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Research Report

Comparison of thrombocyte counts during the post-oral administration of aspirin and the *Holothuria scabra* ethanol extract in Wistar rats (*Rattus norvegicus*)

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ABSTRACT

Background: *Long bleeding time is a risk factor in dental treatment, especially in patients who consume aspirin or other antithrombotic drugs. Holothuria scabra (H. scabra) are mostly echinodermata and have been studied in Indonesia; they contain omega-3 and glycosaminoglycans, with an influence of an antithrombotic drug.* ***Purpose:*** *This study aimed to investigate the thrombocyte counts during the post-administration of aspirin and the H. scabra extract in Wistar rats (Rattus norvegicus).* ***Methods:*** *This study was true experimental with a post-test control group design. The sample consisting of 30 healthy male Wistar rats (R. norvegicus) with a bodyweight of 150–250 g was divided into three groups (n = 10). The rats in Group 1 were given sodium carboxymethyl cellulose (Na CMC). The rats in Group 2 were given aspirin, and the rats in Group 3 were given the H. scabra ethanol extract with a 25 mg/200 g dose as per their body weight (BW). Oral administration was given for seven days. The rats' blood was taken on the eighth day. The amount of thrombocyte was measured using Wright's stain methods. The analysis of variance (ANOVA) and the Least Significant Difference (LSD) tests were conducted for data analysis ($p < 0.05$).* ***Results:*** *The thrombocyte counts (179.00 ± 10.56) in aspirin administration were lower than those in H. scabra (265.00 ± 18.54) and control groups (334.17 ± 13.9), with a significant difference between the groups ($p = 0.0001$; $p < 0.05$).* ***Conclusion:*** *This study indicates that the oral administration of aspirin and H. scabra decreases thrombocyte counts, whereas the administration of aspirin reduces thrombocyte counts to levels lower than those in H. scabra in Wistar rats (R. norvegicus).*

Keywords: *antithrombotic; aspirin; glycosaminoglycans; Holothuria scabra*

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INTRODUCTION

Atherosclerosis is a leading cause of vascular disease throughout the world with manifestations of ischemic heart disease, stroke and peripheral arterial disease. In 2010, there were 665 people per 100,000 population with ischemic heart disease in Central Asia per year.[1] Based on the results of the 2018 Basic Health Research report, the prevalence of heart disease in Indonesia was 1.5%.[2]

Lipoproteins that form in blood vessels lead to plaque formation in specific locations of the arteries through inflammation, necrosis, fibrosis and intimal calcification. The plaque causes coronary thrombosis due to acute rupture, which can result in partial or total blockage of the affected arteries.[3] Dismissal of the thrombus will become an embolism and obstruct the arterial system in pulmonary embolism and brain, for example.[3,4]

One of the medicines used widely in the treatment of atherosclerosis is aspirin, which functions as a long-term antithrombotic option for oral administration for the secondary prevention of myocardial infarction.[5,6] Aspirin in small doses (100 mg per day) shows an anti-thrombocyte activity by irreversibly inhibiting thrombocyte by preventing thromboxane A2 (TXA2) synthesis, which damages thrombocyte secretion and aggregation.[4,7] Problems arise in the administration of aspirin in patients

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with coronary heart disease, which involves bleeding that occurs in the gastrointestinal tract and intracranial due to long-term use of low-dose aspirin.[8] In the field of dentistry, bleeding complications can occur after surgery/tooth extraction, disrupting the healing process.[6,9,10] Therefore, before surgery and/or the extraction of teeth, it is vital to carry out a laboratory examination, which includes a thrombocyte count.[11]

Sea cucumber (*phylum Echinodermata*) has been widely used as medicine in several countries, such as China and Korea. In Indonesia, sea cucumbers are one of the leading marine products that have begun to be commercially cultivated in several regions because of their high economic value. However, there has been limited investigation into their benefits in the health sector.[12] One type of sea cucumber that has been widely cultivated is the sandfish (*Holothuria scabra*). This biota is commonly found across Indonesia's coasts, with straightforward breeding. The results of previous studies have shown that the content of sea cucumbers include lectins, sterols, saponins (triterpen glycosides), proteins, collagen, mucopolysaccharides, glycosaminoglycans (GAGs), chondroitin sulfate, amino acids, fatty acids, vitamin A, vitamin C, riboflavin, niacin, carotenoids, minerals, polyphenols, flavonoids and superoxide dismutase (SOD). The content of these substances can be used as a source of protein and considered as an anti-inflammatory, anticoagulant, anti-cholesterol and antithrombotic agent; it can also accelerate the process of wound healing.[13]

Some sea cucumber content such as GAG sulfate, dermatan sulfate and heparin are important anticoagulants that inhibit clot formation through interaction with antithrombin and heparin cofactors II.[14] These substances have a mechanism that is synergistically useful in the treatment of acute coronary syndrome (ACS).[15] This is used as the basis for selecting sandfish as an antithrombotic that can be used as a substitute for aspirin. The purpose of this study is to compare the administration of aspirin with the *H. scabra* extract, which is given orally to the thrombocyte count in the white rat strain of Wistar (*Rattus norvegicus*).

MATERIALS AND METHODS

This research is an experimental laboratory with a randomised, completed research design. This study was approved by the experimental animal ethics from the Health Research Ethics Commission (KEPK) of the Faculty of Dentistry, Universitas Hang Tuah, Surabaya (EC/082/KEPK-FKGUHT/XII/2019). The parameter of this study involved several thrombocytes taken from blood preparations from mice that had been treated. Thirty research samples were randomly divided into three groups. The sample used had the following inclusion criteria: white rats (*R. novergicus*), male Wistar strain aged 2–3 months, bodyweight 150–200 g. The selection of experimental animals was made based on their fur, eyes and physical conditions, as well as their randomized group design. Monitoring and recording were conducted to determine whether there existed any side effects or the experimental unit experienced any pain to be removed from the sample. Acclimatisation was carried out before and during the study, which involved monitoring environmental conditions, foods and drinks.[16]

The *H. scabra* extract was obtained from a beach in East Kalimantan (Borneo Island), Indonesia. It was transported in freezing conditions; its internal organs were removed, and it was washed thoroughly. Using a blender mixed with distilled water, it was chopped into a ratio of 1:2. The drying process was performed using the freeze–drying method. The freeze–drying results in the form of soft, dry preparations were mashed with mortar and pestle and sifted using a mesh 50.[17] Sodium carboxymethylcellulose was added to ease transfer to the stomach of the rat using the oral gavage.

Research on *R. novergicus* began with acclimatisation for seven days in a laboratory environment. Before being treated, the Wistar rats fasted for about 18 hours, albeit they were given drinking water. Sick rats were excluded from the study. The white rats were divided into three groups. Group 1 consisted of the control group that was given Na CMC. Group 2 included the treatment group and was given an aspirin dose of 1.8 mg/200 g as per BW, which was based on the conversion of an aspirin dose to rats (200 g) as an antithrombotic of 100 mg/day. Group 3 consisted of the treatment group and was given the *H. scabra* extract at a dose of 25 mg/200 g as per BW, which was based on previous research on the benefits of *H. scabra* on thrombocyte counts.[18] The treatment was carried out for seven days by giving a single dose. After blood sampling, the animal was terminated with cervical dislocation.

Thrombocyte count examination was performed on the eighth day, using the Wright wipe method. This method was carried out by adding 0.1 g of Wright powder dissolved with 60 ml methanol to 0.1 g of Wright reagent (Merck® Paint No. 1.01383.0500, Darmstadt, Germany). Afterwards, it was stored in glass bottles and kept in a closed cupboard to avoid sunlight. The solution was used after 10 days of storage. In addition to Wright staining, the use of a buffer solution was needed to fix the Wright smear with a pH of 6.4. The Wright smear procedure was performed by dripping the Wright solution onto the preparation until all smears were inundated. Furthermore, the buffer solution was dripped until all swabs were flooded and left for 5–12 min. The smear was rinsed with water, and the back of the dirty smear was cleaned of the remaining dye. Preparations of peripheral blood smear were left to dry in the air.[18] Observations were made by two people with five visual fields using a light microscope (Olympus® CX21, Japan) with 400 magnification. Using the Statistical Package for Social Sciences version 23 (IBM® 2015, New York, United States), the one-way analysis of variance (ANOVA) was performed ($p < 0.05$), followed by the post-hoc Least Significant Difference (LSD) test ($p < 0.05$).

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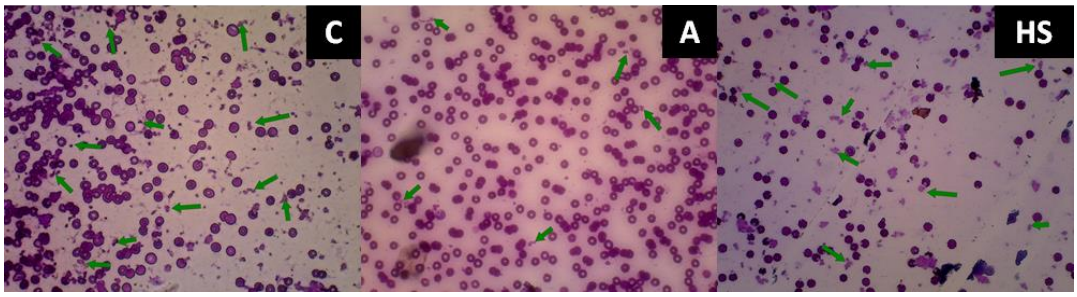

Figure 1. Histological section of thrombocytes (green arrows) during post-administration of aspirin (A), the *Holothuria scabra* extract (HS) and control in Wistar rats. Staining of Wright's methods. Observation using a light microscope at 400x magnification.

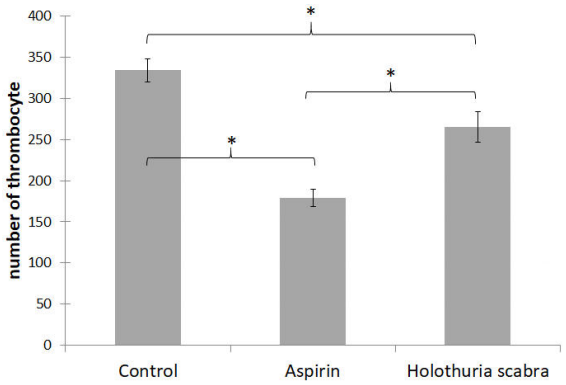

Figure 2. Mean and standard deviation of the thrombocyte counts in each group. *Significance difference: p = 0.05.

RESULTS

The histological section of the thrombocyte in each group can be seen in Figure 1. The mean and standard deviation of the thrombocyte counts in each group describe the differences between the groups (Figure 2). The application of sandfish extract (*H. scabra*) at a dose of 25 mg/200 g as per BW reduced the thrombocyte counts (265.00 ± 18.54) compared with the control group (334.17 ± 13.93). However, it did not reduce the thrombocyte counts similar to the aspirin group (179.00 ± 10.56). The ANOVA test identified a significant difference in the mean of the thrombocyte counts. The LSD test showed that the results were significantly different ($p < 0.05$) between Groups K and A ($p = 0.001$; $p < 0.05$), Group K with HS Group 25 ($p = 0.001$; $p < 0.05$), and Group A with HS Group 25 ($p = 0.001$; $p < 0.05$). These findings illustrate a significant difference between the administration of aspirin and *H. scabra.*

DISCUSSION

This study found that compared with the administration of aspirin, which can prevent bleeding after antithrombotic/anticoagulant medication, some thrombocytes did not decrease significantly. Normally, thrombocytes are associated with the initiation of the coagulation process, whereby their reaction to damage to blood vessels becomes the main target in haemostasis. Thrombocyte hyperactive reaction triggers side effects in coronary artery disease, which results in thrombosis.[4] The antithrombocyte effect of aspirin involves inhibiting the synthesis of thromboxane A2 (TXA2) from arachidonic acid in thrombocytes because of the irreversible acetylation process and inhibition of cyclooxygenase – an essential enzyme in the synthesis of prostaglandins and thromboxane A2.[19] The presence of these obstacles causes a decrease in the number of thrombocytes present in the blood. This is indicated by the significant difference between the control group and the aspirin-administered group, where the thrombocytes in the latter group have thrombocyte counts that are much lower than those in the controls.

Sea cucumbers contain GAGs and omega-3s, which play a vital role in the thrombogenic process.[13] One of the GAGs that plays a role in antithrombotics is dermatan sulfate. Through the formation of complex covalent bonds with heparin-II (HCII) cofactors, dermatan sulfate selectively inhibits thrombin action, thereby preventing vascular thrombosis.[20] Heparin/heparan sulfate contained in GAGs consists of 20–100 units of N-acetate D-glucosamine α disaccharide associated with glucuronic acid. The molecular mechanism of heparin/heparan sulfate as an anticoagulant can bind and increase the inhibitory activity of plasma protein antithrombin against several serine proteases from the coagulation system, the most important of which are factor IIa (thrombin), Xa and IXa.[21] Although the anticoagulant mechanism is more dominant, heparin/heparan sulfate also has antithrombotic properties. Antithrombotic mechanisms occur with the release of Tissue Factor Pathway Inhibitors (TFPI) associated with the molecular weight and sulfate content of heparin.[22] The GAGs found in the echinoderm cannot help in concluding that antithrombotic properties are strong. Some studies summarised by Pavão[23] show that heparin-like polymers in ascidians and molluscs are different from low anticoagulant ability, as well as significant antithrombotic and anti-inflammatory activities; however, they do not cause bleeding.

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Clinical results in humans show that omega-3 from marine biota can function as antithrombotic. Omega-3 fatty acids in marine life containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) consumption 2–4 g/day and cause optimal anti-atherosclerotic, anti-inflammatory and antithrombotic effects.[24] DHA has a quicker start in inhibiting thrombocyte aggregation induced by adenine diphosphate (ADP). However, both EPA and DHA are incorporated into thrombocyte phospholipids by inhibiting the formation of arachidonic acid (AA), which can help in reducing thrombocyte aggregation by lowering thrombocyte procoagulant metabolites arising from AA synthesis. EPA also competes with AA in the cyclooxygenase pathway, which directly and indirectly reduces the formation of TXA2 pro-aggregatory AA metabolites.[24] This was proved in studies with thrombocyte counts in *H. scabra* administration compared with the control group.

A previous study mentioned that the presence of a barrier in TXA2 and thrombocyte aggregation triggers bleeding.[19] In their multinational study in the United States, Akintoye et al.[25] conducted a placebo-controlled trial involving 1,516 patients, who were given perioperative fish oil (EPA-DHA), 8–10 g/day for 2–5 days before surgery and then 2 g/day postoperatively. Compared with the placebo, the administration of fish oil did not show any bleeding.[25] Akintoye et al.'s study is in line with this study's results whereby the administration of the *H. scabra* ethanol extract containing EPA–DHA was not proved to increase perioperative bleeding; conversely, it even reduced the amount of blood transfusion. High omega-3-PUFA administration is associated with a lower risk of bleeding. Conclusions from this study indicate that the oral administration of aspirin and *H. scabra* decreases thrombocyte counts, whereas the administration of aspirin reduces thrombocyte counts to lower levels than those in *H. scabra* in Wistar rats *(R. Novergicus).*

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