Effect of Cacao on Plasma F2-Isoprostane Level, CD34 and ICAM-1 Expression of Coronary Arteries in Cigarette Smoking Exposed Rats

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Abstract

This research aimed to evaluate cardioprotective effects of cacao in smoking exposure condition that the effects were mediated through the anti-oxidant pathway by measure plasma F2-isoprostane level, Endothelial Progenitor Cell (EPC) enhancement by expression of CD34, while dysfunction endothelial condition was measured by expression of ICAM-1 in coronary arteries. This study subjected rats, divided into four groups: the normal control group (2 ml of aqua bidest, air exposure); the cigarette control group (2 ml of aqua bidest, cigarette smoke); cacao group 1 (1205 mg/kg BW/day, cigarette smoke); cocoa group 2 (2410 mg/kg BW/day, cigarette smoke). The oxidant biomarker, F2-isoprostane level was assessed using ELISA; CD34, and ICAM-1 expression in coronary arteries by immunohistochemistry. Cacao 1205 mg/kg BW/day significantly decreases plasma F2-isoprostane level, and ICAM-1 expression of coronary arteries in cigarette smoking exposed rat ($p < 0.05$) but there was not a significant increases CD34 ($p < 0.05$). Cocoa in cigarette smoke-exposed rats can prevent endothelial dysfunction through decrease F2-isoprostane but not increase CD34. The results of this study can be used as a basis for preventing endothelial dysfunction due to cigarette smoke by using cacao.

Keywords: *Cacao, F2-isoprostane, CD34, ICAM-1, Cigarette*

Introduction

Indonesia is the third country with the highest number of active smokers in the world. The smoking habit increases the risk of cardiovascular disease 2-3 times, and a cardiovascular mortality rate in 2008 was 30% of total deaths in the world[1, 2]. Cigarette smoke contains nicotine, CO, tar, and many free radicals that are harmful for health and can trigger various pathological effects on the endothelium[3, 4]. In vitro, the mechanism of endothelial damage by cigarette smoke is known by increasing reactive oxygen species (ROS), which results in lipid peroxidation in endothelial cell membranes with end products. One of the product is F2-isoprostane, that proved as the best marker of oxidative stress in vivo, is very stable, more accurate than other markers, and can be used as an early indicator of the atherogenesis process[5, 6]. Furthermore, ROS cause activation of Nuclear Factor Kappa B (NFkB) which triggers an increase in pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-

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1β) and interleukin-6 (IL-6)[7, 8]. The pro-inflammatory cytokines cause endothelial activation, characterized by increased expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule (ICAM-1). This condition will reduce the anti-adhesive properties of the endothelium which is a sign of endothelial dysfunction. If this condition continues it will cause endothelial damage, triggering the next atherosclerosis process until cardiovascular disease occurs[7, 9].

Cigarette smoke also interferes with endothelial regeneration and maintenance processes carried out by endothelial progenitor cells (EPCs) either through the paracrine system (including Vascular Endothelial Growth Factor, Fibroblast Growth Factor, IL-6, IL-8, IL-11) or differentiate into endothelium to replace the damaged endothelium[10, 11]. EPC markers that are often used are Cluster of differentiation 133+ (CD133+), CD34+ and *Vascular Endothelial Growth Factor Receptor 2+* (VEGFR2+) atau *Fetal Liver Kinase-1* (Flk-1). Research showed that cigarette smoke decreased the amount and functional activity of EPC in blood circulation with CD34+ and VEGFR2 markers[11]. In this research, CD34+ was used to detect progenitor cell in tunica intima coronary artery.

Endothelial dysfunction plays an important role in the pathogenesis of atherosclerosis. Improvement of endothelial function immediately after injury is a key step in an effort to inhibit the development of the atherosclerosis process. Cocoa is a food ingredient that polyphenols rich compared to other food ingredients such as apples, garlic, and grapes. Cocoa polyphenols consist of 29%-38% catechins, 4% anthocyanins, and 58-65% proanthocyanidins. Existing experimental research and clinical trials showed that the cardioprotective potential of polyphenols is mediated through anti-atherogenic, antioxidant, anti-inflammatory effects, and improvement of endothelial dysfunction[12, 13].

Cocoa with high polyphenols content is expected to be the right choice as an herbal medicine in preventing endothelial dysfunction and atherogenesis due to cigarette smoke. Therefore the aim of this study is to explain the prevention of endothelial dysfunction by cacao (Theobroma cacao) in the antioxidant pathway through indicators of plasma F2-isoprostane levels and EPC enhancement by expression of CD34. The condition of endothelial dysfunction was measured by the expression of ICAM-1 in coronary arteries. This research was carried out through an experimental approach using rats (Rattus norvegicus) there were exposed to cigarette smoke. Cocoa powder is used because it is widely used as a base for various food and beverage products and has higher flavonoid content than other forms of processed cacao[14]. The aimed of this study was to evaluate cardioprotective effects of cacao in smoking exposure condition that the effects were mediated through the anti-oxidant pathway by measure plasma F2-isoprostane level, Endothelial Progenitor Cell (EPC) enhancement by expression of CD34, while dysfunction endothelial condition was measured by expression of ICAM-1 in coronary arteries.

Methods

Determination of levels of polyphenol from cocoa powder that will be used using Spectrofotometer examination

The research method was started by determining the level of polyphenol in cocoa powder using the Spectrofotometer method. The level of polyphenol in cocoa powder is 2.22%.

Making cigarette exposure equipment

Making cigarette exposure equipment from acrylic materials through several stages of testing tools and trials in rats and has undergone several improvements. Starting with the manufacture of exposure chambers for 12 rats and cigarettes chamber, and making a mixer room that function to mix cigarette smoke and air. A vacuum and aspirator are also needed. Each part is arranged to start from the cigarette, vacuum, aspirator, mixer, and exposure chamber.

Determination of the number of cigarettes and the length of exposure to cigarette smoke that can cause Rattus norvegicus endothelial dysfunction

Determination of the cigarette number and the time of exposure to cigarette smoke through modification[15]. This stage is through testing tools for exposure to cigarette smoke to rats (Rattus norvegicus), by regulating the amount of cigarette smoke adapted

to the conditions of the experimental animal. This needs to be done to determine the length of exposure to cigarette smoke and avoid rats from monoxide poisoning that often occurs. This stage requires modification of the exposure device by adding an air aspirator for dilution of cigarette smoke that will be given to experimental animals.

Determination of the dosage of cocoa powder given to rat (Rattus norvegicus)

The dose of cocoa powder was adjusted to the intake of high polyphenol (>650 mg/day) and low polyphenol (<500 mg/day)[16]. The dosage of cocoa powder given is based on the spectrophotometer results in the levels of polyphenol in cocoa powder. The dosage of cocoa powder given to treatment group 1 was 1205 mg/kg BW/day and treatment 2 was 2410 mg/kg BW/day.

Treatment of experimental animals

The exposure of cigarette smoke to Rattus norvegicus uses smoking chamber. Twenty four Rattus norvegicus 220-250 g divided into 4 groups, namely: normal control group (2 ml of aquabidest, air exposure); cigarette control group (2 ml of aqua bidest, cigarette smoke); cocoa group 1 (1205 mg/kg); cocoa group 2 (2410 mg/kg). Giving through a sonde, and 2 hours after being given exposure to cigarette smoke 1 cigarette/rat/day. Treatment was given for 14 days. Examination of blood plasma F2-isoprostane levels using ELISA, expression of CD34 and ICAM-1 coronary arteries using immunohistochemical techniques.

Statistical Analysis

Results are expressed as mean ± S.D. The difference between experimental groups were compared by One-Way Analysis of Variance. The results were considered statistically significant if $p < 0.05$. Data analysis used IBM SPSS Statistics software version 23.0 (IBM Corp., Armonk, NY, USA).

Results and Discussion

ELISA Test Results for F2-Isoprostane Plasma

The results of ELISA measurements for plasma F2-isoprostane levels can be seen in Table 1. Based on the results analysis of F2-isoprostane data in table 1 showed that exposure to cigarette smoke increase plasma F2-isoprostane levels but not significant. This result can be explained based on existing research that there is no significant difference in oxidative stress between smokers and non-smokers. The difference in plasma oxidative stress (hydrogen peroxide concentration and the concentration of conjugated dienes) became significant after exercise testing (the standard maximal exercise test and single-sprint anaerobic exercise) [17,18].

Cacao dose 1205 mg/kg BW/day can reduce levels of F2-isoprostane due to exposure to cigarette smoke, approaching the level of F2-isoprostane in the normal control group. This result was supported by several studies have shown that giving cocoa can reduce blood levels of F2-isoprostane in conditions of oxidative stress triggered by exercise, and effectively reduces urine isoprostane in smokers[19, 20]. Polyphenol compounds in cocoa, which are mostly flavonoids, including catechins, epicatechin, and procyanidins, have high antioxidant activity. The tricyclic structure of flavonoids determines the effect of antioxidants that capture ROS, Fe2+ and Cu+ chelates and increase antioxidant defenses[14, 21].

The administration of cacao dose 2410 mg/kg BW/day showed contradictory results, there was a significant increase in F2-Isoprostane levels. The antioxidant potential of phenolic compounds depends on the number and arrangement of hydroxyl groups and the degree of conjugation structures[22]. Flavonoids in cocoa, under certain conditions can be pro-oxidant, such as high concentration of flavonoids and the presence of active redox metals[23]. The results of this study indicate that the cacao dosage of 2410 mg/kg/day, which represent a high intake of polyphenols, are too large so that their antioxidant properties turn into pro-oxidants. The nature of cigarette smoke as free radicals and giving cocoa doses of 2410 mg/kg BB/day which changed its nature to pro-oxidants, could cause an increase in F2-isoprostane levels to be higher than in the cigarette smoke exposure group.

The immunohistochemical Examination Results for CD34 Expression of Coronary Artery

The results of immunohistochemical examination for CD34 expression of coronary artery can be seen in Figure 1 and Table 2. Based on the results analysis of CD34 data in Table 2 showed that exposure

to cigarette smoke significantly decrease CD34 expression. This result was supported by previous studies that smoking can significantly reduce the number of circulating EPCs as measured by flowcytometry as CD45lowCD34CD133 (progenitor cells/PCs) or CD45lowCD34CD133VEGFR2 (EPCs)[24]. Cluster of differentiation 34 in this study is one of the EPC markers that homing on blood vessel walls as a reaction to endothelial dysfunction in the affected area. The paracrine effect of homing EPC is expected to improve endothelial dysfunction that occurs. The decrease in CD34 expression due to exposure to cigarette smoke will affect the endothelial maintenance process.

The administration cacao dose 1205 mg/kg BW/day and 2410 mg/kg BW/day increase CD34 expression due to exposure to cigarette smoke but not significant. In addition, there was no difference between the cigarette smoke exposure group given cocoa and the normal control group. This shows that giving cocoa does not significantly prevent the decline in arterial coronaria CD34 expression due to exposure to cigarette smoke, but giving cocoa can approach the average value of the normal group. The previous studies proved that the role of high flavonoid cocoa for 1 month in endothelial dysfunction in coronary heart patients was a significant increase in FMD (Flow-Mediated Vasodilation) and an increase in the number of circulating EPCs marked by CD34 +/KDR+ -CAC[25].

The immunohistochemical Examination Results for ICAM-1 Expression of Coronary Artery

The results of immunohistochemical examination for ICAM-1 expression of coronary artery can be seen in Figure 2 and Table 3. Based on the results analysis of ICAM-1 data in Table 3 showed that exposure to cigarette smoke significantly increase ICAM-1 expression of coronary artery. This shows that exposure to cigarette smoke has an effect on increasing the expression of ICAM-1 arteria coronaria. Intracellular cell adhesion molecule-1 is one of the adhesion molecules due to inflammation. Adhesion molecules are a central and important component of the immune and inflammatory systems[26]. Many studies have shown that dissolved intracellular adhesion (ICAM-1), P-selectin, and E-selectin are significantly higher in smokers than in nonsmokers[27]. It is known that oxidative stress induced by cigarette smoke is responsible for endothelium activation through expression of adhesion molecules, activation of macrophages and platelets. Oxidizing chemicals, including NO and many free radicals, are present at high concentrations in cigarette smoke and over time mediate endothelial dysfunction[4].

The administration of cocoa dose 1205 mg/kg BW/day and 2410 mg/kg BW/day can reduce ICAM-1 expression due to exposure to cigarette smoke, approaching ICAM-1 expression in the normal control group. The previous in-vitro studies have shown that cocoa polyphenols can modulate transcription and secretion of proinflammatory cytokines in human peripheral blood mononuclear cells (PBMCs)[28]. However, several human studies, focused on the anti-inflammatory effects of cocoa, have shown mixed and conflicting results. Mathur et al have reported that there was no change in inflammatory biomarkers (IL-1, IL-6, TNF-α, hs-CRP and p-selectin) with the consumption of 36.9 g dark chocolate and 30.9 g of cocoa powder drink per drink days for 6 weeks in healthy subjects[29]. Similarly, Farouque et al stated that consumption of flavanol-rich cocoa and cocoa beverages (444 mg flavanol/day) during a six-week period did not change the concentrations of ICAM-1, VCAM-1, E-selectin, or P-selectin in subjects with CHD[30]. In contrast, Kurlandsky and Stote found that consumption of chocolate 41 g day for six weeks significantly decreased the soluble adhesion molecule ICAM-1 but not VCAM-1 and hs-CRP[31]. Monagas et al proved that the concentration of endothelial adhesion molecules soluble P-selectin and serum ICAM-1 in subjects at risk of PKV (smoking, diabetes, hypertension, dyslipidemia), by administering 40 g of cocoa powder in skim milk for 4 weeks, was significantly lower than skimmed milk alone[32].

Table 1. Plasma F2-isoprostane levels (ng/L)

Group	F2-Isoprostane		
	Mean	SD	p
Normal	7.27ab	0.40	0.000*
Cigarette	8.25b	1.00	
Cacao	6.84a	0.66	
Cacao	10.11c	0.92	

Note: *significant at a=0.05 (Oneway Anova); [abc] different superscripts indicate differences between groups

Table 2. Expression of coronary artery CD34

Group	CD34		
	Mean	SD	P
K0	3.33b	1.34	0.024*
K1	1.20a	0.79	
P1	2.16ab	0.73	
P2	1.50ab	1.52	

Note: *significant at a=0.05 (Oneway Anova); [abc] different superscripts indicate differences between groups

Table 3. Expression of coronary artery ICAM-1

Group	ICAM-1		
	Mean	SD	P
K0	1.43a	0.83	0.012*
K1	4.83b	2.87	
P1	1.48a	0.78	
P2	1.10a	0.90	

Note: * significant at a=0.05 (Oneway Anova); [abc] different superscripts indicate differences between groups

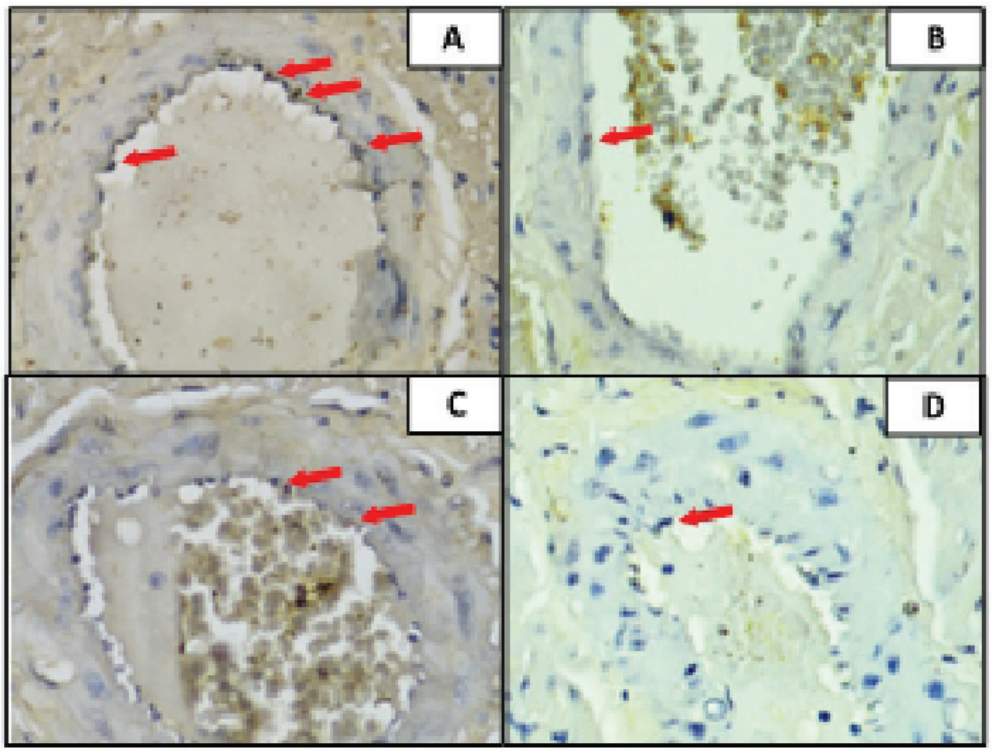

Figure 1. Overview of coronary artery immunohistochemistry of white rats with 400X magnification. Cells expressing CD34 appear brownish in color. In the control group showed more CD34 expressed cells than the other treatment groups.

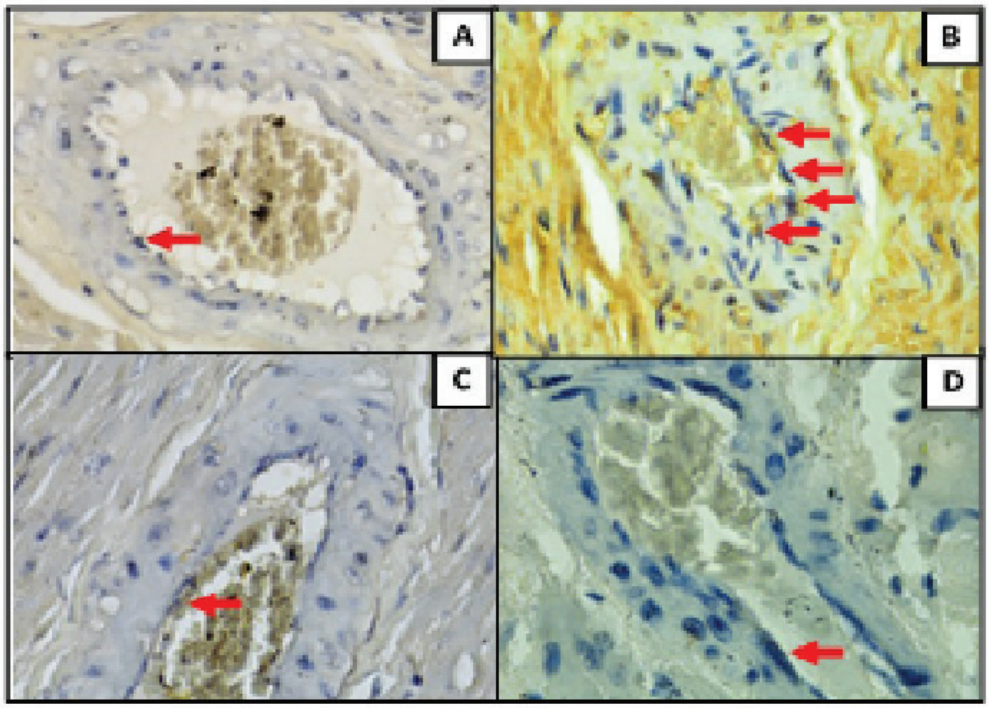

Figure 2. Overview of coronary artery immunohistochemistry of white rats with 400X magnification. Cells expressing ICAM-1 appear brownish in color. In the cigarette group showed more ICAM-1 expressed cells than the other treatment groups

Conclusion

The conclusion of this study is that the administration of cocoa can reduce the negative effects of exposure to cigarette smoke on the endothelium as indicated by a decrease in ICAM-1 expression. This effect is through the anti-oxidant pathway by decrease F2-isoprostane level but not followed by a significant increase in CD34 expression. The results of this study can be used as a basis for preventing endothelial dysfunction due to cigarette smoke by using cacao.

Conflict of Interest: The authors report no conflict of interest in this publish.

Funding: None.

Ethical Approval: All procedures performed in studies involving Veterinary participants were in accordance to the Ethics Committee in Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (2.KE.073.04.2018).

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