The Preventive Effect of Virgin Coconut Oil (VCO) with Lime Acidification (*Citrus aurantifolia*) on Total Cholesterol Levels and Expression of Hepatic TGF-β on Hypercholesterolemic Rats (*Rattus norvegicus*)

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ABSTRACT

Hypercholesterolemia is an excessive level of blood cholesterol condition. Fat and high cholesterol overconsumption cause atherosclerosis, coronary heart diseases, and liver diseases e.g. fatty liver and non-alcoholic steatohepatitis (NASH). One ingredient that can be used to prevent hypercholesterolemia is Virgin Coconut Oil (VCO) with lime acidification. This acidification is to increase lauric acid in VCO. The objective of this study is to determine the prevention effect of Virgin Coconut Oil (VCO) with lime acidification on the increase of serum total cholesterol levels, and the expression of hepatic TGF-β. The study was conducted with an experimental method using a complete randomized design on 20 white rats *Rattus norvegicus*) male Wistar strain aged 10-12 weeks and weight 200-250 grams as samples. Samples were divided into five treatment groups with four replications for each group, namely K- (negative control), K+ (positive control with hyper cholesterol feeding without VCO), P1 (1 ml VCO + 3.02 g hyper cholesterol feeding), P2 (1.5 ml VCO + 3.02 g hyper cholesterol feeding), and P3 (2 ml VCO + 3.02 g hyper cholesterol feeding). Data analysis of serum total cholesterol levels and the expression of hepatic TGF-β was performed by One Way Analysis of Variance (ANOVA) ($\alpha = 0.05$). The results showed that administration of VCO with lime acidification (*Citrus aurantifolia*) using three different dosages could prevent the increasing total cholesterol levels, but had not been proven to have a preventive effect on the increasing hepatic TGF-β expression on hypercholesterolemia white rats.

Keywords: Hypercholesterolemia, Total cholesterol, Hepatic TGF-β, VCO with lime acidification

1. INTRODUCTION

Hypercholesterolemia is one metabolic disorder that is indicated by an abnormal increase of blood fat protein and cholesterol. Hypercholesterolemia is the cause of many diseases in animals such as atherosclerosis, cerebral stroke, coronary heart disease, myocardial infarcts, renal failure, and fatty liver or non-alcoholic steatohepatitis (NASH) which in turn will cause hepatic cirrhosis [1]. Hypercholesterolemia is caused by overconsumption of high-fat feed, obesity, stress, hypertension, and low physical activities [2].

Hypercholesterolemia was reported in 32.8% of cases of 192 dogs in the United States [3]; while it was reported on 13% of cases on cats [4]. Hypertriglyceridemia and hypercholesteremia were reported on 88% of dogs and 78% of hypothyroid dogs [5]. Hypercholesterol became the risk factor of atherosclerosis, coronary heart disease, pancreatitis, diabetes mellitus, renal disease, and hepatic disorders. The factors causing hypercholesteremia include hereditary factors, low activity, and consumption of high-fat food [6].

Blood cholesterol is one of the initial factors of heart and liver diseases. Cholesterol is highly correlated to lipid metabolism and is the main component for steroids synthesis. Cholesterol is absorbed by the small intestine and circulated in kilomicron form. Kilomicron will detach the triglycerides bound in adipose tissue and bring cholesterol to the liver. The liver also produces cholesterol and some will be excreted to the gallbladder in the forms of cholic acid or kenideisicholat acid [7].

The liver, blood vessels, and heart are highly correlated to cholesterol. Low-density lipoprotein (LDL) can attach itself to blood vessel walls and cause atherosclerosis. Lipoprotein cholesterol with high density or high-density lipoprotein (HDL) acts as the transport of LDL from the bloodstream to the liver to be processed [8]. Consuming huge amounts of high fat and high cholesterol food may cause accumulation on hepatic stellate cells and in the long term may cause fatty liver disease. Fatty liver disease increased lipid peroxidation in the liver and may result in hepatic cells damage. This damage may be the cause of non-alcoholic steatohepatitis (NASH) which triggers inflammation in the liver and attracts inflammatory cells and cytokine migration such as TGF-β. The increase of hepatic TGF-β promotes regeneration of damaged hepatic cells. Massive hepatic cells damage will promote hepatic fibrosis which may cause hepatic cirrhosis [9].

Virgin coconut oil (VCO) had the potential to prevent the increase of blood cholesterol levels [10]. VCO is the derivate of pure coconut oil without heating or chemical process and consist of a small number of double chains saturated fatty acid [11]. VCO contains medium-chain fatty acid (MCFA) which consisted of 45-55% lauric acid and will be altered to monolaurin in the body. Monolaurin acts to improve the body's metabolic processes and fat digestibility [12].

VCO with lime (*Citrus aurantifolia*) acidifying is not different from other VCOs [13]. The citric acid in lime can accelerate the separation process of oil and coconut water in coconut milk on VCO making process [14]. Lime addition can also improve the lauric acid profile in VCO by 47.8% as per the Indonesian standard (45.1% - 53.2%), while VCO made without lime addition only contains less than 45.1% lauric acid [15]. VCO production with lime addition can be a cheap and easy-to-do alternative.

Further research is needed to analyze preventive properties of VCO with lime (*Citrus aurantifolia*) acidification on total cholesterol increase and hepatic TGF- β expression in rats (*Rattus norvegicus*) fed with hyper cholesterol diet.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this research were cage, water bottle, digital scale, coconut shredder, lime juicer, tissue, clean cloth, syringe and needle, gut probe, vacutainer, Eppendorf tube, tube rack, centrifuge ABX Pentra C200, alcohol swabs, organ pot, object-glass, cover glass, microtome, water bath, and microscope.

This research also used male Wistar rats weighing 200-250 g, 15 dried coconuts, 5 limes, rice husk, boiled quail egg yolk, pork fat, cholic acid, aqua dest, alcohol, 10% formalin, paraffin, rat anti-TGF-β antibody, diaminobenzidine chromogen (DAB), and CHOD-PAP reagent.

2.2. VCO Production with Lime (*Citrus aurantifolia*) Acidification

The preparations were made by shredding coconut by using the shredder and thick coconut milk was extracted. The coconut milk was then mixed with 1% lime juice using a mixer. The mixture was then kept for one day until it developed three layers: *blondo* (coconut oil dregs), coconut oil, and water. The oil was then moved to a closed sterile container using a 60 ml syringe. The oil was then kept at 23ºC temperature before further use.

2.3. Making of Hypocholesterol Feed

Hyperchlesterol feed was made from freshly boiled quail egg yolk (5%), pork fat (10%), and cholic acid (0.1%) [16]. Aforementioned components were mixed and diluted in aqua dest until 2 ml. The hypercholesterol feed was given to K+ (positive control), P1, P2, and P3 rats, while K- (negative control) rats were given standard feed.

2.4. Giving Hypercholesterol Feed and VCO

The hypercholesterol feed was given for 14 days starting from day-8 by force-feeding 3.02 g feed in 2 ml aqua dest using the gut probe. Rats were fed with standard feed and water. The treatment P1 was given VCO dose 1 (0.6615 g/200 g weight equivalent with 1 ml VCO) 2 hours before hyper cholesterol feed. Treatment P2 was given dose 2 (1.5 ml VCO), and treatment P3 was given dose 3 (2 ml VCO), two hours before hypercholesterol feed. VCO was not given to K+ and K- treatments.

2.5. Rats Euthanasia

Rat euthanasia was done before intracardiac blood sampling. The euthanasia was done by cervical dislocation of the rats. This can only be done to rats

weighed less than 200 g. The cervical dislocation was done by methods used in previous research [17].

2.6. Serum sampling and total cholesterol analysis

The blood samples were taken from each treatment. The blood was obtained from orbital veins and intracardiac. The blood was then transferred to a vacutainer without EDTA and kept until serum was separated from blood cells, then centrifuged at 5000 rpm for 15 minutes. The serum was then transferred to Eppendorf tubes and kept at -20℃ freezer for storage [18]. Total blood cholesterol analysis was done by the standard operating procedure by The Clinical Pathology Laboratory of Faculty of Medicine, Brawijaya University. Before analysis, the serum was thawed, then placed in ABX Pentra C200 tube rack, and put CHOD-PAP reagent. The machine was set to analyze total blood cholesterol. The machine then ran the analysis for 30-45 minutes for 20 samples.

2.7. Liver histopathology specimen

The liver that was kept in 10% formaldehyde was then cut by 2x1x0.5 cm then washed by aqua dest. The liver cube was then put in a tissue cassette for fixation by 10% formaldehyde. The sample was then dehydrated by putting it in increasing alcohol concentration (70%, 80%, 90%, 95%, and 100%). After the dehydration process, the clearing process was done by putting the sample in xylol I, II, and III. The samples were then put in liquid paraffin I and II for the infiltration process. The embedding process was then done by planting the sample in paraffin block and putting liquid paraffin in it and kept until hardened. The hardened paraffin was then cut using microtome in the sectioning process. Each cut was then put in a 40℃ water bath and picked up by using object-glass with Ewit solution [19].

2.8. Immunohistochemistry specimen making and TGF-β expression examination

Immunohistochemistry coloring was done by putting the samples in xylol, then rehydrating by putting it in decreasing concentrations of alcohol (100%, 95%, 90%, 80%, 70%) for 5 minutes each, then put it in sterile aqua dest for 5 minutes for 3 times. The object glass was submerged in 3% hydrogen peroxide solution (bocking solution) at room temperature for 20 minutes. The samples were then incubated in blocking buffer for 60 minutes, and then primer monoclonal antibody which was diluted in blocking buffer was dripped in 4oC temperature and kept overnight. The samples were then put at room temperature and washed with phosphate buffer saline (PBS) for 5 minutes 3 times. The samples were then incubated with secondary antibody for 60 minutes and washed by PBS solution for 5 minutes 3 times. The samples were then incubated with peroxidase for 40 minutes at room temperature and then washed with PBS for 5 minutes 3 times. Diaminobenzidine (DAB) and substrate solution were then dripped and incubated for 10 minutes, then washed with PBS for 5 minutes 3 times. Counterstaining was then done by adding hematoxylin for 5 minutes at room temperature then washed by running water, dried, and mounted in object glass. The object glass was given entellan mounting medium beforehand then covered by coverslip [20]. Immunohistochemistry coloring was done to examine TGF-β expression on a microscope. The expression was determined by examining five areas and evaluating the mean percentage of those areas. TGF-β expression was counted by ImageJ software using plug-in Immunoratio [21].

2.9. Data Analysis

The data obtained were analyzed using one-way ANOVA by SPSS software and continued using the 5% Turkey test to determine significant differences.

3. RESULT AND DISCUSSION

3.1. Preventive effect of VCO with lime acidification on total cholesterol level of hypercholesterolemic rat

Oneway ANOVA and 5% Turkey test analysis showed a significant difference in total cholesterol serum value between control (K- and K+) and treatment (P1, P2, P3) groups. A significant difference was shown between K+ and K- and treatment groups. There was no difference between K- and treatments groups. The results showed that rats given VCO with lime acidification were more adapted to hypercholesterol feed, which resulted in lower total serum cholesterol levels.

Table 1. Total cholesterol, TGF-β expression and their respected decrease value on K+

Treatment	Total Cholesterol	Decrease value on K+ (%)	TGF-β (% area)	Decrease value on K+ (%)
K-	55.50±18.30[a]	-	18.48±4.71[a]	-
K+	127.50±15.33[b]	-	26.31±1.16[b]	-
P1	79.75±14.43[a]	37.45	21.90±2.06[ab]	16.76
P2	68.75±14.93[a]	46.08	19.92±3.17[ab]	24.29
P3	83.50±5.92[a]	34.51	22.18±2.53[ab]	15.70

Note: different superscript shows the significant difference by 5% Turkey Test

Blood cholesterol level in K- group showed in the normal range, which s between 40-130 mgdl-1 [22]. This indicated the normal feed given on K- group without cholesterol addition. The treatment groups (P1, P2, and P3) showed significantly lower cholesterol levels than the K+ group. Treatment P2 showed the lowest (46.08%) total cholesterol decrease compared to K+, but there was no difference between treatments. Treatments of VCO with lime acidification gave a similar preventive effect on serum total cholesterol on any level.

Medium-chain triglycerides (MCT) contents in VCO

metabolized. MCT is not included in the cholesterol cycle thus is not deposited in the body or adipose tissue [15]. Lauric acid has the properties to metabolize fat in any body tissue to energy and increase the body's metabolic activities [10].

The lauric acid content in lime-acidification VCO could decrease total cholesterol level, especially LDL, and increase HDL level. The HDL main role is to bind cholesterol in perifer tissue and blood vessels and transport it to the liver to be excreted to the gallbladder, thus decreasing the blood cholesterol level [10]. Exceed dose of VCO addition could not prevent the increase of
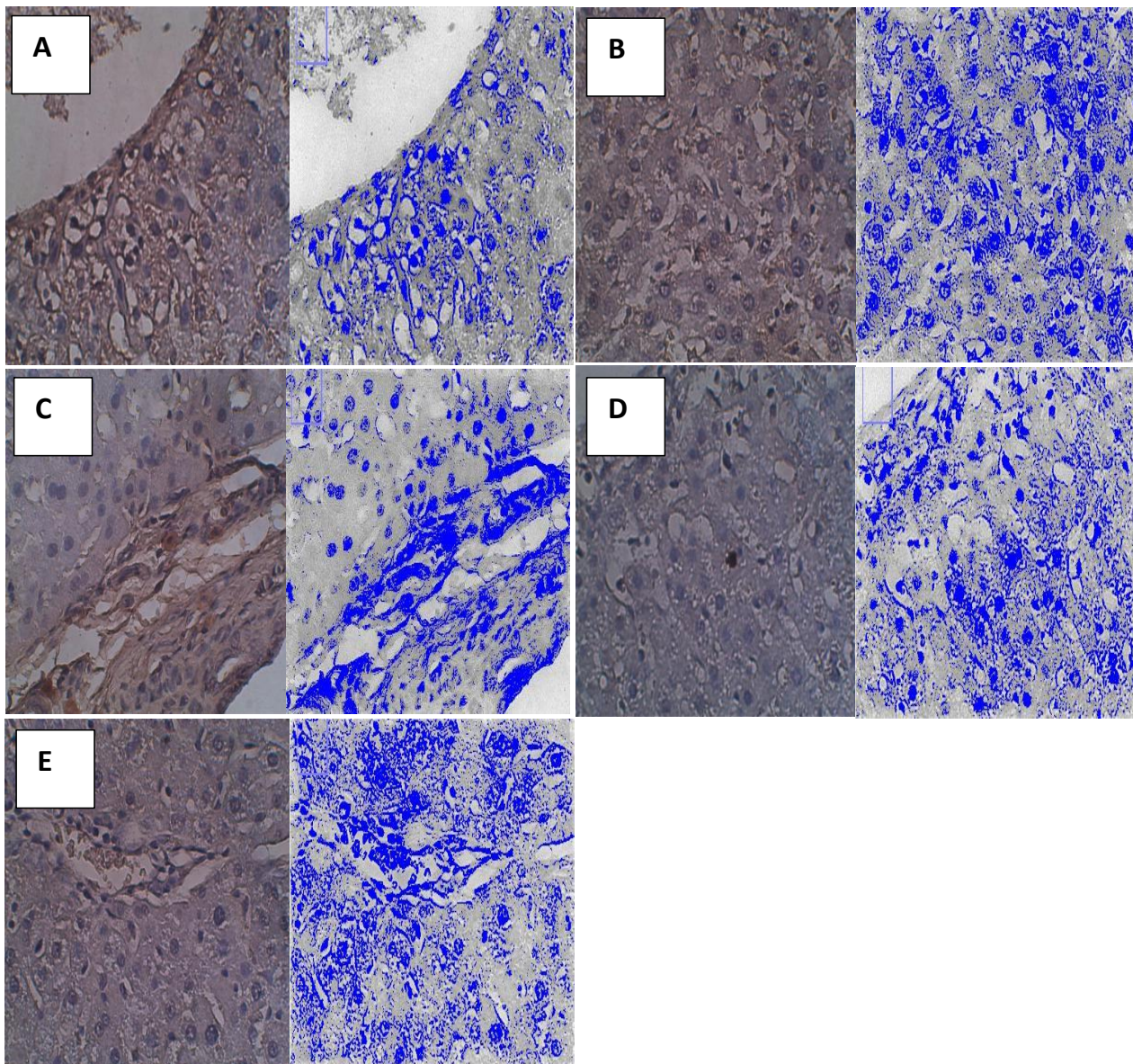

Figure 1. TGF-β expression shown on each group: A) K- group, B) K+ group, C) P1 group, D) P2 group, E) P3 group. Blue area showed TGF-β expression area (immunohistochemistry coloring, 400 X magnifications)

are dominated by lauric acid. MCT is more soluble in water than long-chain triglycerides (LCT) thus MCT can readily be absorbed by hepatic cells via portal veins after absorption by the small intestine and can be rapidly

serum cholesterol level because the saturated fatty acid content in VCO might increase the serum LDL and fat [23].

Table 2. Mean of the percentage of TGF-β expression on white rat liver

Group	TGF-β expression (% area) mean ± DS	Reduction on K+ (%)
K-	18.48±4.71[a]	-
K+	26.31±1.16[b]	-
P1	21.90±2.06[ab]	16.76
P2	19.92±3.17[ab]	24.29
P3	22.18±2.53[ab]	15.70

Note: different superscripts indicated significant differences between treatments (P<0.05). Reduction of TGF-β expression based on positive control group

3.2. Preventive effect of lime-acidification VCO on hypercholesterolemic white rat's hepatic TGF-β

Expression of hepatic TGF-β on hypercholesterolemic male rat marked by brown color on the histopathological view of hepatic tissue under the microscope with immunohistochemistry on TGF-β antibody coloring. The brown color was a result of the reaction between TGF-β antigene in tissue and primary antibody (anti-rat TGF-β) which created a complex bond of antigen-antibody called Streptavidin Conjugated Horse Radish Peroxidase (SA-HRP). SA-HRP bound to secondary antibody added with diaminobenzidine (DAB) substrate [21]. TGF-β expression of hepatic tissue of treatments is shown in figure 1.

Statistical analysis showed significant differences 1n TGF-β expression between control groups (K+ and K-) and treatment groups (P1, P2, P3). A significant difference was detected between K- and K+ groups. Percentages of TGF-β expression area in control groups were significantly different from treatment groups. Lime-acidification VCO could not prevent the increase of TGF-β expression on the hepatic tissue of hyper cholesterol feed-induced rats.

K- Group showed few expressions of TGF-β, this was caused by normal secretion of Kupffer cells, hepatic cells, and stellar cells in normal hepatic tissue. The TGF-β expression will increase if inflammation occurs in hepatic tissue. TGF-β acts in the building of hepatic fibrosis [24]. Mean TGF-β expression area on treatment groups insignificantly decreased compared to K+ groups. Lime-acidification VCO given on any dose could not prevent the increase of TGF-β expression on hypercholesterolemic rats. This might be caused by LCT contents in VCO such as myristate acid (C14) and palmitate acid (C16) with ±16% and ±9% content, respectively [25]. Long-chain fatty acid (LCFA) especially myristate acid and palmitate acid on certain doses could increase lipid peroxidation activities in hepatic tissue and decrease superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activities which functioned in protecting hepatocytes from lipotoxicity effects. This resulted in increased cell destruction risks which triggered inflammation reaction, followed by increasing anti inflammation cytokines.

MCT content in VCO is dominated by lauric acid which is easy to digest and convert into energy. The energy produced then increased metabolic processes in the body including fat metabolism. The increase of metabolic activities might prevent fat and cholesterol accumulation in hepatic cells thus in return decreasing hepatic cell destruction [10].

4. CONCLUSION

Lime-acidification virgin coconut oil given to white rats fed hypercholesterol feed can prevent the increase of total blood serum cholesterol, but is not able to prevent the increase of hepatic TGF-β expression.

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