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Cocoa liquor increases SOD activity in wistar rats experiencing oxidative stress

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ABSTRACT

Chocolate is a healthy food. The active ingredient in chocolate (*Theobroma cacao* L.), especially antioxidants, can counteract the free radicals present in the body. This study aimed to determine the effect of cocoa liquor on increased activity of Superoxyde Dismutase (SOD). The study design was randomized post test only control group with 20 samples of male white rats wistar (*Rattus norvegicus*). The sample was divided into 4 groups: 2 control groups and 2 treatment groups. The treatments provided were instantaneous physical activity and cocoa liquor preparation with a single dose. This study used one way anova and continued with post hoc tukey HSD test for statistic. The results showed that cocoa liquor had an effect on SOD activity. SOD activity increased in treatment group with cocoa liquor either 2 hours or 24 hours before the momentary physical activity.

Keywords: Cocoa liquor, Superoxide dismutase, Oxidative stress

INTRODUCTION

The adverse effects of free radicals have been widely studied, such as the onset of degenerative diseases. Recurrent temporary physical activity during human work can cause illness caused by oxidative stress. Research showed that people who work as much as 55 hours per week are at risk of stroke 33% higher and the risk of coronary heart disease 13% higher when compared with people who work 35-40 hours per week[(1)].

Free radicals have long been studied as the cause of oxidative stress with a variety of triggers. Sources of free radicals exist that come from outside the body and from within the body[(2)]. Redox imbalance in the body in warding off free radicals can cause oxidative stress. Damage due to oxidative stress among others is lipid peroxidation that attacks carbon-carbon double bond lipids especially polyunsaturated fatty acids/PUFAs especially in cell membranes[(3)], protein damage due to amino acid reactions with free radicals and DNA damage that causes modifications and genetic mutations in which guanine is particularly susceptible to free radical attack[(4)].

Instant physical activity, such as when light exercising, working, and moving is the source of free radical formation in the body. Regular and measurable physical activity in exercise is beneficial for the body in adapting to free radicals. In contrast, instantaneous physical activity causes an extreme increase in free radicals and causes oxidative stress[(5)]. Redox ability in the blood decreases significantly post-physical activity[(6)]. Oxidative stress after a false physical activity is more likely to occur in people with sedentary lifestyle which they never exercise or were untrained[(7)].

Endogenous antioxidant defenses can be supported by the addition of oral antioxidants to fight free radicals due to increased physical activity[(5),(8)]. Chocolate (*Theobroma cacao* L.) is one of Indonesian plantations that have been widely used for various health products. Cocoa seeds are one of the foods that have antioxidant capacity/ORAC, total polyphenol content/TP, and a high total flavanol content compared to other foods, especially cocoa seeds from Indonesia[(9),(10)]. Therefore, it is necessary to research how the consumption of chocolate paste can help increase endogenous antioxidant activity in the conditioning of momentary physical activity.

METHODS

This research was conducted at Biochemistry Laboratory, Faculty of Medicine, Airlangga University, Surabaya, in August-September 2017. This research type was laboratory experimental research with randomized post test only control group design. The determination of experimental animals in this study was randomized and compared the treatment groups with the control group. There were 4 groups of experimental animals consisting of 2 control groups (a negative control group and a stress-controlled positive control) and 2 treatment groups. The

experimental animals in this study were 20 male wistar strain rats (*Rattus norvegicus*). Each group consisted of 5 rats with age of rats ± 2-3 months and body weight 150-200 gram.

Psychological Stress

The rats were acclimatized for 7 days then grouped randomly into 4 groups. Each group consisted of 5 male white rats of wistar strains. The Normal Control Group (NC) does not get any treatment. Positive Control (PC) and Treatment Group (T1, T2) are given instantaneous physical activity using Columbus Treadmill Apparatus according to the 4-stage time protocol.

Cocoa liquor

The cocoa liquor used in this study came from a cocoa plantation in Sulawesi, Indonesia which was harvested in March 2017. The cocoa liquor used was 7.2 gr/kg BW and contained 1.26% polyphenol, 2.38% flavonoids, and 0.44% epicatechin. Examination of chocolate paste paste was done at Surabaya Center Health Laboratory in 2017. Cocoa liquor was obtained from cocoa seeds that had passed the process of fermentation of cocoa seeds, drying, roasting, and grinding. The cocoa liquor was dissolved in water and administered to the animal by a sonde. Cocoa liquor was only given for treatment group T1 and T2. Group T1 consumed cocoa liquor 2 hours before physical activity and T2 group consumed cocoa liquor 24 hours before physical activity.

Measurement of Superoxyde Dismutase Activity (SOD)

Blood samples were taken for negative control group immediately after the acclimation was completed, while the blood samples in the positive control group and the T1 and T2 treatment groups were taken as soon as the treadmill was completed. Measurements of SOD activity were performed using ELISA (enzyme-linked immunoabsorbent assay)[(11)]. SOD is an antioxidant enzyme involved in ROS cleansing. SOD converts free radicals (O^{2-}) into H_2O_2[(12)].

Data Analysis

The data were analyzed by descriptive analysis on the characteristics of the study, then different test using One Way Anova statistical test and continued with Tukey HSD test. The difference was considered significant if the value of $p < 0.05$ with a 95% confidence interval. Previously, the data must be met One Way Anova tested the normal distribution criteria with the One Sample Kolmogorov-Smirnov normality test and variation of homogeneous data by performing the Lavene Test.

RESULTS

Based on the study, the average of superoxyde dismutase (SOD) activity of all groups such as Table 1.

Table 1. Mean SOD (U/mL) and Standard Deviation of Control Groups and Treatment Group on White Rats Wistar

Group	n	Superoxyde Dismutase (SOD) (U/mL)
		Mean ± SD
NC	5	89.0501 ± 2.1800 U/mL
PC	5	78.1451 ± 6.9551 U/mL
T1	5	87.5356 ± 6.1502 U/mL
T2	5	90.4485 ± 1.3659 U/mL

Information:
NC: Negative Control (without treatment), PC: Positive Control (only instantaneous physical activity), T1: Treatment Group 1 (chocolate paste paste 7.2 gr/kg BW 2 hours before instantaneous physical activity), T2: Treatment Group 2 (chocolate paste paste 7.2 gr/kg BW 24 hours before instantaneous physical activity).

Based on the table, the activity of SOD in the Negative Control Group (NC) was 89.0501 ± 2.1800 U / mL. The lowest SOD activity is in the Positive Control Group (PC) with a value of 78.1451 ± 6.9551 U / mL. The activity of SOD Group of Treatment 1 (T1) was 87.5356 ± 6.1502 U / mL close to activity of SOD Group Negative Control (NC). The highest SOD activity was in Group of Treatment 2 (T2) of 90.4485 ± 1.3659 U / mL.

SOD activity in all groups had a normal distribution ($p > 0.05$) using a single sample Kolmogorov-Smirnov test. Negative Control (NC) with p value = 0.194, Posetif Control group (PC) value p = 0.256, Treatment Group 1 (T1) value p = 0.223, and Group Treatment 2 (T2) with value p = 0.223. Homogeneity test using Lavene test on SOD data showed homogeneous data in all experimental groups with $p = 0.052$ ($p > 0.05$). The One Way Anova SOD activity test in all the groups showed $p = 0.004 < 0.05$. So, it could be said there are significant differences between groups on the variable activity of SOD.

To determine which groups differed significantly, Tukey HSD test was performed and was significant if the p value <0.05. Tukey HSD test results on SOD activity were shown in Table 2.

Table 2. Tukey HSD T Test Value Superoxyde Dismutase (SOD) Activity

Group	NC	PC	T1	T2
NC	-	0.012*	0.959	0.967
PC	0.012*	-	0.033*	0.005*
T1	0.959	0.033*	-	0.776
T2	0.967	0.005*	0.776	-

Information:
NC: Negative Control (without treatment), PC: Positive Control (only instantaneous physical activity), T1: Treatment Group 1 (cocoa liquor 7.2 gr / kg / bb 2 hours before instantaneous physical activity), T2: Treatment Group 2 (cocoa liquor 7.2 gr / kg / bb 24 hours before instantaneous physical activity)

Based on Table 2, it was known that there was a significant difference between Negative Control (NC) and Positive Control (PC) with significance value p = 0.012. Group of Treatment 1 (T1) was significantly different from Positive Control Group (PC) with p = 0.033. Treatment Group 2 (T2) was also different with Positive Control Group (PC) with p value = 0.005. However, the Negative Control (NC) was not different either with Treatment Group 1 (T1) or Group Treatment 2 (T2) with p = 0.959 and p = 0.967. Treatment Group 1 (T1) was not different from Treatment Group 2 (T2) with p value = 0.776 (Figure 1).

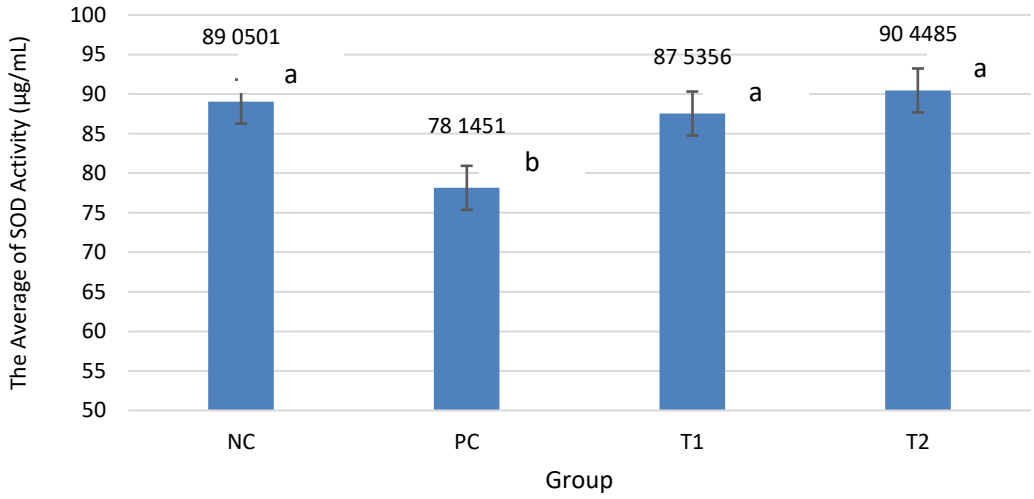

Figure 1. The Average Superoxyde Dismutase (SOD) Activity in All Groups

Based on Figure 1, it was known that positive control group (PC) was different from negative control group (NC), treatment group 1 (T1), and treatment group 2 (T2). There was a striking difference in negative control group in which the SOD activity was lower than for all groups of both negative control (NC), treatment 1 (T1), and treatment 2 (T2) groups. From the SOD activity data in all animal groups in this study, it was found that the Positive Control Group (PC) had the lowest SOD activity compared with the other three groups. While negative control group (NC), treatment group 1 (T1), and treatment group 2 (T2) had mean that did not differ significantly.

DISCUSSION

Instantaneous physical activity using a treadmill running protocol caused oxidative stress[13]. The momentary physical activity of treadmill running had the same rate of lipid peroxidase increase as some other heavy physical activity such as Cross Fit™[14]. During temporary physical activity, there was a high antioxidant mobilization to fight free radicals[15].

During a momentary physical activity, Hypothalamo Pituitary Adrenal (HPA) Axis responded to many stimuli that indicated the regulatory function and integration of the Axis HPA signal associated with hypothalamic corticotropin-releasing hormone (CRH) stimulation and arginine-vasopressin (AVP) secretion (with an important role of CRH), and the synthesis and release of ACTH[16]. The responses lead to energy mobilization to fight stress and result in increased blood pressure, heart rate, and blood sugar levels[17]. A rise in blood pressure in instantaneous physical activity had a response pathway through the hypothalamus to sympathetic output and triggers the adrenal medulla and peripheral sympathetic nerves causing vasoconstriction and raising blood pressure[18].

Blood pressure caused stretching of blood vessels by biological and physical mechanisms where stretching of the circle in blood vessels where this stretch also induces an increase in superoxide production in endothelial cells and smooth muscle cells[19]. NADPH oxidase becomes the primary source of superoxide during temporary

physical activity where the rise in blood pressure causes Rac1 signaling which causes the incorporation of at least 5 components in the vascular tissue of two cystolic subunits (p47phox and p67phox), gp91phox (Nox), p22phox, and G-protein Rac which was small. In the incorporation of this subunit within the cell membrane a superoxide leap was produced on the extracellular side of the membrane by a reduction electron from oxygen via gp91phox using β-nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor[(20)].

Free radicals produced during temporary physical activity were overcome with endogenous antioxidants in the body and some endogenous antioxidants might decrease in blood plasma post-temporary physical activity[(6)]. This was indicated by the decrease of SOD activity in positive control group (PC) which was only treated with treadmill running. The opposite situation could be seen in the group with cocoa liquor in which the treatment group 1 (T1) and the treatment group 2 (T2) were treated with treadmill running but the value of SOD activity remained high and did not differ from the negative control group (NC). Studies had shown increased activity of catalase, glutathione, and superoxyde dismutase (SOD), and also decreased levels of 4-Hydroxynonenal (4-HNE) which was a product of lipid peroxidase in liver tissue of chocolate-fed rats 1/kg kg[(21)].

Epicatechin was a polyphenol derivative with the greatest content in cocoa liquor (*Theobroma cacao* L.) and its contents account for 35% of the total cocoa seed polyphenols that were structurally composed of two aromatic rings and were connected by an oxygenated heterocycle with a 4-hydroxyl group which made (-) - epicatechin compounds with high bioactivity. If taken by oral (-) - epicatechin would be stable during transit in the stomach but would be converted into glucuronid and partly methylated in small intestine. Furthermore, the process in the liver only leaved a level smaller than (-) - epicatechin. Chemical reactions (-) - epicatechin as an antioxidant with reactive oxygen species (ROS) yield (-) - Epicatechin-o-quinone[(22)]. The high activity of SOD in the treatment group 2 (T2) due to the positive effects of epicatechin did not always depend on the presence of epicatechin in the blood after oral daily dosing. Even after 48 hours of epicatechin, antioxidant function is still running[(23)]. This was because epicatechin in chocolate can activate nuclear erythroid-2 like factor-2 (Nrf2). Nrf2 controled the expression of some antioxidants and gene detoxification by binding to antioxidant response elements (AREs) commonly found in the promoter regions of gene antioxidants and in charge of controlling the expression of the gene. Under normal or unstressed conditions, Nrf2 was stored in the cytoplasm by a group of proteins that rapidly decrease it. Under oxidative stress, Nrf2 was not degraded, but switches to the nucleus where it bound to the DNA promoter and initiates the transcription of its antioxidant and protein genes (including peroxiredoxin, glutathione-s-transferase, and superoxyde dismutase)[(24),(25)].

CONCLUSION

Based on the results of this study, cocoa liquor given to experimental animals with instantaneous physical activity could increase the activity of Superoxyde Dismutase (SOD). This increase in endogenous antioxidant activity was demonstrated both in the treatment of chocolate paste 2 hours and 24 hours before the instantaneous physical activity was performed.

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