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THE EFFECT OF BLOOD CLOTTING EFFECTS IN SNAIL, *ACUSTA DESPECTA SIEBOLDIANA*

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ABSTRACT

Snail, *Acusta despecta sieboldiana* (Pfeiffer) is a genus of family Bradybaenidae and a terrestrial pulmonate gastropod mollusk. We investigated the blood clotting activity from *Acusta despecta sieboldiana.* Adenosine diphosphate (ADP) is a platelet agonist that causes platelet shape change and aggregation. When the water extract was added with 40 mg/ml of extract, it had a blood clotting or cohesive effect of about 14%. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) are measured the time it takes for blood to clot. The mean of PT in the water, ethanol, and methanol extracts were 11.2 s, 11.6 s, and 11.7 s, respectively. The time was 32.1 seconds on the 5 mg/ml water extract. Ethanol extract was 33.9 seconds on same concentration and methanol extract was 33.9 seconds. The international sensitivity index (ISI) was 1.3 for three extracts of snail mucus. International normalized ratio (INR) decreased in the high concentration exposure of snail mucus. In this study, snail mucus extract can partially help blood clotting when the wound is low in the agricultural field, but it did not have a significant effect. As a result of this study, it is expected that snail mucus extract can be used for blood coagulation as a hemostatic agent.

KEYWORDS: Snail, *Acusta despecta sieboldiana*, adenosine diphosphate (ADP), Activated Partial Thromboplastin Time (aPTT), Prothrombin Time (PT).

INTRODUCTION

Blood consists of blood cells and plasma, supplying oxygen and nutrients to each tissue in the body and transporting gases or wastes from cells to play an important role in maintaining the homeostasis of each tissue. In vivo, procoagulants and anti-coagulants are balanced in normal conditions, so excessive blood clots are not generated, but damage to the vessel wall breaks the equilibrium state and preagulants are activated, causing platelets in the blood to adhere, activate, and coagulate.[1]

In the early stages of thrombosis, platelets are adhered, activated, and agglomerated due to vascular endothelial cell damage in the wound area, and are promoted by activating the blood coagulation system.[2] Then, adenosine 5`-diphosphate (ADP) and TXA2 (thromboxane A2) are then secreted into the platelet, further activating the platelet, and the platelet Membrane GPIIb/IIIa (Glycoprotein IIb/IIa) receptor in the platelet membrane and fibrinogen are combined to promote platelet coagulation.[3] When platelets are activated, serotonin, Ca^{2+}, TXA2, etc. are advantageous, amplifying the aggregation of other platelets, and reacting with coagulation factors present in plasma to form clots. Through this step, thrombin produced by endogenous and exogenous coagulation-based cascade activity converts fibrinogen to fibrin.[4]

ADP is not a strong factor in human blood clotting but plays an important role.[5] ADP promotes solidification through three receptors (P2Y1, 2Y12, and P2X1). P2Y1 and P2Y12 are G protein complex receptors, and P2X1 is a ligand ion channel receptor.[6,7]

The platelet agglomeration and blood clotting reactions are rapidly performed in the same region, where prothrombin time (PT) means the time until fibrin clots are formed in the extrinsic coagulation system, and activated partial thromboplastin time (aPT) means the time until fibrin clots are formed in the endogenous coagulation system.[8] In normal conditions, activated plasminogen, or plasmin, decomposes fibrin and fibrinogen, dissolves the blood clots and returns to normal blood vessels, but in abnormal conditions causes thrombosis. Many studies have been conducted on blood clotting using PT and aPTT.[9,10]

Mucuses are ubiquitous in animals and appeared roughly 600 million years ago in metazoans.[11] Most higher animals express at least five individual mucin genes and physiological roles are remarkably diverse, suggesting

their function as a fast-evolving.[12, 13] Mucins are a highly glycosylated protein family that are secreted by animals for adhesion, hydration, lubrication, and other functions.[14] Mucin is a biosynthesized macromolecule which can protect the external damage. Intrigue in the mucus slime trails left by snails and slugs date back to ancient Greece, where they utilized the mucus for its ability to reduce inflammation and the signs of aging.[15] Today snail mucus is still used in skin care products by various companies.

Snail mucus contains chemicals such as achatin isolates, heparan sulfate, and calcium.

It is important that wounds be properly cleaned and dressed before any preparations are applied. This prevents infection. Bacterial infection is one of the major factors that affect wound healing. Infection of surgical wounds depends on the bacterial inoculums, the virulence of the bacteria and the duration after contamination.

Snails produce copious mucin which is often referred to as slime. The wound healing property of snail mucus has been reported.[11, 16] Similarly, its physiological and toxicological properties have been documented.[16] Snails gnaw at crops in agriculture, causing great damage.

Pest snails and slugs damage plant seeds, seedlings, underground tubers, leaves and fruit. Damage to seedlings often results in the death of the plant, which means major production losses. While farming, you can scratch your hands or feet and get hurt. In that case, we tried to investigate whether there was a hemostatic effect with nearby snail juice.

MATERIALS AND METHODS

Sample extract

30 snails were collected in the field. The snails were reared at 24 °C for one week. The mucus was extracted by gently poking on foot and mantle lobes. The harvested mucus was filtered through 0.45-µm membrane (Millipore). Distilled water, ethanol, and methanol, which are 10 times different solvents of the sample, were added and stirred for 24 hours to extract active ingredients. The sample was treated with ultrasound at room temperature for 60 minutes. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

Plasma preparation

Blood was prepared from the whole blood of a person aged 30 to 40 years old with a healthy body that has not been drugged in the past month. Vein blood was collected with a syringe to have a ratio of 1:9 (v/v) to blood in a dedicated tube (Vacutainer, Becton Dicknson, UK) containing 0.15 M sodium citrate anticoagulants. Plasma was centrifuged and separated at 4°C 3000 rpm for 10 minutes, and then stored frozen at -20°C. During the experiment, the sample was thawed at room temperature and used.

Platelet agglutination

The test of platelet aggregation was based on the method of Fontana et al.[17] The blood was centrifuged to form platelet rich plasma (PRP), and agglomeration was induced with adenosine 5`-diphosphate (ADP) and epinephrine. In the case of the experimental group, a test solution was added before adding the agglomeration derivative. The agglutination reaction was treated within 2 hours after blood cell collection. 280-µL PRP was constant-temperature at 37°C for 3 minutes, and centrifuged at 1100 rpm for 2 minutes. 20 µL of ADP was added at a concentration of 1, 2, or 5 µmol/L (final concentration).

Agglomeration was measured at 37°C by a photometric method in a 4-Channel aggregate (Regulest). The hemagglutination reaction was determined as a reaction for 5 minutes.

The inhibition rate (%) = (A-B)/Ax100, where A is the maximum cohesion rate (%) of the control group, and B is the maximum cohesion rate (%) of the experimental group.

Measurement of blood clotting (PT, aPTT) activity

A prothrombin time (PT) test measures how long it takes for a clot to form in a blood sample. International Sensitivity Index (ISI) established by the manufacturer. An INR (international normalized ratio) is a type of calculation based on PT test results. The INR is derived from prothrombin time (PT) which is calculated as a ratio of the patient's PT to a control PT standardized for the potency of the thromboplastin reagent developed by the World Health Organization (WHO) using the following formula:

$$INR = \text{Patient PT} \div \text{Control PT}$$

The aPTT is one of several blood coagulation tests. It measures how long it takes your blood to form a clot. Antithrombotic activity was evaluated by measuring PT and aPTT of the sample. Active thrombin plastin time aPTT due to endogenous pathway and prothrombin time PT due to extrinsic pathway during hemagglutination inhibition activity were measured using an automatic hemagglutination analyzer ACL-9000 (Instrumentation Laboratory, Milano, Italy).

aPTT measurement was performed using aPTT agent to treat 200ul of citrated plasma, 100ul of aPTT agent and 100ul of samples, incubated at 37℃ for 5 minutes, and then 25 mM $CaCl_2$ 100ul was added to measure the coagulation time.[18]

PT measurement was performed by treating 100ul of sample in 200ul of citrate plasma, heating at 37℃ for 5 minutes, adding 100ul of PT reactant and measuring the time until plasma coagulation.[19] At this time, saline in which the sample was dissolved was used as the sample control group, heparin (Sigma Co., St. Louis, MO, USA) was used as the positive control group, and each experiment was repeatedly measured three times.

Statistical analysis

Data was conducted using Microsoft Excel and SPSS 21.0 for Windows (Chicago, IL, USA). A one-way and a two-way analysis of variance (ANOVA) followed by the Turkey post hoc test were used to analyze statistical significance (p<0.05). The analysis was carried out at least in triplicate. The results were expressed as the mean±SD. Significance and confidence level were estimated at p<0.05.

RESULTS

Effect on ADP

ADP was used as an aggregation derivative, and the concentrations of snail mucus extract were different to 5 mg/ml, 10 mg/ml, 20 mg/ml, and 40 mg/ml, and there was a slight difference depending on the extraction solvent (Table 1). Blood cohesive effect of snail mucus of water extract was evaluated 4.6% at 5 mg/ml. There was significant difference among four concentration groups (t = 10.919, p > 0.05). When the water extract was added with 40 mg/ml of extract, it had a blood clotting or cohesive effect of about 14%. In other words, there was an inhibitory effect on agglomeration inhibition. The cohesive effect of ethanol extract for snail mucus was evaluated 1.25% at 5.0 mg/ml and 10.59% at 40.0 mg/ml. The mean of ADP in the water, ethanol, and methanol extracts were 10.7%, 6.3%, and 7.3%, respectively. Although methanol extracts were slightly higher in ADP than those of ethanol extracts, there was no significant difference between two extract groups (p > 0.05).

Effect on PT

As a result of examining the effect of three extracts in snail mucus on PT by concentration, the control group was 11.21 seconds, the water extract was 10.28 seconds at 40.0 mg/ml, the ethanol extract was 10.98 seconds at same concentration, and the methanol extract was 11.05 seconds at same concentration (Table 2). The mean of PT in the water, ethanol, and methanol extracts were 11.2 s, 11.6 s, and 11.7 s, respectively. The higher the concentration of all three extracts, the shorter the coagulation time. The international sensitivity index (ISI) was 1.3 for three extracts of snail mucus. International normalized ratio (INR) decreased in the high concentration exposure of snail mucus.

Effect on aPTT

As a result of investigating aPTT by three types of extracts by concentration, it was 31.4 seconds in the control group (Table 3). The time was 32.1 seconds on the 5 mg/ml water extract. Ethanol extract was 33.9 seconds on same concentration and methanol extract was 33.9 seconds. The mean of aPTT in the water, ethanol, and methanol extracts were 29.3 s, 31.3 s, and 30.5 s, respectively. The higher the concentration of all three extracts, the shorter the coagulation time. Although water extracts were slightly higher in ADP than those of ethanol and methanol extracts, there was no significant difference among three extract groups (p > 0.05).

DISCUSSION

Some studies have shown inter-individual differences in ADP-induced platelet aggregation responses ex vivo, but the mechanisms underlying this variability are unknown.[17] In this study, the cohesive effect by ADP showed a positive value compared to the control group, but it was less than 13% (Table 1).

The prothrombin time (PT) and activated partial thromboplastin time (aPTT) are in vitro approximations of the coagulation pathway and they essentially define the extrinsic, intrinsic and common pathways. aPTT is the test of choice for monitoring therapy with unfractionated heparin because it is sensitive to all the coagulation factors this drug targets. PT and APTT are basic coagulation tests which measure integrated actions of majority of coagulation factors in extrinsic and intrinsic pathways of coagulation cascade of blood.[20] During anticoagulant activity, PT and aPTT are the criteria for comparing blood clotting ability, and normal values in adult plasma are PT 10-14 seconds, aPTT 30 to 40 seconds, and INR 1.4 or less. The mean of PT, aPTT, and INR in the water samples in snails were 11.2 s, 29.3 s, and 1.0 s, respectively (Tables 1 and 2). These values were in agreement with the normal values (PT = 13 s, aPTT = 34 s) of the control sample provided by the manufacturer. In this study, snail mucus extract can partially help blood clotting when the wound is low in the agricultural field, but it did not have a significant effect. As a result of this study, it is expected that snail mucus extract can be used for blood coagulation as a hemostatic agent.

Harti et al.[21] reported snail slime and 1.5% chitosan have effective potentials to accelerate the wound healing process. Resanto et al.[22] showed that 24% snail mucus treatment does not significantly affect wound healing (p = 0.488); by contrast, treatment with 48% and 96% snail mucus demonstrated significant effects on angiogenesis (p = 0.01).

This study has several limitations. Firstly, it is an experimental study and results therefore cannot be directly translated to clinical conditions. Although snails do damage to many crops, they had to reduce their sacrifices for experiments. Secondly, thus, the sample volume is small, and should be considered exploratory in nature, although the main findings appear quite clear.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

Table 1: Blood cohesive effect of snail, *Acusta despecta sieboldiana* determined by impedance aggregometry induced by ADP.

Sample	DOSE	Relative clotting (%)	p
Control	PBS	1	0.021
Water	5 mg/ml	4.85±2.50	0.578
	10 mg/ml	8.56±2.73	
	20 mg/ml	12.84±2.39	
	40 mg/ml	12.72±2.51	
Ethanol	5 mg/ml	1.25±1.73	0.444
	10 mg/ml	4.19±1.21	
	20 mg/ml	9.05±1.02	
	40 mg/ml	10.59±3.03	
Methanol	5 mg/ml	2.01±1.84	0.437
	10 mg/ml	4.89±1.50	
	20 mg/ml	9.94±0.69	
	40 mg/ml	12.27±2.45	

PBS: clot induced in phosphate-buffered solution

Table 2. Effect of snail, *Acusta despecta sieboldiana* on prothrombin time.

Sample	DOSE	PT(s)	ISI	PT (INR)
Control	Saline	11.21		1.14
Water	5 mg/ml	11.98±0.72	1.3	1.09
	10 mg/ml	11.48±1.27	1.3	1.03
	20 mg/ml	11.10±1.48	1.3	0.99
	40 mg/ml	10.28±1.43	1.3	0.89
t-test		10.919*	0.0	
Ethanol	5 mg/ml	12.29±0.82	1.3	1.13
	10 mg/ml	11.74±1.01	1.3	1.06
	20 mg/ml	11.18±1.02	1.3	1.00
	40 mg/ml	10.98±1.19	1.3	0.97
t-test		11.119*	0.0	
Methanol	5 mg/ml	12.35±0.87	1.3	1.13
	10 mg/ml	11.86±0.77	1.3	1.08
	20 mg/ml	11.43±1.06	1.3	1.03
	40 mg/ml	11.05±0.77	1.3	0.95
t-test		9.343*	0.0	
Total	*t*-test	0.649	0.0	
Heparin	100 IU/ml	59.0	1.3	8.66

Table 3: Effect of snail, *Acusta despecta sieboldiana* on activated partial thromboplastin time.

Sample	DOSE	aPTT(s)
Solvent	Saline	31.4
Water	5 mg/ml	32.1
	10 mg/ml	30.3
	20 mg/ml	28.0
	40 mg/ml	26.7
Ethanol	5 mg/ml	33.9
	10 mg/ml	32.1
	20 mg/ml	30.5
	40 mg/ml	28.5
Methanol	5 mg/ml	33.4
	10 mg/ml	31.2
	20 mg/ml	29.7
	40 mg/ml	27.7
Heparin	100 IU/ml	43.5

n=3

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