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Ameliorative effects of ark clams (*Scapharca subcrenata* and *Tegillarca granosa*) on endothelial dysfunction induced by a high-fat diet

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Abstract

Endothelial dysfunction is directly involved in consequence of various metabolic syndromes such as diabetes and hypertension. In this study, we investigated the preventive effects of two ark clams [ark shell (AS, *Scapharca subcrenata*) and granular ark (GA, *Tegillarca granosa*)] on endothelial dysfunction induced by a high-fat diet. Wistar rats were divided into four groups as follows: control (normal diet), HF (high-fat diet), AS (high-fat diet + 5% AS powder), and GA (high-fat diet + 5% GA powder) for 12 weeks. AS and GA diets enhanced vascular reactivity of the rat thoracic aorta and significantly increased expression levels of vascular relaxation-related proteins (p-Akt-ser473 and p-eNOS-ser1177). Ark clam supplement reduced endothelin-1 expression level, as compared to the HF group. Additionally, AS and GA showed a trend of improving insulin sensitivity compared to HF. Our results suggest that AS and GA enhance vascular reactivity and ameliorated endothelial dysfunction induced by a high-fat diet.

Keywords: Ark clam, Endothelial dysfunction, High-fat diet, *Scapharca subcrenata*, *Tegillarca granosa*

Introduction

Vascular homeostasis is maintained by the endothelial cells through manifold complex interactions with cells in the vessel wall. Endothelium normalizes vascular tone by balancing vasodilators and vasoconstrictors [1]. Failure of endothelial function is associated with a variety of metabolic syndrome including cardiovascular diseases [2], insulin resistance [3], and hypertension [4]. Nitric oxide (NO), the key regulator of the vasodilatory process, is generated from L-arginine through the catalyzation of endothelial NO synthase [2]. Endothelial dysfunction reduces vascular NO productivity, resulting in various vascular diseases. Interestingly, there is evidence suggesting that the foods can alter endothelial function negatively or positively [5]. Supplements of some foods and nutrients improve an endothelial function [5, 6].

Ark clams, including ark shell (AS, *Scapharca subcrenata*) and granular ark (GA, *Tegillarca granosa*), are edible marine bivalve mollusks. AS and GA are widely consumed as foods in China and Korea. Ark clams are rich in free amino acids such as taurine, glycine, arginine, etc.[7]. Several free amino acids are recognized as useful nutrients for improving endothelial function [8–11]. In particular, taurine that is highly contained in ark clams was reported to have endothelial protective activity [8] and improved vascular function [9]. In addition, several antioxidative and/or anticancer peptides have been isolated and identified from protein hydrolysates of GA muscle [12, 13]. We hypothesized that ark clams could help to prevent endothelial dysfunction. However, the

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preventive effects of ark clams on endothelial dysfunction in an animal model has not yet been assessed.

The chronic high-fat (HF) diet induces endothelial dysfunction and insulin resistance leads to cardiovascular diseases and diabetes [14, 15]. The HF diet has been widely used for assessing the preventive effect of foods on endothelial dysfunction and insulin resistance [16, 17]. In this study, the ameliorative effects of AS and GA on HF-induced endothelial dysfunction were evaluated.

Materials and methods

Materials and chemicals

AS and BC were purchased from the local market located in Boseong County, Korea. The ark clams were steamed for 3 min and the muscles obtained after shucking were dried by hot air dryer at 50 °C for 4 days. The dried muscles were ground by a grinder and stored at − 20 °C until used.

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobin was procured from Millipore Co. (Billerica, MA, USA). The antibodies of phosphorylated Akt-Sers73 (p-Akt-Ser473) and endothelin-1 (ET-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies for eNOS and phosphorylated-eNOS-Ser1177 (p-eNOS-Ser1177) were procured from Cell Signaling (Danvers, MA, USA). Reagent salt, acetylcholine chloride (Ach), sodium nitroprusside dehydrate (SNP), and phenylephrine hydrochloride was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies of insulin receptor substrate-1 (IRS1), phosphorylated IRS1 Serine307 (pIRS1ser307), and glucose transporter 4 (GLUT4) were procured from Upstate Biotech, Inc. (Lake Placid, NY, USA). Rabbit anti-GAPDH polyclonal antibody was obtained from Ab Frontier (Seoul, Korea). All other chemical reagents and solvents used in this study were of analytical grade.

Free amino acid analysis

The free amino acid content in the dried ark clam powders was determined by a Sykam S430 amino acid analyzer (GmbH, Eresing, Germany) [18].

Animal studies

Male Wistar rats (6 weeks old) were purchased from Damool Science (Daejeon, Korea). The rats were housed at room temperature (25 ± 1 °C), humidity (55 ± 5%), and light cycle (12 h; 6:00 to 18:00) and given ad libitum access to food and water. All rats were adapted for 1 week, randomly divided into 4 groups (n = 8), and treated with a special diet for 12 weeks as follows: Control (normal diet), HF (HF diet, 40% lard), AS (HF diet + 5% AS), and GA (HF diet + 5% GA). The animal experiment protocol was approved by the Committee on Animal Care and Use of Mokpo National University (MNU-IACUC-2015–032).

Assessment of vascular reactivity

The thoracic aorta ring was mounted in standard organ baths containing 10 mL of physiological salt solution and maintained at pH 7.4, temperature 37 °C, and continuously bubbled with 95% O_2 and 5% CO_2. The samples were constricted followed by re-equilibration two times using a high-potassium Krebs solution (80 mM KCl), then contracted to 1 μM phenylephrine and exposed to Ach (10^{-9} to 3×10^{-5} M) and SNP (10^{-10} to 3×10^{-5} M). Changes in tension were recorded by using a force–displacement transducer (HugoSachs, Germany).

Western blotting

Protein of thoracic aorta and adipose tissue samples were extracted and determined by the Bradford assay [19]. The protein samples were separated on 10% polyacrylamide gel by SDS-PAGE and transferred to PVDF membranes. After treating with blocking buffer for 1 h, the membrane was reacted overnight with primary antibodies at room temperature. Follow by incubation with secondary antibodies for 1 h and bands were visualized by the chemiluminescence reagent. The bands and their intensities were photographed and analyzed by ImageJ 1.50i software (MD, USA).

Immunohistological study

Samples were fixed in Bouin's solution for 24 h, dehydrated in graded alcohol, and embedded in paraffin. Blocks were cut into 4 μm sections, deparaffined in xylene, and rehydrated in series of alcohol and distilled water. The slides were incubated for 10 min in the antigen retrieval solution (microwave oven), blocked with 3% H_2O_2 solution for 5 min, incubated with 10% normal goat serum for 30 min, and incubated with primary antibody (1:200) overnight at room temperature. The slides were incubated with the biotinylated secondary antibody (1:200) for 2.5 h at room temperature and followed by incubation with avidin–biotin-peroxidase for 30 min. The colors were developed by 0.1% DAB chromogen solution and the photographs were taken by microscopes (Olympus, BX43).

Statistical analysis

The data were expressed as a means ± S.E.M. using SPSS v12.0 (Chicago IL, USA). The statistical significance of mean was determined by ANOVA following the post-hoc Tukey's test. The P value of < 0.05 was considered significant.

Results and discussion

Free amino acid content

The contents of taurine and free amino acids in the AS and GA powders are shown in Table 1. The AS powder showed higher total free amino acid content than the GA powder. Taurine was the most predominant in both AS and GA. The taurine content in the AS powder was higher than that in the GA powder. Additionally, free amino acids, including glutamic acid, glycine, arginine, alanine, β-alanine, and aspartic acid, were abundant in the dried AS and GA powders. Glutamic acid, glycine, and arginine in the AS powder and aspartic acid in the GA powder were more abundant. Ark clams have been found to be rich in taurine, a non-essential amino acid that has been reported to have various health-beneficial effects, including improvement of vascular endothelial function and hypertension [8, 9]. Moreover, glycine and arginine also enhance endothelial function by increasing nitric oxide bioavailability [10, 11]. Therefore, taurine might be one of the key factors that contribute to the preventive effects of AS and GA on vascular endothelial dysfunction.

Table 1 Free amino acid content of cockle powder and raw egg yolk

Component	Ark shell (AS) (mg/100 g)	Granular ark (GA) (mg/100 g)	Raw egg yolk[a] (mg/100 g)
Taurine	1339	811	
Glutamic acid	366	258	117
Glycine	314	55	25
Arginine	307	102	158
Alanine	276	251	63
Aspartic acid	198	387	50
β-Alanine	141	115	
Lysine	33	24	87
Leucine	28	13	74
Valine	22	14	66
Tyrosine	22	10	69
Isoleucine	17	8	39
Phenylalanine	16	5	34
Threonine	15	21	47
Serine	15	11	60
Histidine	12	6	15
Methionine	8	3	16
γ-Amino-*n*-butyric acid	2	3	
Total	3265	2138	976

[a] Data collected from the report by Nimalaratne et al. Food Chemistry 129: 155–161 (2011)

AS improved vascular reactivity

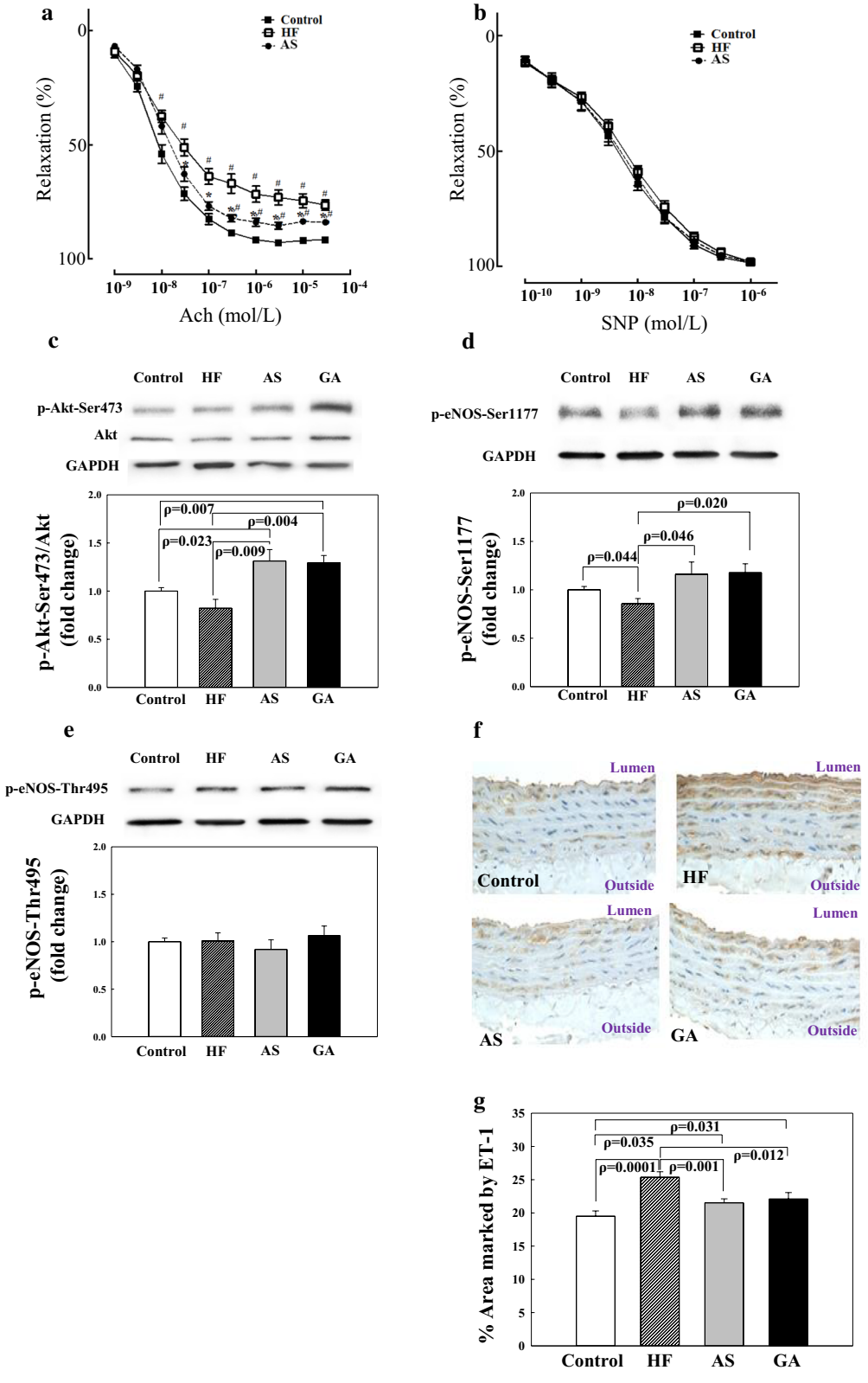
Previous studies have reported that HF causes obesity and leads to endothelial dysfunction [14]. To investigate the potential effects of AS on endothelial dysfunction, arterial segments were collected, and vascular reactivity was determined. As shown in Fig. 1a, the HF group showed a significant decrease in endothelium-dependent relaxation induced by Ach, as compared to the control group. Consistent with our results, previous studies have reported that HF impairs vasorelaxation response to Ach in the rat aorta [14]. The AS supplement significantly increased endothelium-dependent relaxation in the HF diet-fed rat. The half-maximal response concentration (EC50 value) of Ach in the control, HF, and AS groups were 10.9 ± 5.0, 18.0 ±3.1, and 13.5 ±1.7 nM, respectively. In addition, the maximal vasorelaxant effect of Ach in the control, HF, and AS groups were 91.7 ± 1.0, 76.4 ± 2.2, and 84.0 ± 1.4%, respectively. All groups also exhibited dose-dependent relaxation induced by SNP, the NO donor. However, there was no significant difference in EC50 value and the maximal vasorelaxant effect of SNP among all groups, indicating that there was no significant difference in endothelial-independent relaxation. The EC50 value of SNP in the control, HF, and AS groups were 6.8 ± 1.1, 8.0 ± 1.2, and 6.4 ± 1.2 nM, respectively. The maximal vasorelaxant effect of SNP in the control, HF, and AS groups were 98.4 ± 1.0, 98.0 ± 1.3, and 98.16 ± 0.7, respectively. These results suggest that AS improves vascular reactivity by ameliorating HF-induced endothelial dysfunction.

Ameliorative effects of AS and GA on endothelial dysfunction

Acetylcholine induces vascular relaxation by activating the calmodulin-binding domain of eNOS to produce NO [20]. The reduction of NO is the main characteristic of endothelial dysfunction [21]. Thus, the expression of NO-generated proteins were evaluated. As expected, the HF

(See figure on next page.)

Fig. 1 Concentration–response curves of Ach-induced endothelium-dependent relaxation **a** and SNP-induced endothelium-independent relaxation **b** in the phenylephrine-contracted thoracic aorta of the control, HF, and AS groups. The western blot analysis of endothelial dysfunction-related proteins in thoracic aorta tissue and its relative intensities, p-Akt ser473 (**c**), p-eNOS-Ser1177 (**d**), and p-eNOS Thr495 (**e**). Immunohistochemistry staining of ET-1 in the thoracic aorta sample using antibody against ET-1 and visualized with peroxidase displaying different staining intensity (brown color) (f) and their immune positivity expressed as area % (**g**). Data are expressed as mean ± SEM (n = 8). $^{\#}P < 0.05$ vs. Control, $^{*}P < 0.05$ vs. HF


a
Control
HF
AS
Relaxation (%)
0
50
100
10-9 10-8 10-7 10-6 10-5 10-4
Ach (mol/L)
b
Control
HF
AS
Relaxation (%)
0
50
100
10-10 10-9 10-8 10-7 10-6
SNP (mol/L)
c
Control HF AS GA
p-Akt-Ser473
Akt
GAPDH
p-Akt-Ser473/Akt (fold change)
ρ=0.007
ρ=0.004
ρ=0.023
ρ=0.009
Control HF AS GA
d
Control HF AS GA
p-eNOS-Ser1177
GAPDH
p-eNOS-Ser1177 (fold change)
ρ=0.020
ρ=0.046
ρ=0.044
Control HF AS GA
e
Control HF AS GA
p-eNOS-Thr495
GAPDH
p-eNOS-Thr495 (fold change)
Control HF AS GA
f
Lumen
Control
Outside
Lumen
HF
Outside
Lumen
AS
Outside
Lumen
GA
Outside
g
% Area marked by ET-1
ρ=0.031
ρ=0.035
ρ=0.012
ρ=0.0001
ρ=0.001
Control HF AS GA

diet reduced the expression of p-Akt ser473 and p-eNOS-Ser1177 compared to the control (Fig. 1c, d). The AS and GA supplement increased expression levels of p-Akt ser473 and p-eNOS-Ser1177 in the HF-fed rat (Fig. 1c, d) and expressions of those proteins were even higher than expressions of those proteins in control. However, the different expression levels of p-eNOS Thr495 among the groups were not observed (Fig. 1e). Previous studies reported that the NO production is upregulated by the eNOS activity via modulation of p-eNOS-Ser1177 and p-Akt ser473 [22, 23], whereas p-eNOS-Thr495 is a negative regulator and associated with a decreased enzyme activity [24]. The rise in the expression level of p-eNOS-Ser1177 in AS and GA diet resulted in the amplified enzymatic activity of eNOS which was led to increased NO content in vascular endothelial cells. Our results suggest, at least in part, that AS and GA increase vascular reactivity by improving endothelial dysfunction through enhancing Akt-induced eNOS phosphorylation at its active site. ET-1 is a strong vasoconstrictor produced in vascular endothelial cells. Failure of the physiological balance between NO and ET-1 causes endothelial dysfunction [22, 25]. Interestingly, immunohistochemistry staining of ET-1 brown spots in the thoracic aorta (Fig. 1f) and their percentage of the area (Fig. 1g) showed that AS and GA groups have significantly diminished ET-1 expression levels compared to the HF group. As the physiological balance between NO and ET-1 is considered important in endothelial function, AS and GA may ameliorate endothelial dysfunction by decreasing

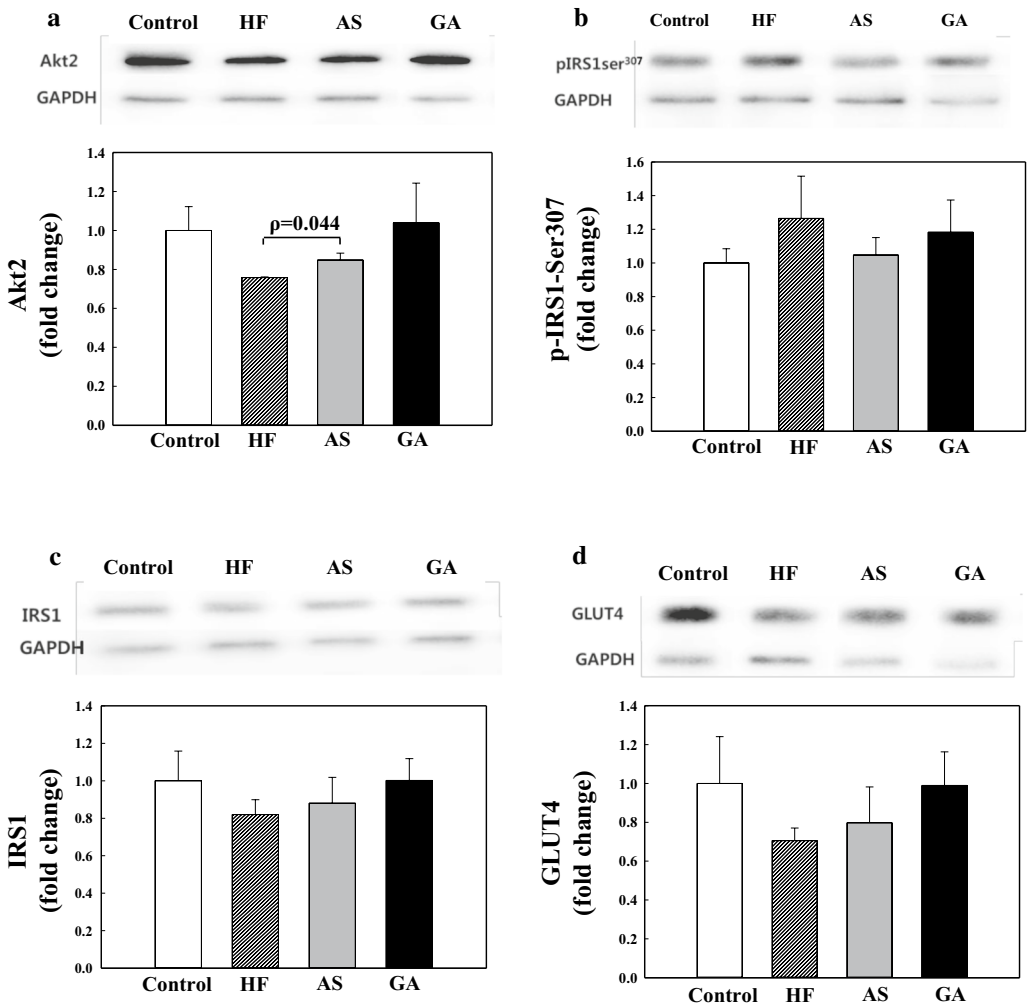


Fig. 2 Western blot analysis of insulin signaling-related proteins in adipose tissue and relative intensities, Akt2 (**a**), IRS1 (**b**), pIRS1ser307 (**c**), and GLUT4 (**d**), in the adipose tissues of rats, fed the diets containing 5% AS and GA powder (w/w) for 12 weeks. Values were expressed as mean ± SEM (n = 8)

ET-1 and improving phosphorylation of NO-production-related proteins, and may lead to increased vascular relaxation.

Effects of AS and GA on insulin sensitivity

Endothelial dysfunction has been reported to be associated with insulin resistance in diabetes [26]. Insulin binding to its specific receptor stimulates the phosphorylation of IRS and activates the IRS/PI3K pathway that regulates glucose uptake in metabolic tissues and controls NO release from endothelial cells [26]. Phosphorylation of IRS proteins on tyrosine residues activates insulin signaling and stimulates the translocation of GLUT4. On the other hand, serine phosphorylation of IRS proteins causes insulin resistance [27]. The HF diet showed lower expression levels of IRS1, Akt2, and GLUT4 and higher expression levels of pIRS1ser307, as compared to the control (Fig. 2). Similar to our results, it was demonstrated that HF induced insulin resistance by impairing insulin signaling [28]. AS and GA showed a trend of improving insulin signaling by increasing of IRS1 and GLUT4 expression (Fig. 2b, d) and by decreasing pIRS1ser307 expression (Fig. 2c). However, significant differences were not observed. The protein expression level of Akt2, a signaling protein in the IRS/PI3K pathway [29], was significantly increased in the AS group compared to the HF group (Fig. 2a).

Here we found that AS and GA enhance vascular activities and ameliorate endothelial dysfunction by increasing the expression levels of vascular relaxation-related proteins and suppressing ET-1 protein expression. Moreover, AS and GA appeared to have the potential to improve insulin sensitivity. Protein hydrolysates of AS and GA have been reported to have in vitro anti-angiogenic, antioxidant, and acetylcholinesterase inhibitory activities [30–32]. Taurine was known to have endothelial protective activity and improve vascular function by the restoration of redox homeostasis [8]. Additionally, glycine and L-arginine have the ability to prevented endothelial dysfunction [10, 11]. In addition to free amino acids including taurine, other components in the clams such as minerals and betaine may contribute to the prevention effects of AS and GA on endothelial dysfunction [33, 34]. Betaines are known to convert homocysteine, which is reported to have adverse effects by disturbing endothelial function, to methionine as a methyl donor [35]. It was reported that both ark clams contain a high amount of betaines [34]. Even though the mechanisms to prevent endothelial function by AS and GA are not clear, our study provides evidence that AS and GA increase vascular reactivity and improved HF-induced endothelial dysfunction.

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Authors' contributions
KSH, MHO, SC, and KP designed experiment. SC, KP, OM, and KHP performed and analyzed data from endothelial dysfunction- and insulin resistance-related proteins. SP performed and analyzed data from immunohistological work. DSG performed measurement of vascular reactivity. KSH, SGK and MHO supervised research. SC, JYC, and KSH prepared the manuscript, with contributions from all authors. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

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