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Anticardiovascular Diseases Effects of Fermented Garlic and Fermented Chitosan

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Abstract

Garlic is a medicinal plant used throughout the world for its anti-inflammatory, antioxidant, and antiplatelet activities. Chitosan is a natural polysaccharide obtained from chitin, and derivatives of chitosan have been shown to inhibit platelet aggregation and adhesion. We hypothesized that fermented preparations of these products may possess stronger antiplatelet effects than the non-fermented forms owing to the increased bioavailability of the bioactive compounds produced during fermentation. Therefore, we compared these compounds via *in vitro* and *ex vivo* platelet aggregation assays by using standard light transmission aggregometry and *ex vivo* granule secretions from rat platelets. We found that fermented preparations exerted more potent and significant inhibition of platelet aggregation both *in vitro* and *ex vivo*. Likewise, ATP release from dense granules of platelets was also significantly inhibited in fermented preparation-treated rat platelets compared to that in non-fermented preparation-treated ones. We concluded that fermented preparations exerted more potent effects on platelet function both *in vitro* and *ex vivo*, possibly as a result of the increased bioavailability of active compounds produced during fermentation. We therefore suggest that fermented products may be potent therapeutics against platelet-related CVDs and can be used as antiplatelet and antithrombotic agents.

Keywords: Cardiovascular diseasess, Fermented garlic, Fermented chitosan, Anti-platelet

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality in developed countries. Multiple risk factors are involved in the pathophysiology, but platelets are considered the main etiological factor of CVDs. Platelet aggregation is a key step in the development and progression of atherosclerotic plaques responsible for narrowed blood vessels that may ultimately lead to stroke and heart attack [1]. Platelet function can be suppressed pharmacologically, and this has been highly successful in decreasing thrombotic events; many clinically approved antiplatelet drugs are now available for the treatment of cardiovascular ailments. Unfortunately, as these drugs can lead to serious complications, such as gastric bleeding, and are ineffective in some patients [2]. There is a need for the development of more effective and safer approaches for the treatment and preven- tion of CVDs. One such approach includes the use of natural products with antithrombotic and anticoagulant activities [3]. Ethnomedicine and natural products have been the focus of many recent studies as potential therapies for CVDs [4], and several dietary and herbal compounds have been shown to contribute to a reduced risk of CVDs [5].

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Garlic has been shown to reduce cardiovascular diseases and its consumption confers significant cardioprotective effects to both animals and humans [6, 7]. Chitosan is a natural bio- degradable and non-toxic cationic polysaccharide obtained from chitin. Its derivatives are known to inhibit platelet aggregation and adhesion [8]. For a long time, fermentation of foods has been appreciated owing to its significant commercial benefits, such as improved nutrient profile and enriched flavor. There are several health benefits of consuming fermented foods beyond the traditionally re- cognized effects on the digestive system [9]. It was previously reported that fermentation enhances the quality and efficacy of food or herbal products through an improved nutrient profile [10]. Therefore, we aimed to compare the effects of fermented garlic and chitosan with those of non-fermented compounds on platelet function.

2. Experiment Materials and Methods

2.1. Reagents

Collagen (native collagen fibrils (type I) from equine tendons), ADP, and thrombin were purchased from Chrono-log (Havertown, PA, USA). The ATP assay kit was obtained from the Biomedical Research Service Centre (Buffalo, NY, USA) and DMSO was acquired from SigmaAldrich (St. Louis, MO, USA). Water was obtained from J.T. Baker (Phillipsburg, NJ, USA). All chemicals were of reagent grade.

2.2. Sample Preparation

The samples to prepare fermented chitosan, chitosan was first dis-solved in hydrochloric acid and chitosanase was added to decompose the chitosan and produce chitosan oligosaccha ride. To prepare fermented garlic, unpeeled garlic was placed in an autoclave and steamed to produce black garlic. Lactic acid bacteria were added for the second round of fermentation.

2.3. Experimental animals and dosage

Male Sprague-Dawley (SD) rats (240~260 g) were purchased from Orient Co. (Seoul, Korea) and acclimatized for 1 week prior to the experiment in an animal room with controlled environmental conditions (12/12 h light/dark cycle, 23℃±2℃ temperature, and 50%±10% humidity). The experiments were conducted in accordance with the IACUC guidelines and the protocols were approved by the Ethics Committee of College of Veterinary Medicine, Kyungpook National University, Daegu, Korea. The rats were randomly divided into six groups (n = 4 in each group) for the *ex vivo* study and received a daily oral administration of vehicle, 300 mg/kg garlic (fermented and non-fermented), and 300 mg/kg chitosan (fermented and non-fermented) for 7 days. Two hours after the final administration, blood samples were collected from rats for testing.

2.4. Preparation of washed platelets

Whole blood was collected from SD rats *via* heart puncture and transferred to a tube containing anticoagulant acid citrate dextrose solution. The blood was centrifuged at 170 ×*g* for 7 min to obtain plateletrich plasma (PRP), which was then centrifuged at 350 ×*g* for 7 min to isolate the washed platelets. The platelet concentration was adjusted to 3×10^8 cells/mL by using Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, and 1 mM $NaHPO_4$, pH 7.4) for use in the platelet aggregation assays. All preparation procedures were performed at room temperature (23℃±2℃).

2.5. Garlic and chitosan inhibit *in vitro* and *ex vivo* aggregation assay

Light-transmission aggregometry (Chronolog, Havertown, PA, USA) was performed in accordance with a previously described method to assess platelet aggregation [11]. Briefly, the washed platelets were preincubated with various concentrations of either fermented garlic (F. garlic), non-fermented garlic (NF. garlic), fermented chitosan (F. chitosan), non-fermented chitosan (NF. chitosan), or vehicle for 2 min at 37℃ in the presence of 1 mM calcium chloride ($CaCl_2$), and then stimulated by the agonist collagen, ADP, or thrombin. The mixture was further incubated for 5 min with continuous stirring.

To achieve *ex vivo* platelet aggregation, washed platelets obtained from vehicle-, F. garlic-, NF. garlic-, F. chitosan-, and NF. chitosan-treated rats were incubated at 37℃ with continuous stirring and stimulated with collagen or ADP for 5 min. To determine the *ex vivo* effects of F. garlic, NF. garlic, F. chitosan, and NF. chitosan on dense granule secretion, an ATP assay was performed as previously described [12]. Briefly, washed platelets obtained from vehicle-, F. garlic-, NF. Garlic-, F. chitosan-, NF. Chitosan-treated groups were incubated at 37℃ with continuous stirring and stimulated with collagen or ADP for 5 min. The aggregation reaction was terminated and the platelet mixture was cen- trifuged. The ATP secretion of the obtained supernatant was determined by using an ATP assay kit (Biomedical Research Service Center) measured using a luminometer (GloMax 20/20, Promega, Madison, WI, USA).

2.6. Nitric oxide & MTT cell viability assay

Nitric oxide assay was carried out using RAW 264.7 cells. Cells were seeded in 96-well plates for 24 hr. Samples with respective concentrations were treated and 30 min later, 0.1 μg/mL of LPS was treated. After 18 hr of incubation, supernatant (100 μL) was collected and mixed with an equal amount of Griess reagent, and the absorbance was measured using a microplate reader (VersaMax, Molecular Devices, USA) at 540 nm. Cell viability was determined using MTT reagent, which was added at a concentration of 0.1 mg/mL, and the plates were incubated for 3 hr at 37°C and 5% CO2. The resulting crystals formed were dissolved in DMSO, and read at 560 nm using a microplate reader (VersaMax, Molecular Devices, USA).

2.7. Statistical analysis

The data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test to measure the statistical significance of the differences observed (SAS Institute Inc., Cary, NC, USA). All data are presented as the mean ± standard error of the mean (SEM), and *P*values < 0.05 indicated statistical significance.

3. Result and Discussion

3.1. Garlic and chitosan inhibit *in vitro* and *ex vivo* agoniststimulated platelet aggregation

The *in vitro* effects of F. garlic, NF. garlic, F. chitosan, and NF. chitosan on platelet aggregation were assessed by using light transmission aggregometry. The effects of the compounds were tested against different ligands (collagen, ADP, and thrombin), and F. chitosan was found to be most effective against ADP-induced platelet aggregation, whereas F. garlic and NF. garlic were more potent and inhibited platelet aggregation against collagen, ADP, and thrombin (Fig. 1A-B). F. garlic and N.F. garlic markedly inhibited agonist-induced platelet aggregation in a dose-dependent manner.

To evaluate the *ex-vivo* effects, the rats were treated with F. garlic, NF. garlic, F. chitosan, and NF. chitosan and the plate- let aggregation assay was performed. Platelet aggregation was inhibited in all treatment groups unlike in the vehicletreated control group. Moreover, F. garlic and NF. garlic showed greater inhibition of collagen-induced platelet aggre- gation than of ADP-induced platelet aggregation (Fig. 1C).

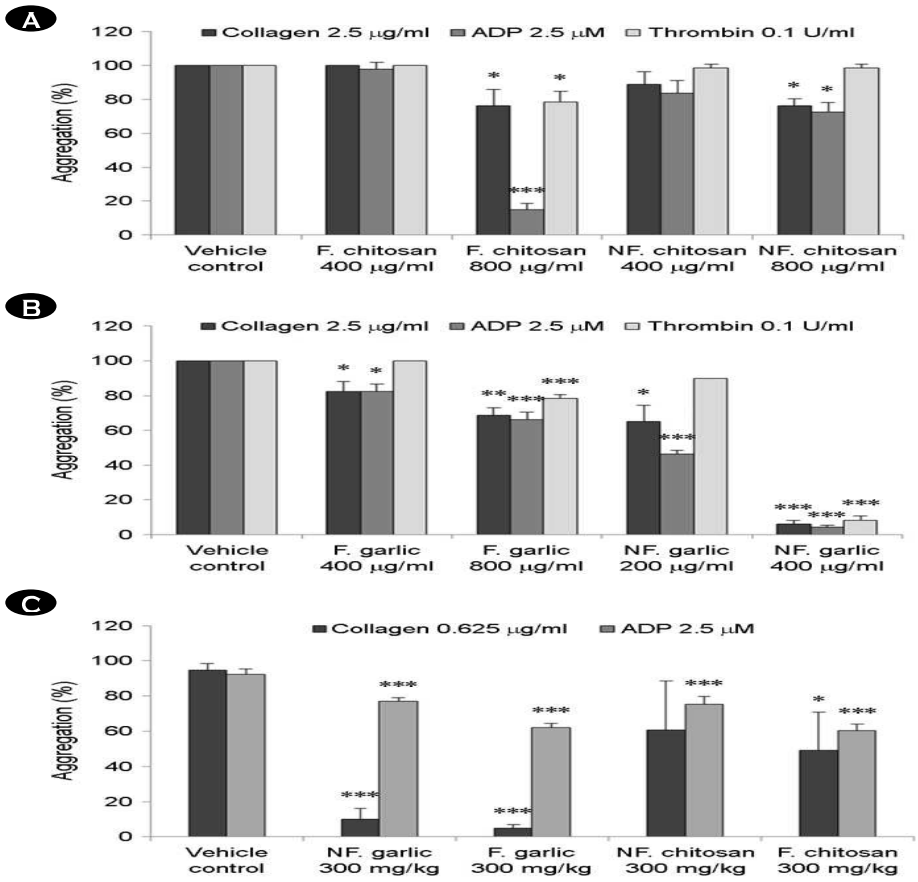

Figure 1. Garlic and chitosan inhibit *in vitro* and *ex vivo* agoniststimulated platelet aggregation.

Garlic and chitosan inhibit *in vitro* and *ex vivo* agoniststimulated platelet aggregation. (A-B) Washed platelets were pretreated with various concentrations of F. garlic, NF. garlic, F. chitosan, NF. Chitosan, or vehicle for 2 min in presence of 1 mM $CaCl_2$, and then stimulated with collagen, ADP, or thrombin for 5 min. (C) Washed platelets obtained from vehicle-, F. garlic-, NF. garlic-, F. chitosan-, or NF. chitosan-treated rats (all treatments at 300 mg/kg) were incubated at 37℃ with continuous stirring and then stimulated with collagen or ADP for 5 min. The graph presents the mean ± SEM of experiments performed on four independent days (n = 1 each day). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the vehicle control.

3.2. Garlic and chitosan inhibit ex vivo ATP secretion.

Activated platelets release the contents of granules such as alpha granules and dense granules; this secretion of granule content enhances platelet activation, including intracellular signaling pathways. The early phases of platelet activation are characterized by the rapid release of ATP [13]. ; therefore, we assessed collagen- and ADP-induced ATP secretion. As shown in Fig. 2, F. garlic, NF. garlic, F. chitosan, and NF. chitosan significantly inhibited ATP release from dense granules in collagen- and ADP-stimulated platelets. Although both were strong inhibitors of ATP release, garlic appeared to be slightly more potent than chitosan (Fig. 2). These data suggest that the compounds exerted antiplatelet effects through the sup- pression of platelet aggregation and granule secretion.

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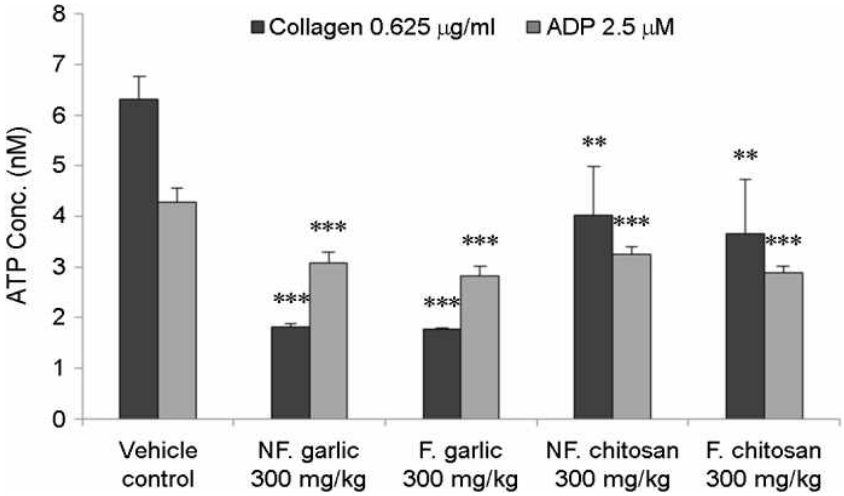

Figure 2. Garlic and chitosan inhibit ex vivo ATP secretion.

Fig. 2 Garlic and chitosan inhibit *ex vivo* ATP secretion. Washed platelets obtained from vehicle-, F. garlic-, NF. garlic-, F. chitosan-, or NF. chitosan-treated rats (all treatments at 300 mg/kg) were in- cubated at 37℃ with continuous stirring and stimulated with col- lagen or ADP for 5 min. Following the termination of the platelet aggregation reaction, the concentration of ATP was assessed by using a luminometer. The results are presented as the mean ± SEM of experiments performed on three independent days (n = 1 each day). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the vehicle control.

4. Conclusion

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality in developed countries. Multiple risk factors are involved in the pathophysiology, but platelets are considered the main etiological factor of CVDs. Platelet aggregation is a key step in the development and progression of atherosclerotic plaques responsible for narrowed blood vessels that may ultimately lead to stroke and heart attack. Platelet function can be suppressed pharmacologically, and this has been highly successful in decreasing thrombotic events; many clinically approved antiplatelet drugs are now available for the treatment of cardiovascular ailments. Unfortunately, as these drugs can lead to serious complications, such as gastric bleeding, and are ineffective in some patients. There is a need for the development of more effective and safer approaches for the treatment and preven- tion of CVDs. One such approach includes the use of natural products with antithrombotic and anticoagulant activities. Ethnomedicine and natural products have been the focus of many recent studies as potential therapies for CVDs , and several dietary and herbal compounds have been shown to contribute to a reduced risk of CVDs .

In the present study, we evaluated the inhibitory effects of fermented and non-fermented preparations of garlic and chitosan on platelet aggregation and granule secretion. We found that fermented preparations significantly inhibited platelet aggregation *in vitro* and *ex vivo* and also inhibited *ex-vivo* ATP release from dense granules. Many studies have shown that platelets are continuously exposed to several factors that cause their activation and aggregation, such as collagen, ADP, thrombin, fibrinogen, von Willebrand factor (vWF), and thromboxane; however, inhibitory factors are also present, such as prostacyclin (PGI_2) and ADPase [13]. Any imbalance in these opposing factors may impair hemostasis; thus, a strong equilibrium is necessary for normal platelet function. Our results indicate that the pretreatment of platelets with garlic or chitosan pre- parations, especially fermented preparations, may help main- tain this balance and hemostasis. As fermentation enhances bioavailability and the absorption of food products. we concluded that the observed antiplatelet effects were a result of the increased bioavailability of bioactive compounds from fermented preparation.

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