Fatty Liver and Liver Malondialdehyde Expression in Severe Malnourished Wistar Rats that given Virgin Coconut oil Compared to Corn Oil in World Health Organization Formula

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Abstract

Objective: There is a lack of antioxidant in severe malnutrition, and it may lead to increase oxidative stress. Fatty liver is one of severe malnutrition (SM) cardinal features. Virgin coconut oil (VCO) contains high antioxidant capacity and medium chain triglyceride. It is supposed to improve those problems. Methods: Posttest only control group design study was done to observe whether VCO could decrease fatty liver and liver malondialdehyde (MDA) expression in 38 male wistar rat with SM. The rats were divided into two group which were VCO Group (Group A) and Control Group (corn oil (Group B)). They were given feeding World Health Organization (WHO) Formula for SM (Formula 75 and Formula 100) that contain VCO or corn oil. After being SM, this formula was fed until 28 days. In the 29th day, they were sacrificed, and liver samples were obtained for fatty liver analysis and MDA expression. Results: Fatty liver was less in Group A (mean 13.74 cell in 5 view field (SD 1.32) than Group B (mean 20.74 cell in 5 view field (SD 2.01), and it was statistically significant (p=0.000). Cut off point for determining low and high liver MDA expression based on o ROC curve were <2.9% (low) and ≥2.9% (high). Low MDA expression (<2.9%) was higher in Group A than Group B (p <0.05). Conclusions: This study found less fatty liver and low liver MDA expression in severe malnourished rat given VCO than corn oil.

Keywords: Virgin coconut oil, Fatty liver, Malondialdehyde, Severe malnutrition.

Introduction

About 20 million children worldwide suffer from severe malnutrition (SM). The mortality rate of SM children is 5-20 times higher than children with well-nourished.1 Extensive fatty liver is one of the SM cardinal features. This is probably due to dysfunction of peroxisome β-oxidation. The initial reaction in peroxisome system will produce radical hydrogen peroxide while antioxidant levels are reduced in SM.2 In kwashiorkor, concentrations of antioxidant reduce.3 Total liver glutathione is decreased to 65% in rat with protein malnutrition compared to rat without protein malnutrition.4 Glutathione depletion may increase liver lipid peroxidation and impaired liver capacity to inactivate reactive oxygen species (ROS).5 Nutrition treatment for SM uses World Health Organization Formula, which are Formula 75 (F75) and Formula 100 (F100).
One of the compositions of F75 and F100 is vegetable oil. Virgin coconut oil (VCO) is one of vegetable oil that contains rich of medium chain fatty acids (MCFAs) (64%), which lauric fatty acid (C12) is the highest (47-53%) one. Linoleic acid is low in VCO (0.90 to 1.72%). Linolenic acid in VCO is ranged from undetectable to 0.2%. Antioxidant activity of VCO is 52-80% when compared to controls tocopherol and BHA.

On SM condition, there are reducing antioxidant levels, increasing free radicals and fatty liver. It suggested VCO that contain a lot of MCFAs, less preformed of inflammatory mediators, and rich of an antioxidant could improve those problems. This study aimed to prove the effect of VCO in SM treatment on fatty liver and liver MDA expression.

Materials and Methods
This was an experimental study using posttest only control group design to observe whether VCO could decrease fatty liver and liver MDA expression in male wistar rat with SM. This study had approved by Research Ethical Committee, Medical Faculty of Udayana University/Sanglah Center General Hospital Denpasar, Bali, Indonesia. We used 38 wistar rats with four-week old age. They were adapted for one week, then treated becoming severe malnutrition by fed low protein diet (5% protein) ad libitum for three weeks.

The low protein diet consisted of 61.5% β cornstarch, 5% mild casein, 10% α potato starch, 8% cellulose powder, 6% soybean oil, 3.5% mineral, 5% sugar, 1% multivitamin. After they become severely malnourished, they were divided into two groups randomly. The first group was treated with F75 and F100 (contain VCO), and the second group treated with F75 and F100 (contain corn oil) for 28 days.

The composition of F75 is skim milk powder 25 g, sugar 100 g, vegetable oil 30 g and 20 ml electrolyte solution, that contain 750 kcal. The composition of F100 is skim milk powder 85 g, sugar 50 g, vegetables oil 60 g and 20 ml electrolyte solution, that contains 1000 kcal. Formula 75 was 416.7 kcalories/kg body weight/day (kcal/kgbw/d) in the first day. They were fed F100 with doses 416.7 kcal/kgbw/d on the second day. In the following days, F100 dose were increased 41.7 kcal/kgbw/d gradually until 916.7 kcal/kgbw/d. After doses achieving 916.7 kcal/kgbw/d, this dose was maintained until 28 days from the first-day treatment.

In the first five days of treatment, all rats were given prophylaxis antibiotic (cefixime 33.3 mg/kgbw/d, divided by two). In the 29th day of treatment, the rats were sacrificed by using ketamine hydrochloride 40 mg/kg dan xylazine 2.5 mg/kg intravenous.

Livers were taken and washed by using NaCl 0.9% and fixation with buffer formalin. Liver samples were processed for staining with hematoxylin and eosin to determine the fatty liver. There are 3 steps to stain the prepared, which are fixation, dehydration, clearing, and embedding.

For fixation, liver tissues were soaked in phosphate buffer formalin 10% for 24 hours, trimming part of the liver tissue, and dehydrated with soaked in 50%, 70%, 90%, 96%, and 100% alcohol respectively for 2 hours every cycle. The next step was soaking the prepared to clearing agent (xylene) for 24 hours until transparent.

Embedding step was started with infiltration 2 times for 1-hour every step with pure liquid paraffin (Histoplast) (60°C), then that tissues were put in liquid paraffin and stayed it there to build block (about one hour). After that blocks were cut using microtome rotary (Jung Histocut Leica 820), 5 µm thickness, and then were plated at object glass and incubated at 60°C for 2 hours. Before it was stained, it was through deparaffinization and rehydration process include soaking in xylene 2x5 minutes, ethanol 100% for 2 minutes, ethanol 96% 2x2 minutes, ethanol 70% for 2 minutes and aquadest for 2 minutes.

Staining was done with Hematoxylin Gill for 5 minutes. The further step was to soak with tap water for 5 minutes, soak with Eosin 1% for 15 seconds, dehydrated in ethanol 70% for 10 seconds, ethanol 96% 2x10 seconds, ethanol 100% for 10 seconds and xylene 2x2 minutes, dry it for 2 hours at room temperature, then mounting in xylene based medium. The slides were analyzed using
digital analysis method, 400 times magnified using Olympus microscope (Japan), captured with Optilab Pro (Miconos, Indonesia). Every prepared was captured 3 times (using JPEG format) using Optilab Viewer 1.0 software (Miconos, Indonesia). Fatty liver was counted based on a percentage of cell that infiltrates with fat in 5 fields with 400 times magnified.

For liver MDA immunohistochemistry examination, paraffin-embedded serial sections of liver were deparaffinized with xylol (2x5 minutes), rehydrated with ethanol (100%, 95%, 90%, 80%, 70% respectively for each in 5 minutes), then wash with Phosphate Buffer Saline (PBS) for 3 x 2 minutes in pH 7.4. Reaction with citrate buffer was done for 20 minutes in 95°C, soaked in 3% H2O2 for 20 at room temperature, and washed with PBS 3 x 2 minutes.

The next step was incubation prepared with antibody Santa Cruz anti-MDA antibody 1:100 for 18 hours in 4°C, then washed with PBS pH 7.4 for 3x5 minutes. Secondary antibody with biotin labeled (Anti rabbit Ig G-Biotin Labelled) was added and incubated for 30 minutes at room temperature, washed with PBS pH 7.4 for 3x5 minutes, added Streptavidin-Horseradish Peroxidase (SA-HRP) for 20 minutes at room temperature, washed with PBS pH 7.4 for 3x5 minutes, added DAB (Diamono Benzidine) for 5 minutes at room temperature, washed with aqua-bidest for 3x5 minutes.

Counter staining with Mayer Hematoxilene was done for 2 minutes, washed with water flow, dried by wind flow, then mounting and covered with a coverslip. A cell that stained by MDA immunohistochemistry was counted in 400 times magnified microscope by using the formula: % cell MDA stained = number of cells that MDA stained/number of whole cell x 100%.

Chi-square test was used to analyze differences in expression of liver MDA (high and low) between Group A and Group B with α = 0.05, two-tailed test. Independent t-test (unpaired t-test) was used to analyze differences between mean fatty liver between Group A and Group B with α = 0.05, two-tailed test.

Results

After 3 weeks fed by low protein diet, the rats became severe malnutrition based on their age. The rats became thin, atrophy of muscles, sparsing hair, and edema similar with kwashiorkor in a human being. At this beginning of the study, characteristic of samples were similar.

One rat had died on the 27th day of treatment (rat from Group B). On the 29th day after being given F75 and F100 with VCO or corn oil content, 37 wistar rat samples had been euthanasia. Liver organs were obtained from both group showing no significant weight different; the median was 5 g (range for Group A = 3.6-7.7 g, Group B = 3.9-7.2 with p value = 0.662).

Normality test was conducted to fatty liver data. Shapiro-Wilk values were 0.54 (Group A) and 0.84 (Group B). Data distribution was normal. It was analyzed by using independent t test. Fatty liver was lower in Group A (mean 13.74 cell in 5 view field (SD 1.32) than Group B (mean 20.74 cell in 5 view field (SD 2.01)) and significantly different (p=0.000).

Characteristic rat tissue histology examination for examining intracellular lipid quantitative on centrilobular liver tissue region (intracellular lipid examination was done on five view field) with HE staining using 400x magnification, showing in Figure A and Figure B. Fatty liver in Group A was less than Group B.

Cut off point for determining low and high liver MDA expression based on ROC curve were <2.9% (low) and ≥2.9% (high). Chi-square test was conducted due to find out whether there were differences between Group A and B in low and high MDA expression. Observed variable values less than five were more than 50%, then we used Fischer Exact test for analysis. Low MDA expression (<2.9%) was higher in Group A than Group B (p <0.05) (Table 1).

Immunohistochemistry examination of rat liver MDA in Group A and Group B can be seen in Figure C below. This examination was performed with 400x magnification microscope. In this examination found that percentage of cell damage (visible colored brownish) less in group A than group B.
Group A rat liver histology for quantitative examination of intracellular lipids on centrilobular liver tissue region (intracellular lipid examination that was carried out at five view fields) with HE staining using 400x magnification. In this examination, fatty liver was obtained by an average 13.74 cells in five visual fields (SD 1.32). Figure A1: 100x magnification, Figure A2: 400x magnification, arrows indicate cells with fatty liver.

Group B rat liver histology for quantitative examination of intracellular lipids on centrilobular liver tissue region (intracellular lipid examination that was carried out at five view fields) with HE staining using 400x magnification. In this examination, fatty liver was obtained by an average 20.74 cells in five visual fields (SD 2.01). Figure B1: 100x magnification, Figure B2: 400x magnification, arrows indicate cells with fatty liver.

<p>| Table 1. Effect of using VCO and Corn Oil on SM rat to Liver MDA expression |
|-----------------------------------------------|----------|----------|----------|</p>
<table>
<thead>
<tr>
<th>Liver MDA expression</th>
<th>X²</th>
<th>P</th>
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<tbody>
<tr>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Group A (VCO)</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Group B (corn oil)</td>
<td>14</td>
<td>4</td>
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**Discussions**

The pathogenesis of fat accumulation in the liver is still controversial in SM. Several hypotheses are predicted as the etiology of this condition. It is most likely due to a decrease in the liver fat breakdown, as the result of β-oxidation dysfunction. Beta-oxidation mainly occurs in mitochondria, and 10% occurs in the peroxisome. Mitochondrial function in SM is still sufficient. It indicates the possibility of peroxisome dysfunction as the etiology of liver fat accumulation. Peroxisome shortens long-chain fatty acids which are then transported to the mitochondria for further oxidation processes. Fat accumulation tends to occur in the longer chains.²
Immunohistochemistry on rat liver MDA Group A and Group B. lipid peroxidation was in coloration that was indicated by arrows. Fatty liver on low-protein diets can also be caused by decreased expression of genes that encode the formation of proteins involved in fatty acid oxidation. The carnitine palmitoyl transferase (CPT) 1α mRNA and CPT 2 mRNA levels decreased significantly in liver from rats that received low-protein diets without MCT compared to rats that received low-protein diets with MCT.

Medium chain fatty acids (MCFAs) may cross the mitochondrial membrane for oxidation through a carnitine-independent mechanism. Accumulation of liver triglycerides can be reduced by MCT administration in mice receiving low-protein diets.10

Medium chain triglyceride (MCT) that replaces polyunsaturated oil in the diet of non-alcoholic fatty liver disease (NAFLD) rat model that consume 70% fat can protect liver pathology. An increase in the ratio of MCT to corn oil reduces concentration of 18:2 and 20:4 liver fatty acids, reduces membrane sensitivity to free radical attack, stimulates β- and ω fatty acid oxidation as the result of peroxisomal proliferator activated receptor (PPAR) α activation, and appears to increase mitochondrial respiration through complex III.11

Virgin coconut oil (VCO) supplementation may also reduce the activity of enzymes involved in lipogenesis (acyl CoA carboxylase and fatty acid synthase (FAS)) and increase mitochondrial oxidation and peroxisome-β oxidation of fatty acids.12 In this study, similar results were also obtained. Fatty liver in rats receiving F75 and F100 with VCO content is less than corn oil. Medium chain triglycerides of VCO can cross the mitochondrial membrane for oxidation through a carnitine-independent mechanism. Accumulation of liver triglycerides can be reduced. Corn oil contains long-chain fatty acids; it makes difficult in fatty acid oxidation under SM conditions where the peroxisome is impaired, then fatty liver becomes more prominence.

Low levels of antioxidants and increasing oxidative stress occurs in SM. Malondialdehyde (MDA) is a product from lipid peroxidase. Increased MDA level suggests increasing lipid peroxidase. Lipid peroxidase leads to fluidity and membrane integrity loss. If this occurs in mitochondria, it will reduce the efficiency of electron transportation for producing ATP, so this will aggravate SM condition. Antioxidant defense mechanism failure is important factor in severe malnutrition pathogenesis.13

Virgin coconut oil can lower MDA level, increase antioxidants level and lower 5-hydroxytryptamine level in the brain of rats that had been given stress challenge.14 If compared to corn oil, the level of oxidation (MDA concentration) in the plasma of rats given VCO is lower than corn oil. This indicated that corn oil increased oxidation rate. Corn oil is mostly composed of unsaturated fatty acids, that is 56% linoleic acid and 30% oleic acid, which is more easily oxidized compared to saturated fatty acids.

Malondialdehyde formation reaction begins with the formation of lipid radicals caused by free radicals from the bonds of unsaturated fatty acids.15 Virgin coconut oil (VCO) supplementation could reduce oxidative stress by decreasing peroxide and MDA level.16 In this study, we also observed low MDA expression in Group VCO was higher than Group Corn Oil. This was corresponding with prior studies above. It could be because of lower oxidation rate of VCO compared to corn oil, or it could be because of VCO can decrease fatty liver, so the oxidative stress in
VCO group become less than Corn Oil Group. These hypotheses need to be explored further. Capacity to revenge oxidative stress in SM can be improved by giving VCO to SM rat.

Conclusions
This study found less fatty liver and low liver MDA expression in severe malnourished rat given VCO than corn oil.

References