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THE EFFECT OF GREEN TEA ON OXIDATIVE STRESS LEVEL AMONG WISTAR SUPLEMENTED BY RECYCLING CANOLA OIL

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ABSTRACT: *Oxidative stress is involved in aging and many diseases, such as neurodegenerative, psychiatric disorders, and various cancers. Recyling oil contains trans fat (TF), one of sources of oxidative stress induce lipid peroxidation resulting in malondialdehyde (MDA) and 4-hydroxynonenal (HNE) generation. Green tea has gained considerable attention because of its antioxidant features. This study focuses on the effect of green tea on plasma MDA levels as one of marker for oxidative stress among wistar fed by recycling canola oil. A post test only control group design study using wistar rat. Samples were randomly divided into five groups (n=5 per group). K1 (negative control), K2 was given recycling canola oil and standard diet for 4 weeks, K3 was given green tea and standard diet for 4 weeks, P1 was given green tea and recycling canola oil diet for 4 weeks and P2 was given recycling canola oil diet for 4 weeks then green tea and standard diet for 4 weeks. Blood samples were collected from abdominal aorta to measure plasma MDA levels using modification of TBARS methods as described by Yagi et al. There is no significant difference between groups on body weight (p=0.310), abdominal circumference (p=0.503) and plasma MDA levels (p=0.398) after giving green tea to wistar fed by standard diet and supplemented by recycling canola oil. We conclude that giving green tea to wistar fed by standard diet and suplemented by recycling canola oil do not influence on body weight, abdominal circumference and plasma MDA levels.*

KEYWORDS: Oxidative Stress, Malondialdehyde, Trans Fat

INTRODUCTION

Oxidative stress has been implicated in aging and many diseases such as neurodegenerative disorders and various cancers. The reactive oxygen species resulted from aerobic metabolism and environmental stressors can chemically change cell proteins and disturb their biological functions (Aiken et al., 2011).

Recent study demonstrated that during food frying, the oldest food preparation methods result in several physical and chemical changes occur in the oil as a result of oxidation, pyrolysis, polymerization, hydrolysis and isomerization reactions, producing numberless substances including trans fat formation that incorporated into foods altering their appearance, aroma and taste of food during this processes (Martin et al., 2007), which could be evaluated using acid value (AV), polar compound content (PC), carbonyl value (CV) and Gardner color (GC) of oil (Totani et al., 2012).

Trans fat (TF), one of product from deep food frying defined as unsaturated fatty acids with at least one carbon-carbon double bond in the trans configuration. It is believed TF is one of sources of oxidative stress (Martin et al., 2007). TF can induce lipid peroxidation, consequentially malondialdehyde (MDA), 4-hydroxynonenal (HNE) and other product such as acrolein and α, β-unsaturated aldehydes are produced (Micha and Mozaffarian, 2008) (Lee and Park, 2013). α, β-unsaturated aldehydes, have been implicated as mediators of inflammation and vascular dysfunction. α, β-Unsaturated aldehydes are toxic because of their high reactivity with nucleophiles and their ability to form protein and DNA adducts without prior metabolic activation. This strong reactivity leads to electrophilic stress that disrupts normal cellular function (Lee and Park, 2013). The rate of lipid peroxidation can be determined using its product such as MDA or HNE, laso protein carbonyl (PCO), total thiol (T-SH), advance oxidation protein products (AOPP), glutathione (GSH) and superoxide dismutase (SOD) activity (Uzun et al., 2013).

Recent studies suggested that MDA is genotoxic due to its ability to produce frame shift mutation. Another point is, MDA also responsible to keep cardiovascular stability since it is known that MDA is involved in atherogenesis result in atherosclerosis (Rio et al., 2005). MDA laso plays an important role in the process of bone loss and is a predictor of osteoclastic activity (Akpolat et al., 2013).

Canola oil have been shown to reduce circulating lipid levels. It influences biological functions that affect various other biomarkers of disease risk (Lin et al., 2013). In addition, canola oil is believed could improve the bone formation for osteoporosis patients, eventhough the proof of this effect still on debate (Azadbakht and Haghighatdoost, 2012, Azemati et al., 2012). The safety of this oil also still questionable regarding the preparation and technique of cooking.

Nowadays, there are growing scientific evidences that green tea (*Camellia sinensis*) has gained considerable attention because of its antioxidant, anti-inflammatory, antihypertensive, antidiabetic, and antimutagenic features (Basu and Lucas, 2007, Gu et al., 2013, Santilli et al., 2013). In green tea, catechins composed 80% to 90% of total flavonoids, with epigallocatechin gallate (EGCG), being the first rank catechin, followed by the other catechins, epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) (Basu et al., 2010). These compounds is exist due to the process procedure of green tea. Green tea is processed without enzymatic oxidation (Chen et al., 2012). EGCG prevent lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors by inhibiting NF-κB expression and activating Nrf2 expression, which were activated and suppressed in the heat stress environment (Sahin et al., 2010). EGCG also suppress breast tumor angiogenesis and growth via inhibiting the activation of HIF-1α, and NF-κβ, and VEGF expression (Gu et al., 2013).

Despite of a large number of studies regarding antioxidant effect of green tea, there is no one published study demonstrated used recycling oil as treatment to determine antioxidant effect of green tea. Regarding these, our study focus on the effect of green

tea on plasma MDA levels among wistar which is fed by recycling oil to obtain better understand on the effect of green tea as one of antioxidant sources.

Understanding effect of green tea as antioxidant related to health is important to enhance the clinical management effort of clinician to provide adjuvant therapy on oxidative stress related diseases such as metabolic syndrome and influence the concept of researchers who are investigating effect of antioxidant for stress oxidative.

METHODS

This study is the post test only control groups design study in the field of biochemistry, correlates with nutrition science and clinical pathology using wistar as a subjects which is done at Biochemistry Department, Faculty of Medicine Diponegoro University for two months. All intervention were carried out with permission of Ethical Committee from Faculty of Medicine Diponegoro University Semarang, Indonesia.

Samples of this research were healthy, male wistar, 1-2 month-old and 200 gram body weight. Sample size of this research was estimated based on World Health Organization recommendation on Research Guideline for Evaluating The Safety and Efficacy of Herbal Medicines.

Five groups contained five subjects for each group were used for this study. K1 as negative control group, K2 was given recycling canola oil and standard diet for 4 weeks, K3 was given green tea and standard diet for 4 weeks, P1 was given green tea and recycling canola oil diet for 4 weeks and P2 was given recycling canola oil diet for 4 weeks then green tea and standard diet for 4 weeks.

Recycling Oil Preparation

Recycling oil was prepared by heating fresh oil from difference sources (canola, corn, rice bran) in the high temperature (over 200^{o}C) which is measured by thermometer for 20 minutes started after temperature reached 200^{o}C, then cooled in room temperature (30^{o}C) until oil reached same temperature (30^{o}C). These preparations were repeated for four times (Martin et al., 2007). Then samples of recycling oil were analyzed to determine the changes of fatty acid components resulted from high temperature heating using gas chromatography (GC).

Analysis of Trans Fat from Recyling Oil

The percentages of fatty acid including TF were detected using gas chromatography (GC) in *Laboratorium Terpadu* Institute of Agriculture Bogor (IPB), Bogor, Indonesia. We focus on percentage of elaidic acid and linolelaidic acid First of all, samples were added by 1 ml sodium hydroxide (NaOH) 0.5 N in methanol then incubated for 20 minutes. 2 ml BF3 16% and 5 mg/ml internal standard then were added then continued by incubation for 20 minutes. Samples were added by 2 ml saturated sodium chloride and 1 ml hexane. Hexane layers were separated and put into tube contained 0.1 g anhydric Na2SO4 then incubated for 15 minutes. Liquid phases

were separated and injected to GC. Analysis started from solution injection to column then continued by injection of 5 AμL standard solutions. Samples were injected after all solution that injected before reached peak and get out. Retention time and peak of samples were measured then compared to standard using formula: Cx = (Ax . R. Cs)/As; Cx is concentration of samples, Cs is concentration of standard, Ax is total area of peak from samples, As is total area of peak from standard and R is time response.

Green Tea Preparation

Green tea infusions were prepared daily as described by Cheng et al by adding 3.0 grams of dry green tea and one spoon of sugar to 1000 ml of boiled water cooled to 75oC. The solution was filtered after 15 minutes, cooled in room temperature, and dispensed into clean drinking bottles that given via drinking water and fed to wistar ad libitum [(Cheng et al., 2004)].

Plasma Malondialdehyde Levels Measurement

After blood collection from abdominal aorta, plasma MDA levels were measured using TBARS Assay Kit from ZeptoMetrix Corporation (cat#0801192). EDTA blood were centrifuged at 3500 rotation per minute (rpm) for 10 minutes in room temperature. Plasma obtained from this method were put into new tube. 125 μl sample and 125 μl SDS solution were took and put into tube then added 3 ml TBA/buffer reagent. This mixture then were incubated for 60 minutes at 95oC. After treatment, cooled at room temperature for 15 minutes, then centrifuged at 300 rpm for 15 minutes. Supernatant were separated and put into cuvete, reading absorbance were using spectrophotometer at 545 nano meter (nm).

Data analysis

The data including body weight and abdominal circumference after treatment, and plasma MDA levels were collected. Furthermore the data were analyzed using SPSS 17 for Windows. Distribution of data were tested using Shapiro-Wilk Test, then data were further analyzed using One way Anova for body weight and abdominal circumference and Kruskal Wallis for plasma MDA levels to determine the difference between groups. The significant differences were concluded at α=0.05.

RESULTS

Analysis of Fatty Acid

Before we started our experiments on wistar, we determined the changes of fatty acid resulting from heating of fresh oil. Our result depicted in table 1 suggests that by heating fresh oil for four times in temperature over 200^{o}C for 20 minutes subsequently changes the component of fatty acid in the oils such as the occurrence of trans fatty acid until 0.13-0.30% in recycling oils. Based on our experiments from difference sources of oil, canola oil produce the highest percentage of TF compared to others (corn and rice bran oils). Regarding this finding, we continued experiments using canola oil.

Body Weight After Treatment

After treatment by giving green tea and recycling canola oil for period as mentioned in methods, body weight was weighted to determine wether there is effect of green tea and recycling oil on changes of body weight. The result for body weight is demonstrated in table 2. It is concluded that by giving green tea to wistar which are fed by standard diet and supplemented by recycling oil, there is no difference on body weight.

Abdominal Circumference After Treatment

Then our experiments were continued. In order to confirm that recycling oil has an effect on central obesity we measured abdominal circumference after treatment, and the result is depicted in table 3. By using one way anova, it is concluded that there is no significant difference between groups. So we conclude that there is no changes on abdominal circumference resulted from giving green tea and recycling canola oil.

Plasma MDA Level

Finally, we measured the level of malondialdehyde on plasma after all of treatment. Table 4 demonstrates our result for the measurement of plasma MDA levels. By using Kruskal Wallis we conclude that there is no significant difference between groups.

DISCUSSION

Green tea contents including polyphenols have strong antioxidant activity (Chen et al., 2012, Basu and Lucas, 2007). These contents are potent scavengers of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide produced by various chemicals. These properties are also considered to be associated with their ability to stimulate antioxidant defense metabolism through the redox-regulated transcription factors and mitogen-activated protein kinases (MAPK)-dependent cell cycle regulation. Furthermore, it has also been reported that green tea has pronounced cell growth inhibitory effects and induce apoptosis in cancer cells(Gu et al., 2013). The exact mechanism this effect is not clearly understood. It has also been demonstrated that the higher concentrations of tea polyphenols in cell culture systems produce H2O2 and lipid peroxidation which may be an important factor responsible for cellular toxicity (Raza and John, 2005).

Antioxidant capacity is an overall ability of organisms or food to catch free radicals and prevent their harmful effect. Antioxidative effect includes protection of cells and cellular structures against harmful effect of free radicals, especially oxygen and nitrogen. Substances with antioxidative properties are called antioxidants. They are contained in food and food supplements, most commonly in fruits, vegetables, rice, wine, meat, eggs, and other foodstuff of plant and animal origin (Nekvapil et al., 2012).

Our results from KI as negative control groups and KIII which is given green tea and standard diet for 4 weeks demonstrates that green tea decrease plasma MDA levels

even it is not significant (KI 34.9 nmol/ml and KIII 31.2 nmol/ml). These results is in line that green tea has an effect as antioxidant (Chen et al., 2012).

Recent study demonstrated that TF formed during food frying, the oldest food preparation methods. Several physical and chemical changes occur in the oil as a result of oxidation, pyrolysis, polymerization, hydrolysis and isomerization reactions, producing numberless substances that incorporated into foods and that alter their appearance, aroma and taste of food during this processes (Martin et al., 2007).

The formation of TFA during food frying is closely related to the process temperature and oil use time. Trans unsaturations started to increase at 150^{o}C and became much more significant from 250^{o}C. After heating for 20 minutes at 200, 250 and 300^{o}C, increasing of 356.5%, 773.9%, and 3026.1%, respectively, in the concentration of trans isomers in relation to the initial values (Martin et al., 2007). Analysis of percentage of TF from samples after heating in over 200oC for 20 minutes and repeated for four times suggested that there is TF formation during oil high temperature heating.

Consumption of such industrial TF raise the total HDL, cholesterol ratio in blood, lead to systemic inflammation, endothelial dysfunction, as consequence coronary heart disease, and insulin resistance thorough oxidative stress effect resulted from TF metabolisms (Micha and Mozaffarian, 2008). Emerging evidences also suggest that TF consumption may increase weight gain and fat accumulation, particularly of visceral fat (Kuipers et al., 2011). Eventhough our results do not prove these phenomenon, we still believe that TF consumption result in increase of weight gain and fat accumulation. We hypothized that because of small amount of TF in our recycling oil, so we cannot confirm this phenomenon.

Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems (Romero et al., 1998). Once this imbalance appears, cellular macromolecules may be damaged by the predominant free radicals. This leads to oxidative modifications of the genome, proteins, structural carbohydrates, and lipids; in the later case, lipid peroxidation (LPO) occurs (Romero et al., 1998). LPO is a free radical-related process, that in biologic systems may occur under enzymatic control such as generation of lipid-derived inflammatory mediators, or nonenzymatically (Maritim et al., 2003). Lipid peroxidation is a radical chain reaction in which hydrogen atoms are abstracted from unsaturated fatty acid, yielding alkyl radical that react at near-diffusion limited rates with molecular oxygen to give lipid hydroperoxyl radicals (Lambert and Elias, 2010). This latter form is, as mentioned above, associated mostly with cellular damage as a result of oxidative stress, and a great variety of aldehydes is formed when lipid hydroperoxides break down in biological systems, among them, MDA and HNE (Maritim et al., 2003, Janero, 1990).

In physiological state, at neutral pH, MDA is present as an enolate anion and is of low chemical reactivity. Nevertheless, this molecule is able to interact with nucleic acid bases to form several different adducts. The main product of this reaction is known to

be pyrimido-[1,2-α] purin-10(3H)-one deoxyribose (M1dG) which is in equilibrium with the open formN2-oxopropenyl-guanosine. M1dG are able to induce sequence-dependent frame shift mutations and base-pair substitutions (Rio et al., 2005, Sosa et al., 2006). On the other hand, an alternative mechanisms of genotoxicity is involving the ability of MDA to create interstrand cross-links in DNA which have potent biological effects. It is demonstrated that MDA also have the ability of MDA to generate DNA-protein cross links. All of these potentially genotoxic activities of MDA may lead to mutations and subsequently to cancer (Rio et al., 2005).

MDA toxicity also is directed toward cardiovascular disease. It is reported that MDA react with primary amines to form the Nε-(2-propenal)-lysine and generate lysine-lysine cross-links with 1-amino-3iminopropene and pyridyl-dihydropyridine type bridges. These reaction products have been detected in apoB fractions of oxidized lipoproteins (LDL) and are thought to be involved in the impaired interaction of the modified lipoproteins and macrophages. This phenomenon is the basis of atherogenicity (Rio et al., 2005).

Results of epidemiological studies in Asian countries have shown chronic green tea consumption to be significantly associated with reduced risks of cardiovascular disease due to its high content of polyphenolic flavonoids, mainly the catechins, that has been shown to reduce surrogate markers of atherosclerosis and lipid peroxidation, particularly LDL oxidation and malondialdehyde concentrations, in several in vitro, animal, and limited clinical studies (Basu et al., 2010, Basu and Lucas, 2007).

Green tea extract and tannin mixtures have been shown to scavenge nitric oxide and superoxides, and the flavan-3-ol linked to gallic acid is an important structural property that confers this activity. The effects of green tea catechins on copper-induced oxidation of low-density lipoprotein (LDL) have been investigated in several in vitro studies. The activity of green tea catechins against copper-induced oxidation of LDL is in the order of EGCG=ECG>EC=C>EGC. Moreover, green tea catechins have been shown to prolong lag time, inhibit formation of oxidized cholesterol, and decrease linoleic acid and arachidonic acid concentrations. Studies also show that green tea catechins reduce LDL oxidation, thiobarbituric acid reactive substances (TBARS) formation, cellular oxidation, and superoxide production (Chan et al., 2011).

The potent antioxidant activities of catechins in green tea are due to their three adjacent hydroxyl (OH) groups on the B-ring as in EGCG, GCG, EGC, and GC which are more effective in scavenging free radicals than the two adjacent OH groups as in ECG, CG, and EC (Chan et al., 2011).

Regarding lipid peroxidation, Basu et al (2010) suggested that green tea beverage lowered lipid peroxidation depicted from biomarker of oxidative stress such as MDA and HNE among obesity and metabolic syndrome patients (Basu et al., 2010). Sahin et al (2010) demonstrate that EGCG prevent lipid peroxidation and enhances antioxidant defense system via modulating hepatic nuclear transcription factors by inhibiting NF-κB expression and activating Nrf2 expression, which were activated and suppressed in the heat stress environment (Sahin et al., 2010).

Despite our results demonstrated that there is no significant difference between groups, if we see comparison between KI and P1 and P2 groups, our treatment is in line with our hyphotheses even it is not significant. And herewith we recommend to do further study with extension of duration of treatment and increase the dosage of recycling canola oil to determine its effect in lipid peroxidation.

These finding might contributes the evidence of effect of canola oil on the health and also green tea as antioxidant related to health is important to enhance the clinical management effort of clinician to provide adjuvant therapy on oxidative stress related diseases such as metabolic syndrome and influence the concept of researchers who are investigating effect of antioxidant for stress oxidative.

CONCLUSION

Regarding our experiments, we conclude that giving green tea to wistar which is fed by standard diet and supplemented by recycling canola oil does not influence on body weight, abdominal circumference and malondialdehyde levels in plasma.

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TABLES

Table 1. Level of trans fatty acid in recycling oil

Parameter	Result (% w/w)			
	Canola Oil	Corn Oil 1	Corn Oil 2	Rice Bran Oil
Elaidic Acid, C18:1n9t	0.25	0.11	0.08	0.19
Linolelaidic Acid, C18:2n9t	0.05	0.06	0.05	0.03
Total Trans Fatty Acid	0.30	0.17	0.13	0.22

Table 2. Body Weight after Treatment

Group	Body Weight in Mean ± SD (gram)	p Value *)
Kontrol 1	157 ± 31.11	
Kontrol 2	183.8 ± 12.05	
Kontrol 3	183.8 ± 10.64	0.310
Perlakuan 1	186.4 ± 17.57	
Perlakuan 2	174.25 ± 22.25	

*) One Way Anova

Table 3. Abdominal Circumference After Treatment

Group	Abdominal Circumference in Mean ± SD (cm)	p Value *)
Kontrol 1	16.85 ± 2.75	
Kontrol 2	16.12 ± 3.20	
Kontrol 3	17.16 ± 1.96	0.503
Perlakuan 1	15.22 ± 0.67	
Perlakuan 2	15.12 ± 0.17	

*) One Way Anova

Table 4. Plasma MDA Levels

Group	Plasma MDA Levels in Mean ± SD (nmol/ml)	p Value *)
Kontrol 1	34.9 ± 31.11	
Kontrol 2	14.8 ± 6.55	
Kontrol 3	31.2 ± 14.04	0.398
Perlakuan 1	23.0 ± 11.44	
Perlakuan 2	29.6 ± 25.85	

*) Kruskal Wallis

FIGURES

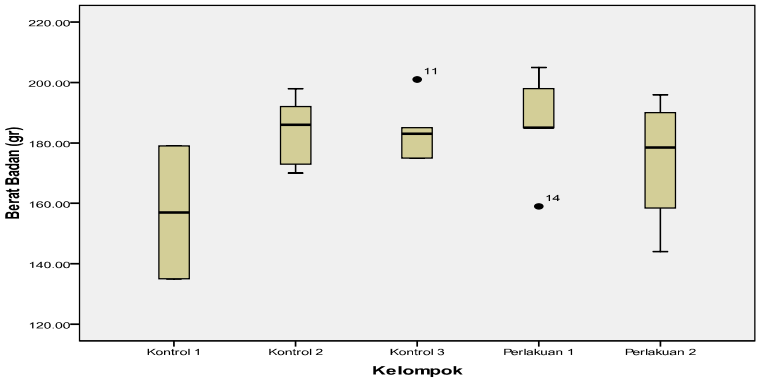

Figure 1. Boxplot of Body Weight After Treatment

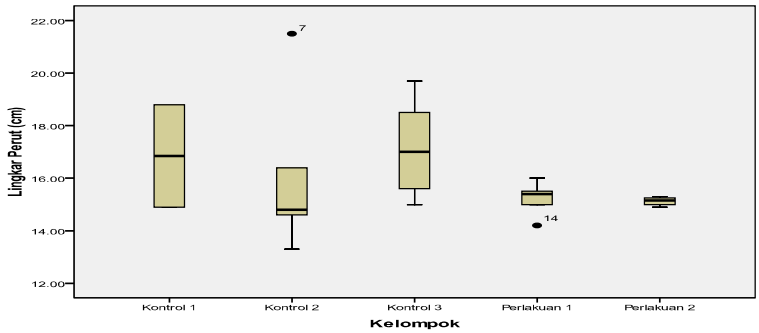

Figure 2. Boxplot of Abdominal Circumference After Treatment

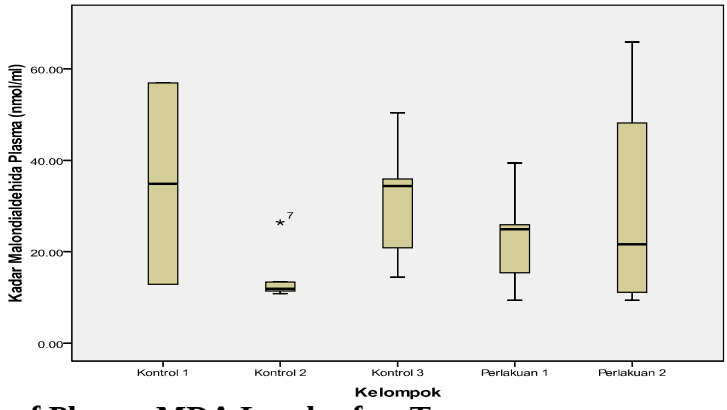

Figure 3. Boxplot of Plasma MDA Levels after Treatment

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