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Effect of heparin administration on heparin-induced thrombocytopenia (HIT) in atherosclerotic New Zealand Rabbit (Oryctolagus cuniculus)

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ABSTRACT

Background: Platelets have been associated with the pathogenesis of atherosclerosis by excreting various chemokines (i.e. PF4), which systematically interact with multiple molecules. The formation of circulating heparin-platelet factor-4 (PF4) complexes have been observed in heparin-induced thrombocytopenia (HIT) cases, raising questions about the role of PF4-derived atherosclerosis in the predisposition of HIT.
Methods: An in vivo model of atherosclerosis was generated by feeding rabbits with a high-cholesterol diet for six weeks and a normal rabbit as a control group. HIT was induced by injecting heparin subcutaneously for ten days twice daily. Platelets count before and after heparinization was performed by hemocytometer, PF4 measurement was determined by ELISA assay. Histopathology examinations for artery thickness, foam cell, and endothelial denudation were conducted by Hematoxylin and Eosin (H&E) staining.
Results: There was no significant difference in the IgG PF4/heparin complex between the atherosclerotic and the normal groups (43.84±2.07 ng/mL vs. 41.87±3.44 ng/mL), platelet count was significantly reduced only in the atherosclerotic group (p=0.01; mean differences 7162.00±57311.02/mm^3), endothelial denudation and foam cell formation were observed in the atherosclerotic group. Also, a significant difference observed in endothelial thickness between the atherosclerotic group compared to the normal group (470.32 ± 131.15 µm vs. 332.05±50.82 µm; p=0.032).
Conclusion: Our results suggest that atherosclerosis was significantly associated with the development of heparin-induced thrombocytopenia. However, atherosclerosis is not a risk factor for the formation of PF4-heparin complex in HIT.

Keywords: Heparin, PF4, atherosclerosis.
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INTRODUCTION

Cardiovascular diseases (CVD) is one of the most prominent cause of death in several countries, causing 16-17 million deaths annually in 2008 and is a contributor to 30% of all deaths worldwide. Of these deaths, 7.3 million were due to coronary heart disease and 6.3 million to strokes. More than 80% of deaths from cardiovascular disease occur in low- and middle-income countries. Moreover, it is estimated by 2030, deaths from cardiovascular disease could reach 23.3 million.[1]

The underlying pathological process of CVD is associated with atherosclerosis. Atherosclerosis is a slow, chronic and progressive disorder in the large or moderate arteries that can lead to intravascular thrombosis. Recently, the role of platelets in inflammation and atherosclerosis have been reported. Various molecules, either on the surface or stored in the platelet alpha granules mediate the communication between platelets and other inflammatory cells during the vascular inflammatory process which is involved in the development and progression of atherosclerosis.[2,3]

During platelet activation, platelets will release many chemokines from their negligent granules which will affect a broad spectrum of biological processes. One of the chemokines produced is platelet factor-4 (PF4). PF4 plays a role in thrombosis and hemostasis, especially when it binds to heparin. PF4 has a strong affinity for the negative pole of heparin resulting in the PF4/heparin complex. In patients exposed to heparin, this PF4/heparin complex can produce antibodies to the PF4/heparin complex, which leads to platelet activation and increase the risk of arterial and venous thrombosis. This syndrome is known as heparin-induced thrombocytopenia (HIT).[4] The use of heparin in some clinical situations has been around for more than 50 years. As an anticoagulation agent, heparin is widely used in various procedures and conditions. These are including

surgery, acute coronary syndrome, venous thromboembolism, atrial fibrillation, peripheral artery occlusion disease, dialysis, and circulation outside the body.

It is estimated that in America, about 12 million people who are hospitalized receive some form of heparin. Half of the heparin recipients were medical patients, whereas the rest were patients with surgery/undergoing interventional procedures. Heparin has several serious side effects, the best-known side effect of which is thrombocytopenia.[5] Heparin is the second anticoagulation agent besides warfarin which is frequently used in therapy and surgery. Significant heparin side effects can occur when heparin binds to platelet factor 4 (H-PF4) and forms a complex that triggers antibodies against this complex, leading to endothelial and platelet activation followed by heparin-induced thrombocytopenia (HIT).[6]

HIT is initiated by antibodies that recognize PF4 associated with polyanionic molecules which present on the surface cell membranes of blood vessels. These antibodies are generated by the multimolecular complex that is formed when heparin is administered. Heparin binds to PF4 induces recognition and response by the immune system. The antibody has a binding site on PF4. These antibodies can only be triggered by the presence of PF4 to generate thrombocytopenia and/or thrombosis.[7]

HIT is a severe drug-mediated side effect of heparin that can lead to a life-threatening vein or artery thrombosis. More than 50% of patients suspected of HIT have decreased platelet counts that appear within 5 to 14 days after administration and develops a thrombotic event while receiving anticoagulant therapy. The onset of HIT can be rapid (within 24 hours after heparin administration) or slow. Various clinical features of HIT include lesions on the skin where heparin is injected and/or a rapid acute systemic reaction to heparin intravenous bolus administration. HIT occurs due to the activation of immunoglobulin (Ig)G antibodies mediated platelets aggregation during heparin administration (unfractionated or low molecular weight heparin). Activated platelets release some micro-particles which cause thrombin formation and subsequently lead to a prothrombotic state.[8]

Based on the presence of an immunological process in atherosclerosis that causes platelet activation, the platelets secrete various chemokines, including PF4. This study aimed to prove that atherosclerosis is associated with the development of heparin-induced thrombocytopenia and whether atherosclerosis is a potential risk factor for the formation of platelet factor-4 (PF4)/heparin complex in animal models.

MATERIALS AND METHODS

Animals and Experimental design

Sixteen healthy male New Zealand rabbits (*Oryctolagus cuniculus*) aged 6 months, healthy, with a bodyweight of 2,500-3,000 grams were used. Rabbits were housed individually in cages in temperature-controlled room (24 °C) under a 12-h light/dark cycle with free access to food and water in the Physiology Laboratory Faculty of Medicine, Brawijaya University. Food and water consumption were recorded daily, while the body weight was measured regularly. The Ethical Committee Faculty approved the experimental protocol of Veterinary, Universitas Airlangga with ethical clearance reference number 373-KE. All study procedures for animal study according to universal declaration of animal rights.

The in vivo study was carried out for 8 weeks. Rabbits were randomly assigned to two equal groups: the atherosclerosis group (AT group) and the normal group (N group). The atherosclerosis was induced by 0.2% cholesterol given for the period of 6 weeks, whereas the normal group was given standard laboratory diet; water was provided *ad libitum*. Following a one-week adaptation period, heparin sodium was given for 10 days at a dose of 70 units/kg body weight for two times a day subcutaneously.

Analysis of IgG PF4-heparin complex and platelet count

Blood samples were collected at 0 and 45th day via ear marginal vein into EDTA tubes for platelet counts (standard complete blood count) and at day 11th for IgG PF4-heparin complex (Elisa kit by Bioassay technology, China). At the end of the study, the rabbits were euthanized with pentobarbital (100 mg/kg body weight) through intravenous injection.

Histopathologic studies

The aortic arch and its branches (brachiocephalic artery, left common carotid, and left subclavian artery) of the rabbits was removed, cleaned, dried, and fixed in 10% neutral buffer formalin. The tissues were embedded in paraffin blocks and stained with hematoxylin-eosin. These samples were analyzed for the atherosclerotic lesions: artery denudation, foam cells, and tunica media thickness. The endothelial thickness was measured from lumen to the edge of the artery wall using Image Reiter software. Measurements were taken from three sections of the aorta and the average of these measurements was applied for analysis.

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Statistical analysis

The numerical data obtained are expressed as mean values ± standard deviation (SD), then categorical data are displayed as frequency and percentage. An independent t-test was used to compare IgG-PF4/heparin complex, aortic wall thickness, foam cells, and endothelial denudation between atherosclerosis and normal control groups. If the data did not fit the constraints of this parametric test, data were analyzed with the Mann-Whitney test. Paired sample t-test for platelet counts pre and post heparin intervention was performed and the Wilcoxon test is used as an alternative if the data does not have a normal distribution. Statistical significance was set at $p < 0.05$. All analyses were carried out using SPSS program version 25.0 (IBM Corporation, Armonk, New York, USA).

RESULTS

Table 1 shows the IgG PF4/Heparin complex between the atherosclerotic and normal groups (43.84 ± 2.07 ng/mL vs 41.87±3.44 ng/mL). However, this parameter was not statistically significant between the atherogenic rabbits compared to the normal control group. In contrast, the

Table 1 Comparison of the IgG-PF4/heparin complex in normal and atherosclerotic rabbits

	IgG-PF4/heparin complex (ng/mL)			
Sample	**1st assay**	**2nd assay**	**Mean ± SD**	**p-value**
AT1	42.25	47.00	43.84 ± 2.07	0.206
AT2	47.81	47.67		
AT3	44.35	40.72		
AT4	43.55	43.48		
AT5	45.00	43.93		
AT6	44.90	44.71		
AT7	42.12	40.06		
AT8	42.16	41.87		
N1	45.25	41.64	41.87 ± 3.44	
N2	44.25	43.87		
N3	36.19	34.19		
N4	39.64	42.93		
N5	42.80	42.90		
N6	45.03	43.71		

Table 2 Comparison of the endothelial wall thickness in the experimental groups

Sample	Body weight (gram)	Endothelial wall thickness (µm)	Mean ± SD	p-value
AT1	3500	422.94	470.32 ± 131.15	0.032
AT2	2900	381.11		
AT3	3200	376.89		
AT4	2500	423.47		
AT5	2600	434.32		
AT6	3200	380.04		
AT7	3500	738.07		
AT8	3600	605.74		
N1	2600	238.34	332.05 ± 50.82	
N2	3100	329.43		
N3	2600	381.11		
N4	2800	353.26		
N5	3100	323.54		
N6	3200	366.67		

Table 3 Effect of cholesterol exposure on aortic atherosclerotic lesions in the experimental groups

Sample	Foam cells	Mean rank	Sum of rank	P value	Endothelial denudation	Mean rank	Sum of rank	p-value
AT1	++	10.38	83	0.02	++	10.5	84	0.01
AT2	+++				+++			
AT3	+++				+++			
AT4	+++				+++			
AT5	++				++			
AT6	++				++			
AT7	+				+			
AT8	+				+			
N1	-	3.67	22		-	3.5	21	
N2	-				-			
N3	-				-			
N4	-				-			
N5	+				+			
N6	-				-			

Table 4 Effect of heparin administration on platelet profile of control and experimental rabbit groups

Sample	Platelet count (1000/mm³)		Delta platelet changes (Mean ± SD)	P value
	Pre-treatment	Post-treatment		
AT1	384,000	268,000	7,162 ± 57,311.02	0.01
AT2	324,000	288,000		
AT3	468,000	282,000		
AT4	302,000	246,000		
AT5	289,000	196,000		
AT6	268,000	234,000		
AT7	238,000	226,000		
AT8	223,000	183,000		
N1	270,000	396,000	44,000 ± 527.41	0.97
N2	323,000	322,000		
N3	270,000	346,000		
N4	362,000	423,000		
N5	362,000	354,000		
N6	350,000	360,000		

endothelial thickness was significantly correlated with atherosclerosis (p=0.032). The endothelial wall of atherosclerotic rabbits was significantly thicker than the normal group (470.32 ± 131.15 μm vs 332.05 ± 50.82 μm; p = 0.032) (Table 2).

In addition, this study demonstrated that atherosclerotic lesions were also significantly corresponded with cholesterol exposure (p=0.01 and p=0.02 for endothelial denudation and foam cells formation, respectively). It was found that endothelial denudation was more abundant in the atherosclerosis group than in the normal group (10.35 vs 3.5; p = 0.01) (Table 3). Similar results were obtained in the foam cells formation of the atherosclerotic rabbits. The atherosclerotic rabbits had a more significant number of foam cells compared to the normal control rabbits (10.38 vs 3.67; p= 0.02) (Table 3). Comparisons between groups concerning the conditions of endothelial cells, foam cells, and endothelial thickness also showed significant

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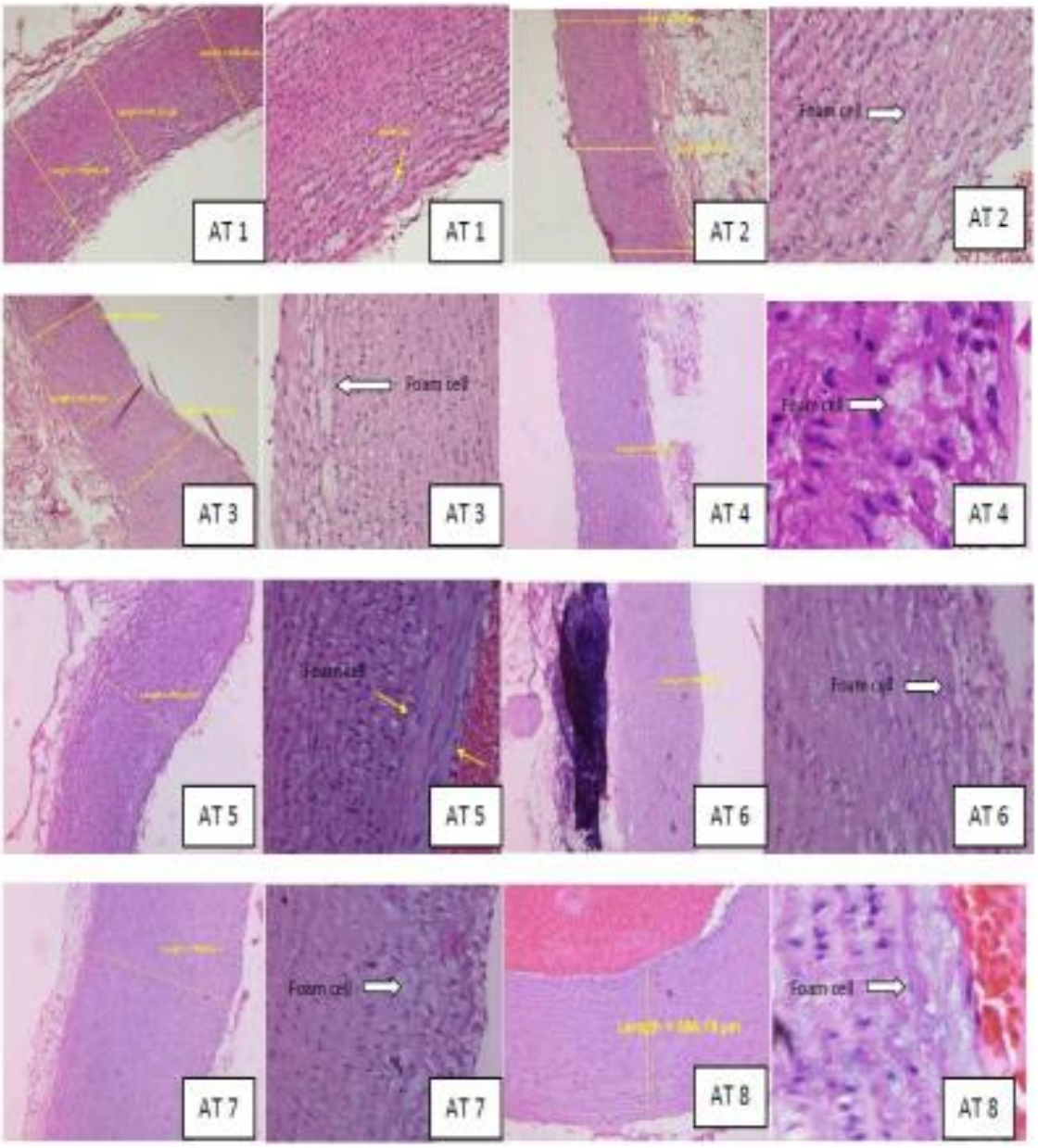

Figure 1 Photomicrographs showing the histologic appearance in the atherosclerosis group. Atherosclerotic lesions were evaluated as arterial wall thickening, as well as foam cells and endothelial denudation formation. (Hematoxylin eosin stain, 400x magnification; Nikon H600L microscope; 300 megapixels DS Fi2 camera) (White and yellow arrow indicating foam cell)

alteration in the cholesterol treated group than in the normal control group, as seen in Figure 1 and Figure 2.

The summary of the platelet count at baseline and post-intervention is presented in table 4. The baseline parameter did not show a statistically significant difference across the two groups. The post-intervention parameter showed a significant difference in the AT group compared with the normal control group ($p = 0.01$; mean differences 7,162 ± 57,311.02 and $p = 0.97$; mean differences 44,000 ± 527.41, respectively).

DISCUSSION

Several studies have documented the development of HIT in various pathological conditions. However, the causal role of atherosclerosis in the development of HIT remains to be proven. Endothelial cell denudation is frequently found as one of the

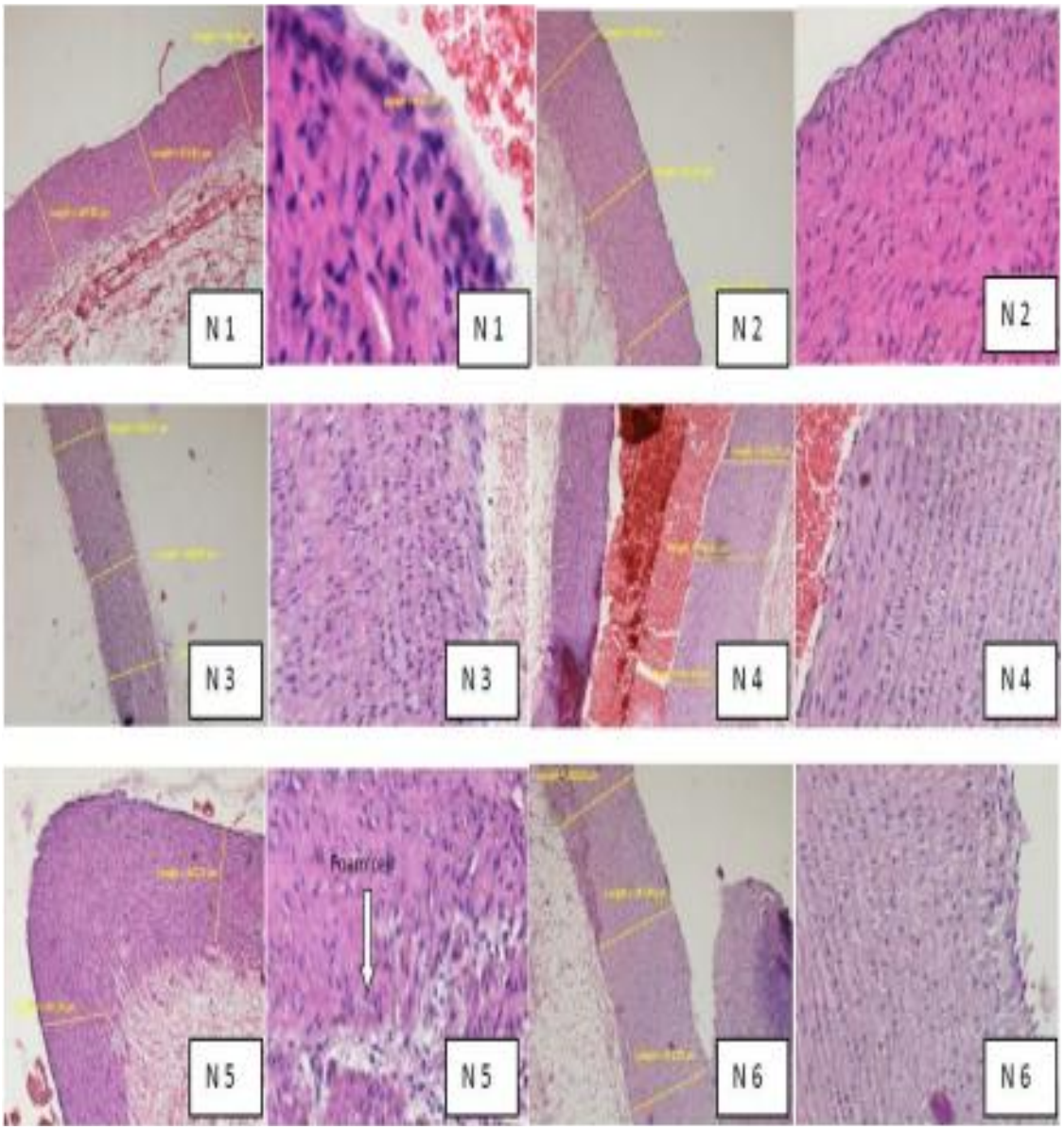

Figure 2 Photomicrographs showing the histologic appearance in the normal control group. No endothelial wall thickening, foam cells and endothelial denudation formation were observed, but foam cells were found in sample N5. (Hematoxylin eosin stain, 400x magnification; Nikon H600L microscope; 300 megapixels DS Fi2 camera) (white arrow indicating foam cell)

atherosclerotic lesions.[9] The main initiating factors in Watanabe heritable hyperlipidemic (WHHL) rabbits are adhesion of leukocytes and platelets to the endothelium and accumulation of lipids on the aortic wall. The progression of atherosclerosis in WHHL rabbits and diet-induced atherosclerosis in rabbits are the same. Damaged endothelial cells cause arterial vasospasm as a consequence of reduced endothelial-derived relaxing factor.[10]

In arterial walls, LDL molecules become susceptible to oxidation by free radicals. Macrophages, which are primary immune cells involved in the atherosclerosis process, have a high affinity for oxidized LDL (OxLDL). OxLDL known by macrophage scavenger receptors induces these cells to take up lipids via phagocytosis. Macrophages are unable to process oxLDL and cause the accumulation of lipids in immune cells which transforms them into a type of cellular debris known as foam cells. The formation of foam cells from macrophages with the formation of fatty streaks has a role in the pathogenesis of atherosclerosis. Foam cell formation is thought to be triggered by LDL, including oxLDL or minimally modified LDL (mmLDL).

Heparin-induced thrombocytopenia (HIT) is caused by the presence of IgG antibodies against PF4/heparin complex. IgG antibodies recognize heparin-induced changes in PF4 tetramer in chain

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length and degree of heparin sulfation. This feature explains the difference in the incidence of HIT with different heparin regiments. Theoretically, the optimal heparin concentration that causes favorable conditions for HIT is a prophylactic rather than a therapeutic dose of heparin. The IgG / PF4 / heparin complex binds and activates platelets via the FC receptor and also activates thrombin via other pathways and causes prothrombotic conditions associated with venous and arterial thrombosis.[11]

This study used unfractionated heparin with a prophylactic dose. Antibodies to PF4/heparin usually form between five and ten days after initiation of heparin therapy. Although many patients are being treated with heparin, antibody generation to PF4/heparin is more frequent in certain patient groups. However, not all antibodies activate platelets and most patients with antibodies to PF4/heparin do not cause HIT clinically.

In this study, heparin was given for ten days in both the atherosclerosis group (AT) and the control group (N), then the PF4/heparin complex antibodies were examined in the eleventh day. Elisa examination of complex IgG PF4/heparin with mean levels in the AT group 43.38±2.07 and the N group 41.87±3.44 (p=0.206), respectively. No significant difference between the IgG PF4/heparin complex in the atherosclerotic (AT) group and the control group (N) was observed. This finding is in line with Nakamoto *et al*. study that the formation of PF4/heparin antibodies complex does not ultimately cause HIT.[12-14]

Laboratory tests can help diagnose HIT; however, not all antibodies to PF4 / Heparin are pathological. Only a subgroup of patients with positive antigen tests have antibodies that activate platelets, whereas only a few patients experience thrombocytopenia and end in thrombosis.[15,16] In this study, there was no examination for the occurrence of thrombosis, although the ELISA test showed IgG to PF4/heparin complex was high in both groups.

It has been reported that a significantly higher incidence of HIT is observed in patients receiving bovine heparin compared to porcine heparin. Moreover, an increased risk of HIT is also observed in patients exposed to unfractionated heparin than low molecular weight heparin (LMWH).[17]

This study used bovine-derived heparin which is more antigenic than heparin derived from porcine, which may explain why the IgG values against the PF4/heparin complex in both of the two experimental were high. Interestingly, Lebenow et al. found that patients undergoing major surgical procedures have a greater risk of developing an immune response to PF4/heparin complex when compared to patients undergoing minor surgical procedures regardless of the type of heparin given. The risk of developing HIT is also lower when using LWMH than UFH in trauma cases. Thus, non-drug factors, such as severe trauma, undergoing major, or minor surgical procedures, are markers for an immune response that can cause HIT, which is a reaction to drug administration (heparin).[18]

Patients undergoing cardiopulmonary bypass have frequently experienced a decrease in platelet count within 72 hours postoperatively. In this patient, platelet recovery followed by a secondary reduction in platelet count between days 5-14 after surgery had a high suspicion for HIT when compared to day four after surgery. Unfractionated heparin (UFH) administered during cardiac-pulmonary bypass is highly immunogenic. Approximately 25% to 50% of post-cardiac surgery having heparin-dependent antibodies for 5-10 days. The risk of HIT is 1-3% if UFH is continued until the first postoperative week.[19] A retrospective study in North America demonstrated that several independent predictors are closely related to the risk of HIT in patients undergoing cardiopulmonary bypass. These factors are including female, congestive heart failure, cardiac insufficiency, atrial fibrillation, liver disease, and chronic kidney failure. HIT is associated with an increased risk of adverse surgical outcomes such as death, stroke, amputation, acute kidney failure, respiratory failure and the need for tracheostomy. HIT is also associated with a 50% increase in early mortality and the majority of patients diagnosed with postoperative morbidity or functional deficits.[20] Notably, patients who were previously known to have HIT had lower intensive care unit (ICU) and hospital mortality and duration of stay in the ICU than patients who were newly diagnosed with HIT. These finding indicate that an adequate diagnosis of HIT can reduce mortality and reduce ICU length of stay admissions.[21]

The delta platelet changes in the atherosclerosis group (AT) was statistically significant compared to the normal control group (7,1625 ± 57311.02/mm^3 with a value of p = 0.01 and 44,000 ± 52767.41/mm^3 with p-value = 0.97), indicating that atherosclerosis is a possible risk factor for the development of HIT. These findings suggest that heparin administration should be used with caution in patients with atherosclerosis to prevent further mortality.

CONCLUSION

The current study demonstrated that the use of a high cholesterol diet for six weeks resulted in the induction of atherosclerotic lesions in experimental rabbits. The administration of unfractionated bovine heparin generated anti

PF4 heparin antibodies complex in experimental rabbits, although no significant difference between the atherogenic diet and the normal control was observed. It was also confirmed that platelet count was significantly reduced in the atherosclerotic rabbits compared to the normal group following heparin used. These results are indicating that atherosclerosis contributes to the pathogenesis of heparin-induced thrombocytopenia, even though the mechanisms remain elusive. This present study also showed that atherosclerosis is not a risk factor for the production of anti-PF4 heparin antibody complex.

DISCLOSURE

The materials contained in this paper were previously reported in the thesis of V.J.N. who conducted a specialist study program in Thoracic, Cardiac, and Vascular Surgery in Faculty of Medicine, Universitas Airlangga. The author declares there is conflict of interest regarding publication of this article. This study doesn't receive any specific grant from government or any private sectors.

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