Korean J. Food Preserv.
Vol. 14, No. 4. pp. 431-437, August 2007

사단법인 한국식품저장유통학회
THE KOREAN SOCIETY OF FOOD PRESERVATION

The Abanones, *Haliotis discus hannai*, Exhibit Potential Anticoagulant Activity in Normal Sprague Dawley Rats

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정상 Sprague Dawley 쥐에 대한 전복의 항응고능에 관한 효과

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Abstract

The primary objective of this study was to determine the effects of abalone in reducing blood pressure and increasing anti-coagulant capacity. The serum angiotensin-converting-enzyme (ACE) activities of rats on an abalone-supplemented diet did not significantly differ from the ACE levels of rats on a normal diet, at any time (before the experiment, or 1 week, 2 weeks, 3 weeks, and 4 weeks, after commencement of the abalone diet) during the experiment. This result showed that abalone-supplemented diets had no effect on the activity of ACE, which controls blood pressure. To determine if an abalone-containing diet might increase anti-coagulant capacity, both prothrombin (PT) and activated partial thromboplastin time (APTT) levels were measured. The PT levels of control rats remained constant throughout the experiment. In rats fed the abalone-containing diet, PT levels increased with time, and the increase became statistically significant after 2 weeks, when compared to pre-trial PT levels. Control rats showed no significant change in APTT levels over time. The rats fed abalone, however, showed significant differences in APTT levels. Specifically, when pre-trial APTT levels were compared with 4-week levels, and when 1-week levels were compared with 4-week levels, the differences attained statistical significance. These results indicate that an abalone-supplemented diet may inhibit blood coagulation in normal rats. The results of this study prove the inherent health value of abalone, and may encourage investment in the seafood industry. Future studies will explore other possible beneficial effects of abalone, apart from the anti-hypertension and anti-coagulant effects examined above.

Key words : *Haliotis discus hannai*, animal study, serum ACE activity, prothrombin time, activated partial thromboplastin time

Introduction

Abalones are marine gastropod mollusks which can be found down to a depth of 5~50 m of low tide waterlines of open sea(ocean) islands and reefs. Abalones feed on macrophytes that grow in clean water. Among more than 100 types of abalones, South Korea is a habitat for *Haliotis discus hannai, H. gigantea, H. discus discus, H.sieboldii,* and *H. diversicolor superfexta*(1,2)

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Abalones are valued for their rich ingredients and positive effects on human health. They are rich in both protein and vitamins and have been shown to foster healthy skin, improve energy, and speed recovery after delivery. The significant quantity of taurine in abalones aids healthy liver function, recovery from fatigue, and the prevention of myocardial infarction. Abalones are often referred to as the "royal family" of shellfish, due to their nutrition and taste, which are thought to be superior to the majority of other marine products. The price of abalone, consquently, is among the highest. Abalones feed on brown and red algae such as brown seaweed and kelp, and the physiologically active materials in those algae appear to enhance the positive qualities of abalones. Sea algae facilitates physiological activation to a higher degree than do terrestrial plants(3), and brown algae evidences physiological activation characteristics superior to those of green and red algae. Brown seaweed, kelp, and green laver are known for their anti-tumor activities and ability to repress hypertension, and laver is known for its ability to reduce cholesterol and for its anti-ulcer functions(4, 5). Brown algae is rich in acid polysaccharides, which contain neutral polysaccharide, laminaran, and sulfuric acid. Representative acid polysaccharides that contain sulfuric acid include fucoidan and alginate, which have been shown to exert anti-coagulation, anti-cancer, and anti-AIDS effects(6,7). While many studies of sea algae have been conducted, the functions of abalone are supported only by ancient documents, and there currently exists a lack of scientific corroboration for these claims(8). The overall value of the abalone has also declined, due to an amelioration of the animal's scarcity via mass production by farming, in addition to the aforementioned lack of scientific corroboration for the abalone's positiveeffects. Thus, a scientific effort is clearly necessary to determine the functions of abalone for people who seek to utilize it for their health and wellness.

The purpose of this study is to assess the clinical effects of abalone with regard to its ability to reduce blood pressure and increase anti-coagulant capacity. This might facilitate the development of the abalone farming industry and may also effect improvements in public health in general.

Materials and Methods

Sample

Final samples were prepared via the freeze-drying and powdering of cultured whole abalone (including body and viscera) collected from Wandogoon, Jeonnam, Korea. The freeze-dried abalone powder was added to animal feed at a proportion of 5%, and this modified feed was used as the experimental rat diet.

Animal Care

Sixty Sprague-Dawley male rats were purchased from Samtaco Bio Korea Inc. (Osan, KyunggiDo). They were NTacSam: Sprague-Dawley rats, 6 weeks old, and all weighed 200±15 g upon purchase. They were adapted to be fed with Rodent superfed food composed of protein 22.1% above, fat 3.5% above, fiber 5.0% below, ash content 8.0% below, calcium 0.6% above and phosphorus 0.4% above etc. (Kangwondo, Superfed Co. Korea), and divided into normal control and experimental group considering their weight. The abalone supplement group(ASG) consisted of 30 experimental rats fed on a diet to which 5% of abalone supplement had been added, and the normal control group(NCG) consisted of 30 controlled rats fed on a diet without abalone supplements.

The rats were allowed to adapt to the laboratory environment prior to the experiment; temperature(20℃), moisture (50-60%), and a 12-hour photocycle. Water and meals were provided ad *libitum*, and the rats' weights were measured every week.

Among the total (experimental and control) of 60 rats, 1), rats fed with abalone-supplemented feed (n=30): 6 rats were sacrificed week for 4 weeks, 2) rats fed with normal feed (n=30): Six rats were sacrificed prior to the experiment (0 week) and another rats were sacrificed every week for 4 weeks. Care and treatment of experimental animals were in accordance with the guidelines published in the NIH Guide for the care and use of laboratory animals.

Abalone Food Supplementation.

Throughout the experimental process, the rats remained free to eat feed and drink water, and their dietary calories were not controlled. The experimental group rats were provided with feed to which was added a 5% proportion of abalone supplement.

Animal Sacrifice Procedures and Blood Collection

The rats were not fed for 12 hours prior to their sacrifice and were used after cervical vertebral separation. Incisions were made from the abdomen to the chest, and blood was sampled from the heart while the heart was still beating. The blood samples were centrifuged for 15 min at 3,000 Xg,

normally and in a vacutainer treated with sodium citrate. The blood plasma and serum were maintained in a freezer at -70℃ prior to use.

Measurement of Angiotensin I-converting enzymes (ACE) activity

ACE activity was determined via a modification of the method developed by Cushman and Cheung. 50 μL of serum collected from the rats was mixed with 100 μL of 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl. This was mixed with the substrate, 50 μL of 5 mM Hippuryl-Histidyl-Leucine, and pre-incubated for 10 min at 37℃. After the reaction was halted via the addition of 1 M HCl 200 μL to the mixture, 2 mL of ethyl acetate was added to it and vortexed for 15 sec. The liquid was then centrifuged for 5 min at 1,000 Xg. The ethyl acetate layer (1.5 mL) was volatilized in a double boiler, then vortexed after the addition of 1 mL of 1N NaCl. The ACE activity was determined in accordance with the absorbance at 228 nm with the final liquid.

Anti-Coagulant activity measurement

In order to determine the antithrombus functions of the rats in the ASG group, a blood coagulation analyzer, COAG-A-MATE® XM BIOMRIEUX, INC. (USA), was employed to measure prothrombin time (PT) and activated partial thromboplastin time (APTT). The PT was measured by mixing 0.1 mL of blood plasma and 0.1 mL of abalone extract, which were warmed for 180 sec at 37℃ prior to the addition of 0.2 mL of thromboplastin reagent, after which the clotting time was measured. APTT was measured by mixing 0.1 mL of blood plasma and 0.1 mL of abalone extract and warmed for 60 sec at 37℃ prior to the addition of 0.1 mL of activator reagent. It was activated for 300 sec before the clotting time was measured after the addition of 0.1 mL of $CaCl_2$.

Statistics

SPSS (v.12.01) was used for the statistical analysis of the data. Mean and standard deviations were calculated and two-way ANOVA by repeated measures were employed for comparisons between groups and between different times during the experiment (before, 1, 2, 3, and 4 wk). Post-hoc tests (Newmann-Keuls) were run for variables evidencing significant differences from the ANOVA. The significance level was $p<0.05$.

Changes in the weight and water intake of the normal rats during the experiment

Table 1. shows the change in the body weights of the rats in the ASG and NCG groups during the experiment: prior to the experiment (before), after 1 week (1 wk.), after 2 weeks (2 wk.), after 3 weeks (3 wk.), and after 4 weeks (4 wk.).

Table 1. Change of body weight (kg) of the rats in ASG and NCG

(week)

Period / Group	Before[a]	1[b]	2[c]	3[d]	4[e]	F-value	post-hoc
ASG[2)]	283.82[1)] ±5.59	330.92 ±10.97	352.30 ±23.56	370.47 ±17.51	405.40 ±17.50	46.767***	a-b,c,d,e b-d,e e-c,d
NCG[3)]	278.45 ±12.66	318.72 ±16.55	329.75 ±19.39	367.97 ±17.82	390.68 ±22.59	35.876***	a-b,c,d,e b-d,e c-c,e

[1)]Values are mean and standard deviation of 6 numbers.
***p<0.001, significant difference between period.
[2)]ASG; Abalone Supplement group.
[3)]NCG; Normal Control group.

The rats in the ASG evidenced a steady increase in weight during the experiment, and also manifested a statistically significant (F=46.767, p<.001) difference between the weeks (before, 1, 2, 3, and 4wk). Post-hoc tests evidenced a significant difference: before vs. 1, 2, 3, and 4wk; 1 wk vs. 3 and 4 wk; 4 wk vs. 2 and 3 wk. Rats in the NCG also evidenced significant increases in weight over the different times of the experiment (F=35.876, p<0.001). Post-hoc tests indicated significant differences: between before vs. 1, 2, 3, and 4 wk; 1wk vs. 3 and 4 wk; 2 wk vs. 3 and 4 wk. No significant differences were detected in the weights of the rats in the two groups over the different time points of the experiment. This result shows that the rats fed on the abalone-supplemented diet evidenced no significant gains in weight as compared to the rats fed on a normal diet.

Table 2. shows the change in the quantity of daily water intake for the rats in the ASG and NCG groups. Neither the ASG nor the NCG group rats evidenced any statistically significant differences in their water intake over the 4 weeks (each F= 2.560[ns], F= 1.095[ns], respectively). Additionally, no significant differences were detected in water intake between the two groups over the experimental period.

Table 2. Change of daily water intake (mL) of the rats in ASG and NCG (week)

Period / Group	Before	1	2	3	4	F-value
ASG[2)]	33.83[1)] ±1.13	40.33 ±2.29	41.50 ±2.24	36.67 ±5.16	40.00 ±77.5	2.560[ns)]
NCG[3)]	35.83 ±3.42	37.50 ±2.96	40.42 ±7.44	40.00 ±4.47	37.50 ±2.24	1.095[ns)]

[1)]Values are mean and standard deviation of 6 numbers.
[ns)]No significant difference between period.
[2)]ASG; Abalone Supplement group.
[3)]NCG; Normal Control group.

Effect of abalone supplement on decreasing blood pressure of normal rats

Table 3. shows the change in serum ACE activity occurring during the experiment for the normal rats in the ASG and NCG groups. Serum ACE activity evidenced no statistically significant differences in both the ASG and NCG group rats over the different time points of the experiment (each F=1.032[ns], F=0.934[ns], respectively), Additionally, no significant differences in ACE activity were detected between the two groups. This result indicates that abalone supplements had no significant effect on the activity of angiotensin-I converting enzymes, which control blood pressure.

A great deal of recent research has focused on the effects of extracts from plants, sea algae, and Chinese medicine on ACE activity as a factor in decreasing blood pressure(10-12). This trend constitutes a response to the observed current increase in chronic degenerative illnesses such as obesity, diabetes, circulatory diseases, and high blood pressure, as the result of the increasing consumption of animal food, which contains a great deal of fat(13). High blood pressure has been identified as an important cause of coronary heart disease, strokes, and circulatory diseases, and as a cause of death in more than 50% of patients(14). High blood pressure is explained physiologically by the Renin-Angiotensin system, an increasing amount of research is focused on the search for materials and methods by which ACE activity can be controlled directly(15). ACE is known for its ability to transform inactive Angiotensin-I to active Angiotensin II by participating in angiotensin and bradykinin metabolism and by forming inactive bradykinin-1-7 and bradykinin-1-5 via the hydrolysis of the C-terminal depeptide of bradykinin, a vasodilator(16, 17). Increased angiotensin II levels exert influence on the AT receptor, and the AT1 receptor triggers blood vessel contraction, Aldosterone and Vasopressin emission, sodium absorption of the renal tubules, and a reduction in bloodstream to the kidney which results in different kinds of diseases in the cardiovascular system, the kidney, and the nucleus(18). The AT_1 receptor also inactivates Bradykinin, which accelerates blood vessel expansion, blocks platelet adsorption, and proliferates into the smooth muscle cells via the B_2 receptor in the Kallikrein-kinin system. The inhibition of ACE activity can reduce tension and blood pressure by increasing Na emission and the half-life of blood vessel expansion peptides, which results in the expansion of blood vessels in the kidney and reduces blood vessel contraction(20). There has been a great deal of research conducted with an eye toward controlling ACE activity. These studies have determined that soybean fermentation foods and their hydrolysis products(21, 22), processed marine products including salted fish(23), microorganisms(24), Chinese medicine(25), and plants(26) have a substantial capacity to control ACE activity. Our prior study(27), in which in vitro experiments were conducted with 80% ethanol extracts and water soluble extracts determined that the abalone body and viscera evidenced a significant capacity to inhibit ACE activity. This contradicts the results from this study, which determined that a 5% abalone supplemented rat diet evidenced no significant effects with regard to the control of ACE activity. This may be due to the difference between in vitro and in vivo conditions, and the rats employed in the current study evidenced normal blood pressure. It appears that in future studies, it may be advantageous to add the element of SHR, which is induced hypertension.

Table 3. Serum ACE activity (ug/mL) in ASG and NCG rats (week)

Period / Group	Before	1	2	3	4	F-value
ASG[2)]	5.195[a1)] ±2.409	3.449[a] ±0.675	3.318[a] ±0.698	4.617[a] ±1.234	4.520[a] ±2.222	1.032[ns)]
NCG[3)]	5.048[a] ±2.101	4.895[a] ±1.242	4.954[a] ±1.554	4.546[a] ±0.884	4.132[a] ±1.401	0.934[ns)]

[1)]Values are mean and standard deviation of 6 numbers. A value sharing the same superscript is not significantly different at $p<0.05$.
[ns)]No significant difference between period.
[2)]ASG; Abalone Supplement group.
[3)]NCG; Normal Control group.

Effect of abalone supplement on anti-coagulant activity of normal rats

Table 4. indicates the change in prothrombin time (PT) during the experiment with the ASG and NCG rats. No significant differences in PT were detected between times for the NCG rats. The ASG rats evidenced increased PT as time elapsed, and the increase became statistically significant

after 2 wk as compared to before. Significant differences were detected (F=4.779, p<0.01) before vs. 2 wk (16.42±0.66 sec.), 3 wk (16.58±0.85 sec.), and 4 wk (16.52±0.86 sec.). This result indicates that abalone-supplemented diets exert a positive preventive effect against blood coagulation.

Table 4. Prothrombin time (sec.) in ASG and NCG rats

(week)

Period / Group	Before	1	2	3	4	F-value
ASG[2)]	14.83[a1)] ±1.18	15.67[a,c] ±0.38	16.42[b,c] ±0.66	16.58[b,c] ±0.85	16.52[b,c] ±0.86	4.779[***]
NCG[3)]	14.83[a] ±1.18	318.72[a] ±16.55	329.75[a] ±19.39	367.97[a] ±17.82	15.05[a] ±0.31	0.284[ns)]

[1)]Values are mean and standard deviation of 6 numbers. A value sharing the same superscript is not significantly different at p<0.05.
[**]p<0.01, significant difference between period.
[ns)]No significant difference between period and group.
[2)]ASG; Abalone Supplement group.
[3)]NCG; Normal Control group.

Table 5. shows the changes in activated partial thromboplastin time (APTT) occurring during the experiment for both the ASG and NCG rats. The rats in NCG evidenced no significant differences in APTT over time. However, the ASG rats evidenced significant differences in APTT levels (F=4.997, p<0.01) before vs. 4 wk and 1 wk vs. 4 wk. The significant increases in APTT, coupled with the increased PT levels, suggests that an abalone-supplemented diet exerted a positive delaying effect on blood coagulation in normal rats.

Abalones are considered to be a high-quality marine product, largely due to the fact that they are rich in protein and vitamins. They are also renowned for their positive effects on healthy skin, energy, fast recovery after delivery, recovery from fatigue, and the prevention of myocardial infarction(8). Although there have been many studies conducted concerning sea algae, abalone's functions are supported only by ancient documents and there is a current lack of scientific verification in this regard(8). Even though a variety of studies have been conducted on the effects of sea algae on anti-coagulation and antithrombosis, few studies have been conducted regarding abalone's nutritional qualities and functions in the human body. There has also been a decided reduction in the value of abalones, due to a lessening of their scarcity via mass production by farming. This study constitutes a response to this demand to determine the functions of abalone for people who seek greater health and wellness.

Abalones feed on a variety of sea algae, which is known for its anti-coagulation effects(31). Therefore, this study attempted to determine the anticoagulant capacity of abalones and conducted animal experiments via measurements of PT and APTT. The results indicated that a diet to which powdered abalone body and viscera had been added evidenced a significantly higher anticoagulation effect on the rats as compared to those of the control group. This shows that abalones may habror materials with an antithrombotic effects. This suggests that future research should attempt to uncover what constituents of abalones exert this antithrombosis function. The results of this study may be related to the results of another previous *in vitro* study which elucidated the positive effects of abalone on anticoagulation capacity(32).

In conclusion, this study determined that while abalone evidenced high blood pressure-ameliorating effects, we also discovered a positive effect on antithrombosis by promoting anticoagulation. These results indicate a verification of abalones' positive antithrombotic functions, not only by in vitro, but also by *in vivo* experiments.

Table 5. Activated partial thromboplastin time (sec.) in ASG and NCG rats

(week)

Period / Group	Before	1	2	3	4	F-value
ASG[2)]	18.57[a1)] ±0.34	18.69[a,c] ±0.73	20.74[a,b,c] ±1.83	20.61[a,b,c] ±1.64	21.58[b] ±2.01	4.997[**]
NCG[3)]	18.84[a] ±0.34	19.08[a] ±1.18	18.88[a] ±1.24	19.11[a] ±1.71	19.28[a,#] ±1.71	0.884[ns]

[1)]Values are mean and standard deviation of 6 numbers. A value sharing the same superscript is not significantly different at p<0.05.
[**]p<0.01, significant difference between period.
[ns)]No significant difference between period.
[#]p<0.05, significant difference between group
[2)]ASG; Abalone Supplement group.
[3)]NCG; Normal Control group.

Acknowledgements

This study has been supported by a grant from the Jeonnam Research Service on Examination of abalone function (2004) with technological help from the Solar Salt Biotechnology Research Center and Food Industrial Technology Research Center (RRC) of Mokpo University.

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(접수 2007년 3월 21일, 채택 2007년 6월 22일)