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Aged Garlic Extract and Its Components Inhibit Platelet Aggregation in Rat

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Many clinical trials have demonstrated the beneficial effects of garlic (*Allium sativum*) on general cardiovascular health. Aged garlic extract (AGE) is known to display diverse biological activities such as in antioxidant, anti-inflammatory and anticancer activities. However, few studies have been directed on the effect of AGE on cardiovascular function. In this study, we aimed to investigate the effect of AGE and its components on platelet activation, a key contributor in thrombotic diseases. In freshly isolated rat platelets, AGE and its components have shown inhibitory activities on thrombin-induced platelet aggregation. These *in vitro* results were further confirmed in an *in vivo* platelet aggregation measurement where tail vein injection of garlic oil and S-Allylmercapto-cysteine (SAMC) significantly reduced thrombin and ADP-induced platelet aggregation. Potential active components for antiplatelet effects of AGE were identified to be SAMC and diallyl sulphide through agonist-induced platelet aggregation assay. These results indicate that aged garlic extract can be a novel dietary supplement for the prevention of cardiovascular risks and the improvement of blood circulation.

Key words : Aged garlic extract (AGE), antiplatelet activity, diallyl sulphide, S-Allylmercapto-cysteine (SAMC)

Introduction

Garlic has been used for many centuries, as both a flavoring and a folk medicine. At present, the potential therapeutic and health promoting effects of garlic are attracting considerable interest. The pharmacological effects of garlic are associated with antihypertensive, antimicrobial, anticancer, anticoagulant, as well as, hypoglycemic effects [5,25,33,36]. Aged garlic extract (AGE) is well known for its ability to decrease parameters associated with cardiovascular disease [25]. It has been shown to protect the oxidation of human LDL and to reduce oxidative stress [11,12,21] and blood pressure in smokers [3]. AGE has also been shown to be effective in lowering plasma cholesterol and triglycerides and LDL cholesterol in hyperlipidemic subjects [29].

Platelets can be stimulated by various agonists, including adenosine diphosphate (ADP), and have 3 receptors to which ADP can bind [14,15]. Platelets adhere to the exposed collagen, laminin, and von Willebrand factor in the injured vessel, a process that is known as platelet activation. This result can also be achieved through agonists, such as ADP, collagen, and thrombin. Once ADP binds to these receptors, platelets change shape and aggregation takes place, including the secretory processes and the liberation of arachodoinic acid, which is rapidly converted to prostaglandins and lipoxygenase products such as thromboxane A_2 (TXA_2).

It is now recognized that garlic and its various components have the ability to inhibit platelet aggregation both in vivo and in vitro [3,13,26]. The antiplatelet activity of garlic has usually been studies with raw, dehydrated, or extracted preparations. Aqueous extracts of raw garlic, garlic oils, and other components of garlic inhibited human platelet aggregation *in vitro* [1,6,35]. *In vivo*, chronic intake of raw garlic, garlic powder, garlic oil, and AGE inhibited platelet aggregation in human subjects [7,16,20,21]. A single dose of garlic powder also demonstrated significant in vivo antiplatelet activity in humans [20]. Animal feeding studies reported in vitro and in vivo platelet inhibitory activity, induced by raw extracts of garlic [32]. One such garlic component is an AGE, which inhibits platelet aggregation in vivo [5, 31]. AGE also inhibits platelet aggregation when platelets are stimulated by agonists such as ADP, collagen, and epinephrine [24,29,30,31].

However, it remains unknown which components of AGE are able to inhibit platelet aggregation. Our objective

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was to study the protective effect of S-allylmercapto-L-cysteine (SAMC) and diallyl sulphides from AGE against thrombin, collagen and adenosine 5′-diphosphate (ADP)-induced platelet aggregation. The results will provide further insight into the medical benefit of these sulfur agents on diabetic complications.

Materials and Methods

Materials

Trisodium citrate, ethanol, ethyl acetate, methanol, dimethyl sulfoxide, adenosine, NaCl, KCl, $MgCl_2$, HEPES, glucose, $NaHCO_3$, Na_2HPO_4, $CaCl_2$, glutaraldehyde, EDTA, urethane, and bovine serum albumin were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Collagen was from Chrono-log (Harvertown, PA, USA). AGE was donated by Do-Wool nongsan. Diallyl sulphide (DAS) was obtained from Fluka and diallyl disulphide (DADS) was obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Diallyl trisulphide (DATS) was obtained from LKT. S-allylmercaptocysteine (SAMC) was obtained from Sejong University (Prof. Kyung).

Experimental animals

Male Sprague-Dawley rats, 5-6 weeks old, weighing 250-300 g (Orientbio, Seoul, Korea), were used for *in vitro* aggregation. Animals were housed in polyethylene cages in the animal room with controlled temperature at 24±2°C, a constant humidity of 50±10%, and a 12 hr light-dark cycle. Animals had free access to a standard diet from Purina Korea and UV-sterilized tap water *ad libitum*. The animals were acclimatized for at last 1 week prior to experiments and were randomly assigned to treatment groups. Protocols were approved by the institutional animal care and use committee (IACUC) of Amorepacific R&D Center prior to every experiment.

Preparation of rat platelets

Rat blood was collected male Sprague-Dawley rats, 5-6 weeks old, weighing 250-300 g. Blood was anticoagulated with 3.8% trisodium citrate solution (1:9 citrate/blood, v/v). All procedures were conducted at room temperature, and the use of glass containers and pipettes was avoided. Platelet-rich plasma (PRP) was prepared by centrifugation at room temperature for 15 min at 150× *g*. Platelet-poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation at room temperature for 20 min at 2,000× *g*. The platelet count in PRP was adjusted to 3×10^8 platelets/ml using PPP.

Platelet aggregation measurement

Platelet aggregation was determined by the turbidometric method using an aggregometer (Chrono-log). After incubation with soybean extracts or fractions for 10 min at 37°C, PRP was loaded on the aggregometer and stimulated with various agoinsts for 5 min. Platelet aggregation was measured by light transmission, with 100% calibrated as the absorbance of PPP and 0% calibrated as the absorbance of PRP.

In vivo experiments

Male Sprague‐Dawley rats (SamTako, Osan, Korea) weighing 200-300 g were used for animal studies. Before the experiments, animals were acclimated for 1 week, and food and water were provided *ad libitum*. All the protocols were approved by the ethics committee of the Chonnam National University Animal Service Center. SAMC (150 mg/kg body weight), Garlic oil (1 and 3 ml/kg body weight) and control as corn oil was administered through tail vein injection. For measurement of *in vivo* platelet aggregation, 3 day after single oral administration of SAMC and garlic oil, whole blood was collected from abdominal aorta anticoagulated with 3.8% trisodium citrate solution (1:9 citrate/blood, v/v) under anesthesia. PRP preparation and platelet aggregation measurements were done as described above except for the concentration of ADP used (8-10 μg/ml).

Statistical analyses

All experiments were performed with triplicate independent samples and were repeated at least twice, giving qualitatively identical results. Results are expressed as mean±standard error of the mean. Data were analyzed using Student's t-test, with p values <0.05 taken to indicate statistical significance.

Results

In vitro antiplatelet effects of fractions of AGE

To examine the effect of fractions of AGE on platelet aggregation, we treated freshly isolated rat platelets with various fraction of AGE for 5 min and then initiated platelet aggregation with thrombin (0.3 U). AGE showed inhibitory

effect against thrombin induced platelet aggregation in a concentration dependent manner and achieved significance between 1-10% (v:v) (Fig. 1A and E upper panel). Notably, S-allylcysteine (SAC), major sulfur compound, showed very weak inhibitory effect against thrombin induced platelet aggregation (Fig. 1B and E middle panel). But, water soluble fraction and oil fraction of AGE showed the strongest inhibitory effect against thrombin-induced platelet aggregation than other extraction conditions (Fig. 1C and D). Oil fraction of AGE inhibited thrombin-induced platelet aggregation significantly in a concentration-dependent manner (Fig. 1E lower panel).

Identification of active compoenets of AGE

For the identification of active components for antiplatelet effects of AGE, individual components of AGE (garlic oil, SAC, SAMC, DAS, DADS, DATS) were tested for their effects on thrombin induced platelet aggregation. Total percentage aggregation of platelet was reduced concentration-dependent manner (Fig. 2). Garlic oil and DATS showed that the significant inhibitory effect on platelet aggregation than DAS, DADS. Water soluble sulfur compound, S-allylmerceptocysteine (SMAC) showed that the inhibitory effect on platelet aggregation than followed by SAC. Also, individual components of AGE were tested for their effects on ADP or collagen induced platelet aggregation. SAMC, garlic oil and three dially sulphides inhibited ADP induced platelet aggregation in a dose-dependent manner (Fig. 3). And garlic oil and three dially sulphides inhibited collagen induced platelet aggregation (Fig. 4). As a result, water soluble sulfur compound, SMAC and three dially sulphides showed the most potent inhibitory effect on agonists-induced platelet aggregation.

In vivo antiplatelet effects of SAMC and garlic oil

To confirm the antiplatelet effect of SAMC and garlic oil, we conducted an *in vivo* platelet aggregation measurement after single or multiple oral administration of SAMC (150

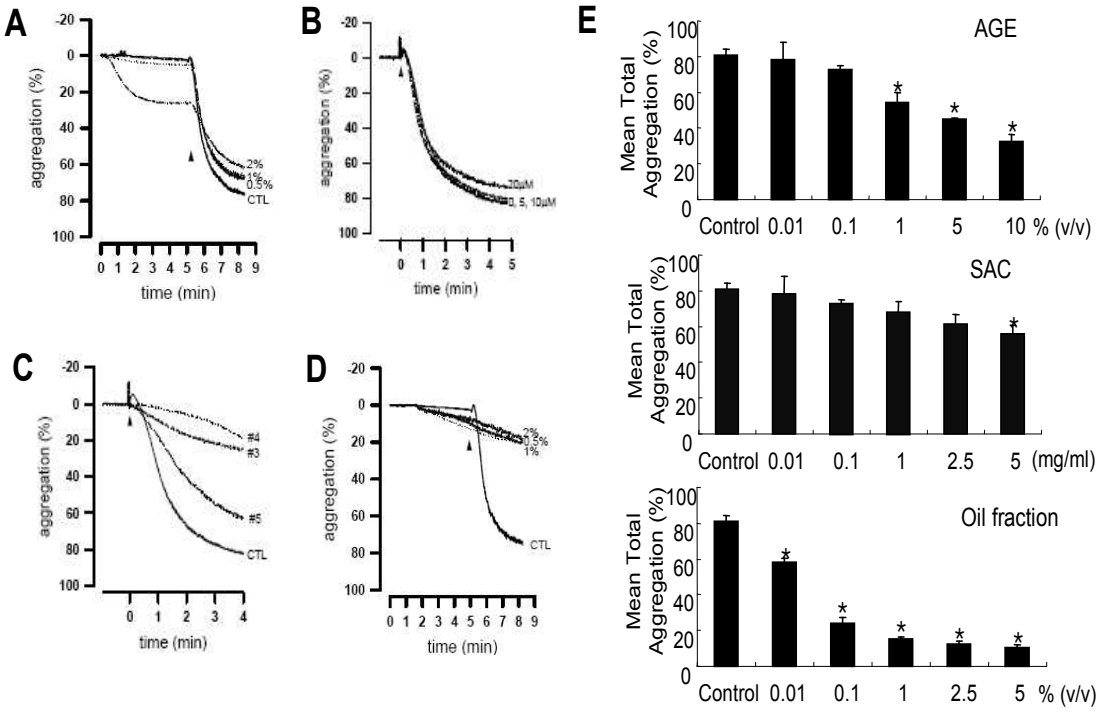

Fig. 1. Inhibition of thrombin induced platelet aggregation by aged garlic extract and its components. Washed platelets were pretreated with various concentrations of garlic compounds in aggregometer for 5 minutes. And thrombin (0.3 U) was treated. (A) AGE, dose dependently, inhibited thrombin-induced-platelet aggregation. (B) SAC weakly inhibited thrombin induced platelet aggregation. (C) AGE (#5, 2%), water-soluble fraction (#4) and oil fraction (#3) of AGE inhibited thrombin-induced platelet aggregation. (D) Oil fraction of AGE, dose dependently, inhibited thrombin-induced platelet aggregation. (E) AGE, SAC and oil fraction of AGE, dose dependently, inhibited thrombin-induced platelet aggregation. Values are mean±SD of six determinations. *p<0.05 vs. agonist-induced control.

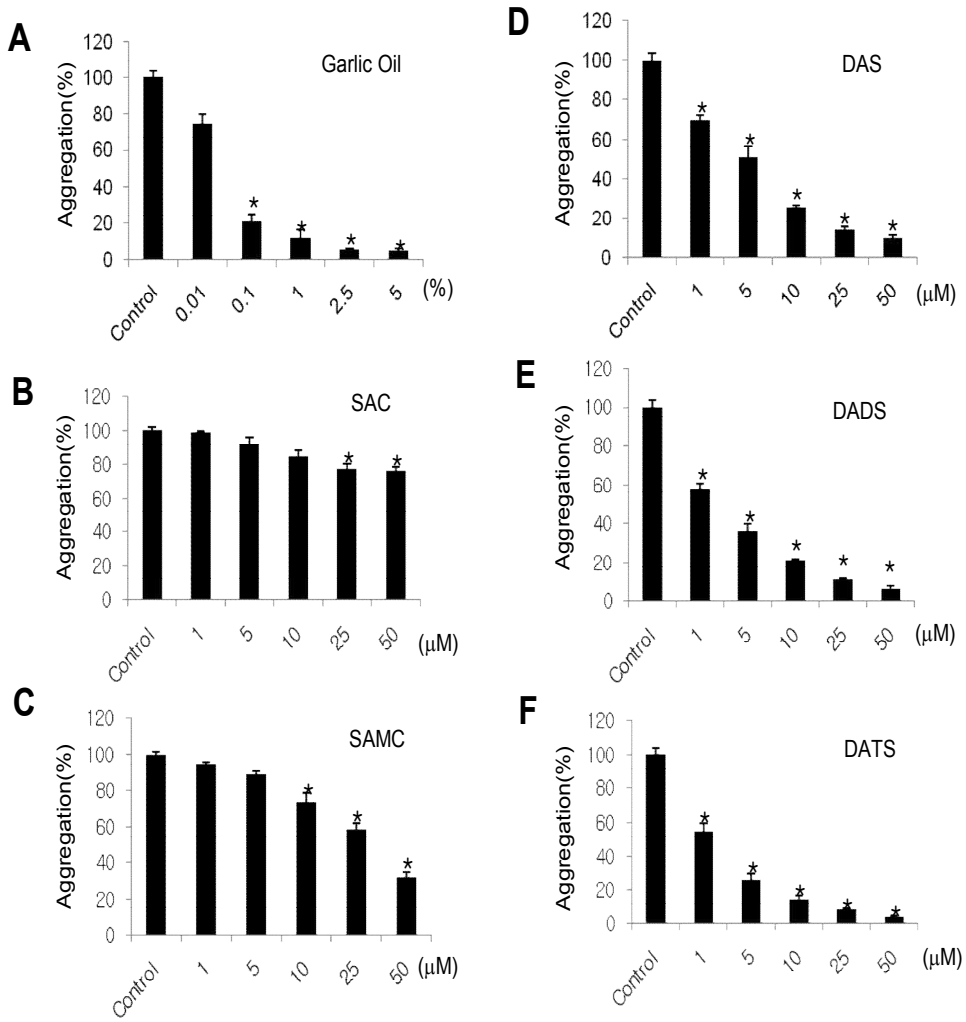

Fig. 2. Effect of Garlic oil and components of AGE on thrombin-induced platelet aggregation (A-F). Washed platelets were pretreated with various concentrations of garlic compounds in aggregometer for 5 minutes. And thrombin (0.3 U) was treated. Values are mean±SD of six determinations. *p<0.05 vs. agonist-induced control.

μg/kg body weight once daily) and garlic oil (1 ml or 3 ml/kg body weight once daily) as control corn oil. As a result, thrombin and ADP-induced platelet aggregation was significantly inhibited by SAMC and garlic oil (Fig. 5A and B). However, treatment of SAMC, AGE and garlic oil not inhibited food intake and body weight (Table 1).

Discussion

In this study, we demonstrated that AGE and its components can inhibit agoinsts-induced platelet aggregation. These *in vitro* results were further confirmed in an *in vivo* platelet aggregation where single oral administration of garlic oil reduced platelet aggregation. In addition, we identified SAMC and three diallyl surphies as a potential active ingredient for antiplatelet effects of AGE through agoinsts-induced platelet aggregation.

Commonly used antithrombotic drugs are frequently associated with adverse effects, and their benefit for prevention of cardiovascular risks is often being questioned [4,18]. In support of this concern, an analysis of 22 clinical trials using aspirin, a representative antiplatelet drug, as a preventive treatment for thrombosis has demonstrated that bleeding risk increased by aspirin intake from 0.07% to 0.1%, while the rate of serious thrombotic events decreased minutely from 0.57% to 0.51% [2]. For this reason, food materials have gathered huge attention as an alternative and preventive measure against cardiovascular risks, especially for thrombotic events, and many efforts have been made to develop functional foods with antithrombotic activities. In 7

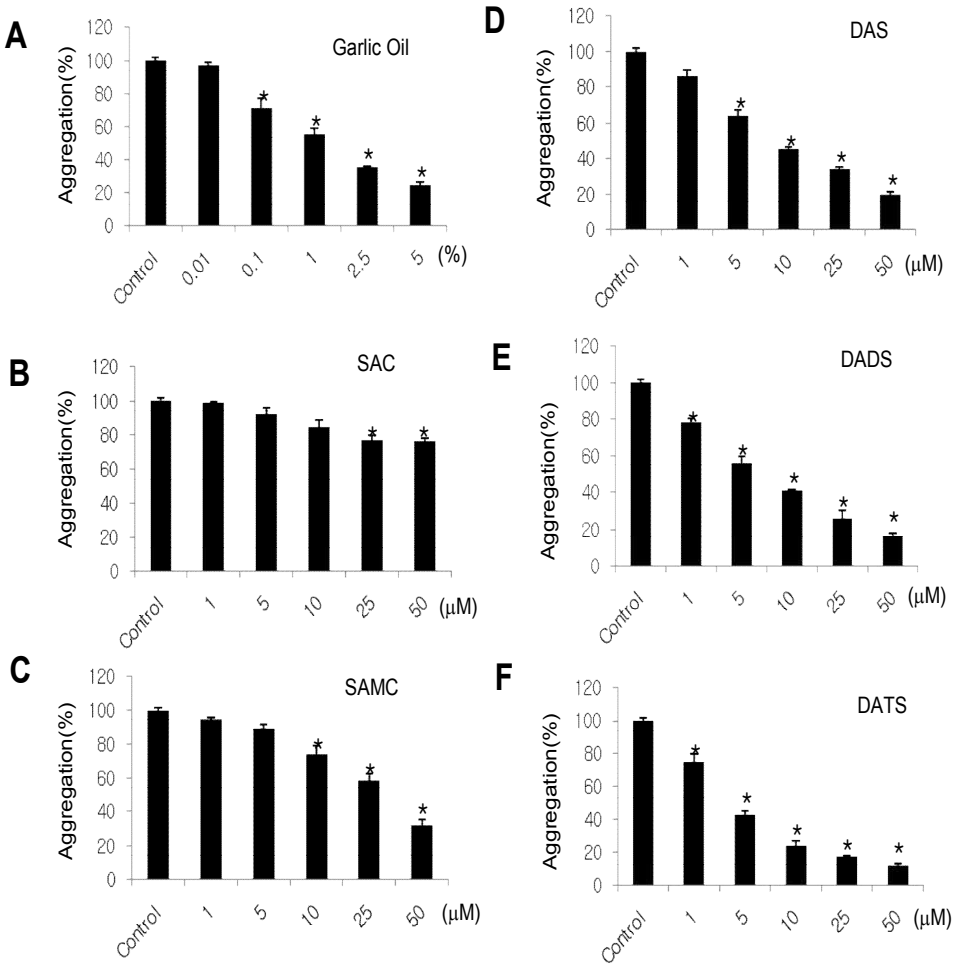

Fig. 3. The effect of garlic oil (A), water soluble sulfer compound, SAC (B), SAMC (C) and diallyl sulphide, DAS (D), DADS (E) and DATS (F) on ADP (50 μM) induced platelet aggregation at various concentrations. Garlic oil, water soluble sulfer compounds, and diallyl sulphide was incubated with platelets for 5 min. Platelet aggregation was initiated by ADP and aggregation was measured for 5 min in aggregometer. Values are mean±SD of six determinatios. *p<0.05 vs. agonist-induced control.

clinical trials, garlic (*Allium sativum*), a common spice, has shown antiplatelet activity in both healthy people and patients with cardiovascular disease [27,28]. The antithrombotic effect of onions (*Allium cepa*) has been also demonstrated in *in vivo* thrombosis animal models [9,38].

AGE has many biochemical properties associated with a reduction in risk factors for cardiovascular disease. One of risk factors in this disease is the increased ability of platelets to aggregate. Previous report shown that dietary supplementation with AGE reduces the ability of platelets to aggregate in healthy subjects. AGE is a complex mixture with relatively high concentrations of water-soluble compounds and low concentrations of oil-soluble compounds, and it is standardized by SAC, its major organosulfur constituent. In this study, AGE significantly inhibited platelet aggregation between 1 – 10% (v:v) (Fig. 1A and E upper panel). Because water-soluble fraction and oil fraction of AGE show inhibition of thrombin-induced platelet aggregation, it is highly likely that the water-soluble compounds and oil soluble compounds present in AGE are the ones responsible for its antiplatelet aggregation reaction. The SAC, water-soluble major organosulfur compound failed to show any significant inhibition of platelet aggregation.

Other studies have indicated that DAS, DADS and DAT were able to inhibit thrombin, collagen and ADP induced platelet aggregation via interfering intracellular Ca^{2+} mobilization [8,19,23]. Our present study clearly showed that three test diallyl sulphides could markedly inhibit thrombin,

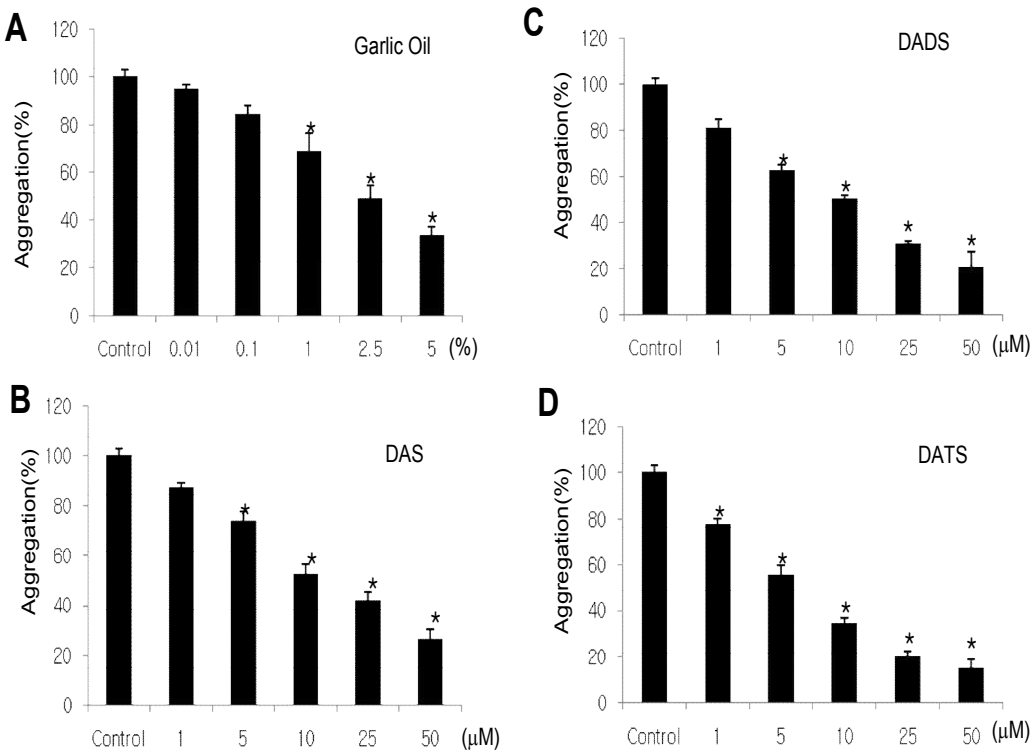

Fig. 4. The effect of garlic oil (A) and diallyl sulphide, DAS (B), DADS (C) and DATS (D) on collagen (0.5 mg/ml) induced platelet aggregation at various concentrations. Garlic oil and diallyl sulphide was incubated with platelets for 5 min. Platelet aggregation was initiated by collagen and aggregation was measured for 5 min in aggregometer. Values are mean±SD of six determinatios. *p<0.05 vs. agonist-induced control.

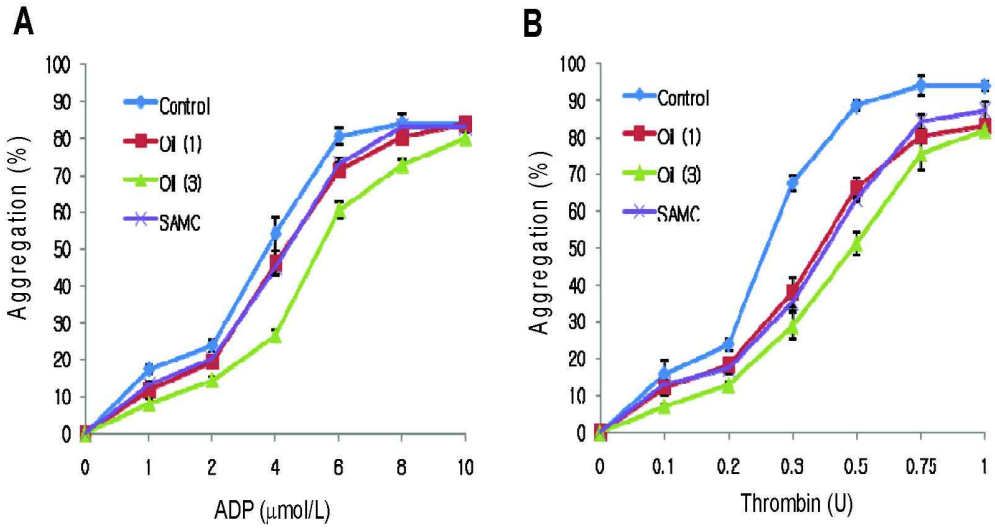

Fig. 5. *In vivo* effect of SAMC and garlic oil on agoinst induced platelet aggregation. (A) The effect of SAMC (150 μg/kg) and garlic oil (1 ml/kg or 3 ml/kg) on various concentrations of ADP (μM) induced platelet aggregation. Garlic oil was incubated with platelets for 5 min. Platelet aggregation was initiated by ADP and aggregation was measured for 5 min in aggregometer. (B) The effect of SAMC (150 μg/kg) and garlic oil (1 ml/kg or 3 ml/kg) on thrombin (U) induced platelet aggregation. Platelet aggregation was initiated by thrombin and aggregation was measured for 5 min in aggregometer. Values are mean±SD of three determinatios.

Table 1. Food intake and body weight in Sprague-Dawley rats treated with SAMC, AGE and garlic oil

	Food intake (g/day)	Body weight (g)
Control (n=3)	20.36±0.53	272.52±12.05
Garlic oil (1 ml/kg)	18.26±0.89	276.21±16.25
AGE (3 ml/kg)	18.26±0.89	276.21±16.25
SAMC	19.89±0.21	270.2±11.56

ADP and collagen-induced human platelet aggregation. It is known that collagen, ADP and thrombin are three of the most important agonists of platelet aggregation [22]. Therefore, our present study and those previous studies supported that both DADS and DAT than DAS were effective agents in antiplatelet aggregation. Our present study found that three test diallyl sulphides were effective antiplatelet agents; therefore, use of these agents could benefit thrombotic prevention and therapy. Like α-tocopherol, the three test agents were natural lipid-soluble antioxidants, and these agents (DAS, DADS and DAT) are compounds naturally formed in garlic, Chinese leek and onion [13,17,37]. The content of DAS, DADS and DAT in garlic was 250-480, 2,600-4,100 and 1,250-1,970 μg/kg of garlic, respectively [34]. In other words, 28-45 g of garlic could provide 10 μM of DAT. Therefore, based on the natural and dietary property, the use of these agents at these concentrations as antioxidative and/or antiplatelet agents should be safe and acceptable.

In summary, we have demonstrated that AGE and its compoenents inhibited agonists-induced platelet aggregation and activation, leading to reduced thrombus formation. In addition, repeated oral administration of SAMC and garlic oil manifested more potent antiplatelet aggregation effects *in vivo*, indicating that long-term intake of SAMC and garlic oil could be more effective in the prevention of thrombotic events. More importantly, this study has provided, we believe, important evidence for the utility of the common food materials for the effective prevention of cardiovascular diseases.

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초록 : 흰쥐에서 흑마늘 추출물과 그 성분들에 의한 혈소판 응집억제 효과

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많은 임상 실험을 통해 마늘(*Allium sativum*)이 심혈관 질환에 유효한 효과가 알려져 있다. 흑마늘(AGE)은 항산화, 항염증, 항암 효과 등 다양한 생물학적 효과가 보고되었다. 그러나, 흑마늘의 심혈관계 질환과의 상관관계에 대한 연구는 아직 미흡한 실정이다. 흑마늘과 그 유래 성분이 혈소판에 미치는 영향을 조사하였다. 백서의 혈액을 얻어, 흑마늘과 그 유래 성분의 thrombin에 의해 유도되는 혈소판 응집에 대한 억제 효과를 조사한 결과, 흑마늘과 diallyl sulphides는 농도의존적으로 혈소판 응집을 억제함을 관찰하였다. 또한 *in vivo* 실험에서도 마늘 오일과 S-Allylmercapto-cystein (SAMC)는 thrombin과 ADP에 의해 유도되는 혈소판 응집을 유의성 있게 억제하는 것을 관찰하였다. 흑마늘 유래 SAMC와 diallyl sulphides가 혈소판 응집 억제 효과를 나타내는 것을 확인할 수 있었다. 이러한 결과는 흑마늘이 심혈관계 질환의 예방을 위한 건강보조식품으로의 새로운 가능성을 제시한다.