels of VCAM-1 and

ICAM-1 were measured in aortic tissue (Fig. 2). Although statistical significance was not reached, COL/EP group had a tendency for increased VCAM-1 expression levels and all treated groups had it for decreased VCAM-1 levels. In ICAM-1, the COL/EP group significantly increased ICMA-1 mRNA expression compared to CON and G significantly decreased. GT and T showed decreasing pattern in ICAM-1 levels. Representative H&E staining results of aorta and lung tissue are shown in Fig. 3. A significant decrease in the thickness of aorta was observed in G and T groups (Fig. 3A). Also, prominent occlusions were found in lung tissue of the COL/EP group, whereas, G, T, and GT groups showed less prominent occlusions in lung tissue ($p < 0.0001$) (Fig. 3B). The consumption of a fresh clove of garlic for 26 weeks reduced serum thromboxane by approximately 80% (Ali and Thomson, 1995). Garlic ethanol extract, containing compounds such as diallytrisulfide, 2-vinyl-1,3-dithiene and ally 1,5-hexadienyltrisulfide, inhibited platelet aggregation in vitro (Apitz-Castro et al., 1983). Tomato is considered a representative anti-thrombotic food (Palomo et al. 2012; Yamamoto et al., 2003). In agreement with previous studies, garlic powder and tomato extract showed increasing APTT which is intrinsic pathway and decreased the expression of adhesion molecule, ICAM-1, in aorta. This meant that these treatments might help to maintain endothelial function in blood vessels by the delayed coagulation time, altered vascular tone and decreased expression of ICAM-1. However, in the GT group, the expected synergistic effect was not statistically significant, although GT showed similar prolongation of APTT to G and T groups and alleviated occlusion in lung tissue. Although each food has capacity for regulating thrombosis, the mechanisms might be different. However, to reach significant results, the amount and treated time should be procured. Further investigation for searching an effective combination might be also needed. In the present study, collagen and epinephrine were used as inducing agents for the thrombosis model. This model represents a simple and efficient tool for atherothrombosis research. However, the severity of the disease phenotype likely prevents the ability to demonstrate a synergistic effect of garlic and tomato combination (Lim et al., 2016). The other limitation of this study was the absence of a platelet aggregation measurement, notwithstanding its key role in thrombosis (Nesbitt et al., 2009). Despite these limitations, garlic and tomato represent promising anti-thrombotic food ingredients. Further studies including elucidation of mechanisms of action and human intervention trials should be proceeded.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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