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Purple Corn Anthocyanin Extract Improves Oxidative Stress of Rats Fed High Fat Diet via Superoxide Dismutase Mechanism

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Abstract: ***The objective of this study was to investigate the ability of Purple Corn Anthocyanin Extract (PCAE) in improving oxidative stress of rats fed High Fat Diet (HFD) as well as possible mechanism involved. Twenty-four Wistar rats were divided into 6 groups. Five groups were given HFD for 4 weeks to induced oxidative stress and added the following injection orally for 6 weeks later: pioglitazone 1.35 mg/kg body weight (bw) (positive control group, PC), PCAE at dose 6.25, 12.5, and 25 mg cyanidin-3-glucoside (CGE)/kg bw (LA, MA, and HA groups, respectively), and nothing (negative control group, NC). Normal control group (NLC) was given AIN93M for along 10 weeks. Plasma malondialdehyde (MDA), total antioxidant capacity of plasma and liver, body weight, liver weight, plasma superoxide dismutase (SOD), liver histopathology were measured, while lipid peroxidation and liver index were calculated. High fat diet increased plasma and liver malondialdehyde (MDA), lipid peroxidation index of plasma and liver, liver weight, liver index, and liver mitochondria SOD, while reduced total antioxidant capacity of plasma and liver, and liver SOD activity. Supplementation of PCAE improved oxidation stress by reducing plasma and liver MDA, lipid peroxidation index in plasma and liver, liver weight, liver index, and liver mitochondria SOD, moreover increasing plasma and liver total antioxidant capacity and liver SOD activity. The best dose of PCAE supplementation to improve oxidative stress induced by HFD was 12.5 mg CGE/kg bw as well as pioglitazone 1.35 mg/kg bw. PCAE could improve oxidative stress induced by HFD via SOD mechanism.***

Keywords: anthocyanin, high fat diet, oxidative stress, purple corn, superoxide dismutase

1. Introduction

Fat plays an important role in the diet. Fat plays a role in the texture, flavor, and aroma of various types of food. In general, good food is one that has high fat content because the taste, aroma, mouthfeel and hedonic properties of fat contribute to taste. However, fat is also a high energy source [1]. The incidence of cardiovascular disease is correlated with diets high in saturated fatty acids (SFA). Animal fats, which contain higher proportions of SFA, increase the risk of vascular system diseases [2].

Report from Mokdad et al. stated that a high-fat diet accompanied by lack of physical activity was the second leading cause of death in the United States in 2000 [3]. A high-fat diet causes oxidative stress [4-7]. Even the research of Tsai et al. showed that consumption of high-fat foods in healthy people within two hours can increase blood triglyceride levels and oxidative stress as indicated by a decrease in antioxidant enzymes and an increase in oxidation products [8].

By observing some of the negative effects of a high-fat diet, the United States Department of Agriculture (USDA) made the Dietary Guideline fat consumption for adults is 20-35% with saturated fat a maximum of 10% of total calories. A person is categorized to consume high fat if in the diet contains more than 30% of calories from fat [9].

Fatty acids directly influence the increase in reactive oxygen species (ROS) through peroxidation reactions and through production in mitochondria [10]. Research Ming et al. concluded that high-fat diets cause decreased enzymatic antioxidant activity (SOD, GSH-Px, and CAT), decreased levels of non-enzymatic antioxidants (GSH, vitamin C, and vitamin E), and increased lipid peroxidation (levels TBARS) which indicates an oxidative stress condition [11]. Oxidative stress is involved in several diseases, i.e. cardiovascular diseases [CVDs], diabetes mellitus, chronic obstructive pulmonary disease, chronic kidney disease, neuro-degenerative diseases, cancer, sarcopenia and frailty [12].

Antioxidants are defined as substances which, if they are at lower levels than the oxidized component, will slow down or prevent oxidative damage caused by ROS. ROS at high levels will be detrimental because it is pathophysiological, whereas at low levels it is beneficial for normal physiological work. Human antioxidant defense systems include endogenous antioxidants (both enzymatic and non-enzymatic) and exogenous antioxidants derived from the diet [13]. The primary antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). The non-enzymatic antioxidants are molecules that interact with ROS and terminate the free radical chain reactions, i.e. bilirubin, α-tocopherol (vitamin

E), and β-carotene. Exogenous antioxidants include vitamin C, vitamin E, and phenolic antioxidants (including phenolic acids and flavonoids), etc. [12]. Anthocyanin is a flavonoid compound, which is a widely distributed plant polyphenol.

Anthocyanins are responsible for the colors of red, purple, and blue in fruits and vegetables. Anthocyanins sources include berries, currants, grapes, and some tropical fruits, also red to purplish blue-colored leafy vegetables, grains, roots, and tubers. Purple corn is one of the anthocyanins sources in grains [14]. Some researchers found that purple corn anthocyanin could improve oxidative stress in isolated mouse organ [15] in diet-induced obese mice [16], and had an antioxidant activity of plasma in sheep [17].

The objective of this study was to investigate the ability of Anthocyanin Extract of Purple Corn (AEPC) in improving oxidative stress of rats fed High Fat Diet (HFD) as well as possible mechanism involved, i.e. SOD pathway.

2. Materials and Methods

2.1 Purple Corn Anthocyanin Extract (PCAE) Preparation

Purple corn (Zea mays) seed was cultivated and cropped at 3 month-old, and subsequently sun-dried (before 12 pm) to a water content of ca. 12%. Furthermore, it was ground and sieved using a 60-mesh size screen. The unsieved part powder was then extracted using ethanol-citric acid 3% (1:10, m/v), mascerated using magnetic stirrer at 1000 rpm for 3 hours at ambient temperature. The extract was then centrifuged at 3000 rpm at ambient temperature for 20 minutes. The supernatant was evaporated at 40^{o}C with rotary evaporator Buchi R-3000 (Buchi Labortechnic AG, Flawil, Switzerland) for 4-5 hours. The thick extract was sealed and stored in dark condition at -10^{o}C until use and called as Purple Corn Anthocyanin Extract (PCAE).

The characteristic of anthocyanin extract of purple corn was 2.55 mg cyanidin-3-glucoside equivalent (CGE)/ ml and 11.2 µmol Trolox Equivalent (TE)/ ml of total antioxidant capacity. Cyanidin-3-glucoside and peonidin-3-glucoside, which were anthocyanin components in PCAE, were 27.8 ppm and 10.4 ppm, respectively (data not published).

2.2 Animals

Twenty-four male Wistar rats age 2 months were maintained at 21-25^{o}C and 50-60% of humidity and kept on a 12 h light-12 h dark cycle with free access to food and water. The research protocol was approved by The Committee of Ethical Clearance for Pre-clinical Research of The Integrated Laboratory of Research and Testing, Gadjah Mada University, Yogyakarta, Indonesia Ref: 441a/KEC-LPPT/III/2016.

2.3 Oxidative stress induction by high fat diet (HFD)

After three days of adaptation, the rats were randomly divided into six groups. One group (n=4) were fed normal diet (AIN93M), whereas five groups (n=4 per group) were fed high fat diet (HFD) for 4 weeks. High fat diet contained carbohydrate, fat, and protein i.e. 52.4, 35.4, and 12.2 % of total energy, respectively (18).

2.4 Treatment protocols

Twenty-four male Wistar rats were randomly divided into six groups, i.e.:

1) NLC was a normal control group; rats were given AIN93M and distilled water per oral (po) for along 10 week-treatments
2) NC was a negative control group; rats were given HFD and distilled water po for along 10 week-treatments
3) PC was a positive control group; rats were given HFD for 10 week-treatments and pioglitazone 1.35 mg/kg body weight (bw) po for the last 6 week-treatments
4) LA was a low dose of PCAE group; rats were given HFD for 10 week-treatments and 6.25 mg CGE/ kg bw po for the last 6 week-treatments
5) MA was a medium dose of PCAE group; rats were given HFD for 10 week-treatments and 12.5 mg CGE/ kg bw po for the last 6 week-treatments
6) HA was a high dose of PCAE group; rats were given HFD for 10 week-treatments and 25 mg CGE/ kg bw po for the last 6 week-treatments

The changes in body weight were recorded weekly, while distilled water was prepared fresh daily. Place table titles above the tables.

2.5 Plasma and serum collection and tissue samplings

After the ten-week experiments was ended, the rats were fasted overnight (12 h) and then anesthetized using sodium pentobarbital. Blood were collected from retro-orbital sinus of rats. The blood was collected into heparinized or non-heparinized tubes and centrifuged at 2000 rpm for 20 min, respectively the plasma and serum was collected and stored at -80^{o}C until analysis to determine the biochemical parameters. After weighing, the liver organs were removed, washed in ice-cold PBS 0.01M pH 7.4, and the tissues were collected and fixed in 10% formalin for histopathological analysis. For SOD analysis, after washing, liver tissues were collected and kept at -80^{o}C until analysis. Liver index was calculated by liver weight divided by body weight.

2.6 Thiobarbituric acid reactive substances (TBARS) assay

The TBARS level of plasma and liver were determined using an MDA ELISA kit in accordance with the manufacturer's protocol (Fine Test, Wuhan, Hubei, China) and TBARS value was expressed as µmol malondialdehyde (MDA)/ml of plasma or µmol MDA/g protein.of liver.

2.7 Total Antioxidant Capacity (TAC) Assay

The total antioxidant capacity (TAC) was determined by the Ferric Reducing Antioxidant Power (FRAP) according to Benzie and Strain with modification. Liver and phosphate buffer saline (PBS, 1:10) were homogenised for 10 sec and

centrifuged at 5000g for 10 min at 4oC and the supernatant removed. A mixed solution of 50 µl of plasma or liver extract and 50 µl of distilled water was added to 900 µl of FRAP reagent and incubated at 37oC for 25 min. The change in absorbance was monitored at 539 nm. The control contained 50 µl of PBS, 50 µl of distilled water and 900 µl of FRAP reagent. Calibration curve was prepared with trolox standard ranging from 0 to 500 µM. Values for TCA plasma and liver were reported as trolox equivalent (TE) in mmol/ml and mmol/g protein, respectively [19], [20]. Protein content of the liver were measured using Lowry methods.

2.8 Lipid peroxidation index

Lipid peroxidation index of plasma and liver were measured with the calculation of TBARS level/TAC level.

2.9 Superoxide dismutase (SOD) analysis

The SOD activity of liver was determined using an SOD activity colorimetric assay kit (BioVision, Milpitas, CA, USA) in accordance to the manufacturer's protocol and the value was expressed as % inhibition. Subcellular SOD levels in the mitochondria and cytoplasm of the liver were carried out using differential fractionation as described below. The SOD level of mitochondria and cytoplasm was determined using an ELISA kit according to the manufacturer's protocol (Fine Test, Wuhan, Hubei, China) and the value was expressed as ng/g protein.

2.10 Differential Fractionation

The differential fractionation procedure can be seen in Figure 1. A hundred mg of frozen tissue were carried out differential fractionation using the freeze-thaw method for protein lysis as a substitute for the sonication process. Tissue slices were placed in a microcentrifuge tube and 100 µl STM buffer was added and grinder for 5 minutes in cold conditions and a 200 µl STM buffer was added (all buffers for extraction were supplemented with a mixture of protease inhibitors). Then stored in the refrigerator for 30 minutes and divortex 15 seconds. The tissue was centrifuged at 8000 rpm for 10 minutes and supernatant (S1) and pellet (P1) were obtained. The supernatant was centrifuged at 11,000 rpm for 10 minutes and separated to obtain a supernatant (S2) and pellet (P2).

For S2 thereafter, 250 µl acetone is added to make precipitation occur and stored in the freezer -20oC for 1 hour. Subsequently the S2 was centrifuged at 12,000 rpm for 5 minutes and the liquid was separated to obtain a supernatant (S3) and pellet (P3). The P3 fraction was added with 200 µl STM buffer and called the cytosol fraction and ready to be tested for SOD levels by the ELISA method according to the manufacturer's protocol.
For the P2 fraction, 100 µl buffer STM was added and centrifuged 11,000 rpm for 10 minutes. Once separated, a supernatant (S4) and pellet (P4) will be obtained. The P4 fraction is added 200 µl SOL buffer and is called the mitochondrial fraction. For the mitochondrial fraction, it is necessary to make a process to make lysis cells, namely the freeze-thaw method, which is the freezing cycle -20^{o}C and thawing as much as 3 times carried out for 3 days ([21], [22] with modifications). After that, samples of the mitochondrial fraction were ready to be tested for ELISA for SOD levels.

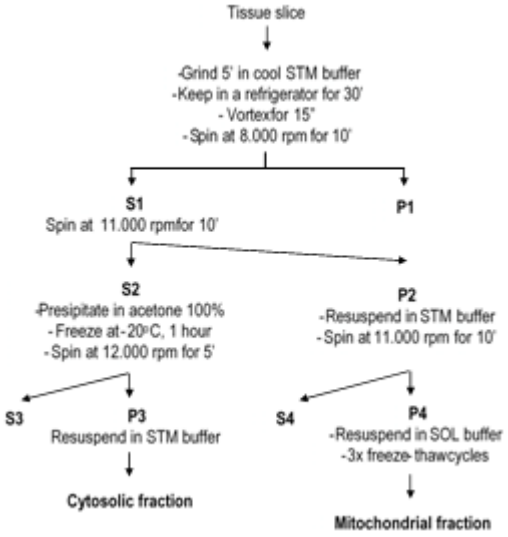

Figure 1: Differential fractionation protocol scheme. The protocol describes two subcellular fractions, i.e. cytosolic and mitochondrial fraction (S = supernatant; P = pellet) (Source: [21], [22] with modification)

2.11 Histopathology Analysis

This study used organ samples (liver) from six groups of rats, each with four rats so that there were a total of 24 liver organs. All livers were fixed with normal formalin, buffered 10% for 24 hours and then processed for hematoxylin-eosin staining. Histopathological preparation of the liver was carried out in the Pathology Laboratory, Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada.

2.12 Statistical Analysis

All data were expressed as mean and standard deviation (SD). The differences between the groups were evaluated by ANOVA followed by Duncan's multiple range test (DMRT). Correlations were analysed by the Pearson rank test. All analyses were carried out with SPSS software, IBM version 18.

3. Results

3.1 Lipid peroxidation

Figure 2 illustrates the effect of PCAE administration on rats fed a high-fat diet on plasma and liver MDA levels. The negative control group showed an increase in plasma TBARS of 108% compared to the normal control group. Provision of pioglitazone or PCAE together with a high-fat diet can reduce plasma MDA production by 33-39%.

More striking changes occur in liver TBARS. High-fat diets cause an increase in liver MDA production by 300%, but this condition can be improved by giving pioglitazone or PCAE in mice high-fat diets, as evidenced by a decrease in liver MDA levels by 12-65%. The effect of PCAE in decreasing

the MDA production of the liver is influenced by the dose, the moderate dose PCAE provides the greatest decrease in MDA production compared to higher or lower doses.

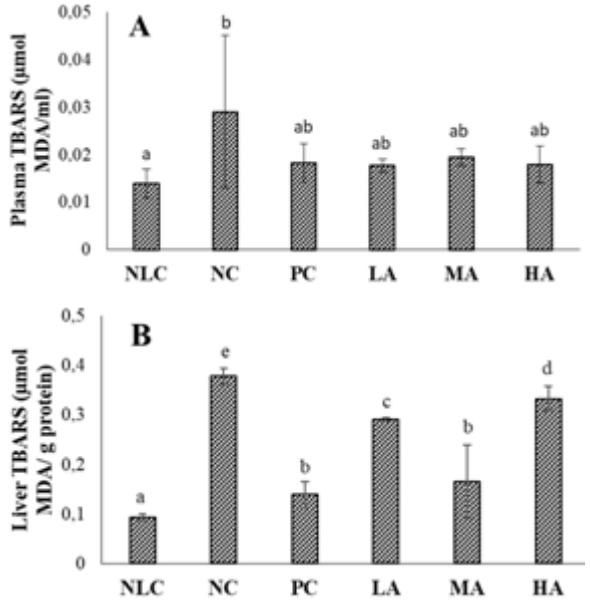

Figure 2: Effect of administration of purple corn anthocyanin extract (PCAE) on plasma lipid peroxidation (A) and liver lipid peroxidation (B) in rats high in fat diet (NLC = normal control, standard feed; NC = negative control, high fat diet; PC = positive control , high fat diet + pioglitazone; LA = high fat diet + PCAE 6.25 mg / kg body weight (BB); MA = high fat diet + PCAE 12.5 mg / kg bw; HA = high fat diet + PCAE 25 mg / kg bb; different letters show significant differences between groups at 5% significance level; P = 0.05; n = 3)

3.2 Total antioxidant capacity

Figure 3 shows the total antioxidant capacity of the plasma and liver of high-fat diet rats treated with PCAE and without PCAE treatment. The total antioxidant capacity of plasma has decreased by 90% due to the provision of a high-fat diet, but this condition can be improved by providing pioglitazone or PCAE along with the high-fat diet resulting in an increase in total antioxidant capacity of 300-770%. A similar condition occurs in the liver, where the feeding of high-fat feed in rats causes a decrease in total antioxidant capacity of the liver by 40%. Improvement of total liver antioxidant capacity can be done by providing pioglitazone or PCAE treatment together with a high-fat diet which results in an increase in total liver antioxidant capacity of 46-133%. The effect of PCAE in increasing the total antioxidant capacity in plasma and liver is affected by the dose.

3.3 Lipid peroxidation index

Figure 4 shows the effect of PCAE administration on plasma lipid peroxidation index and liver in rats fed high fat diet. Lipid peroxidation index is a score obtained by dividing the TBARS value by the total antioxidant capacity value. The higher the lipid peroxidation index score, the higher the oxidative stress in the plasma or liver. High-fat diets cause an increase in plasma lipid peroxidation index scores by 2,470% compared to normal diets, but with the administration of pioglitazone or low, medium and high dose of PCAE, plasma lipid peroxidation indexes decreased by 92, 88, 92, and 94%, respectively.

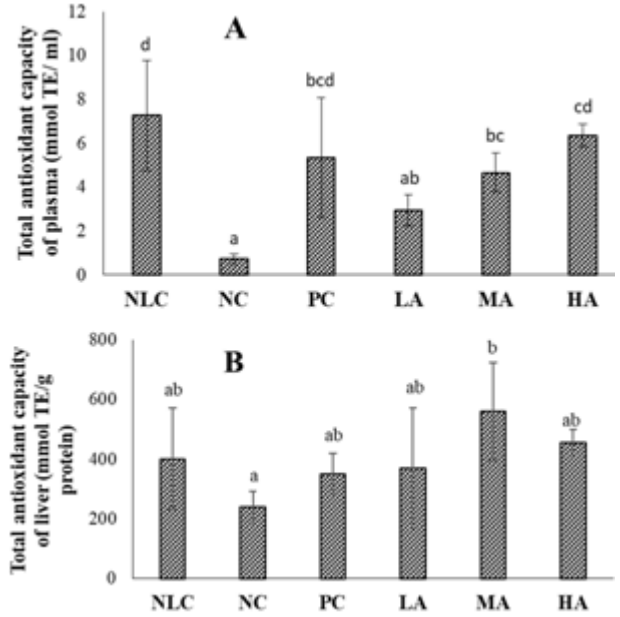

Figure 3: Effect of administration of purple corn anthocyanin extract (PCAE) on plasma total antioxidant capacity (A) and liver total antioxidant capacity (B) in rats fed high-fat diet. See Fig. 2 for further explanation

A high-fat diet causes an increase in the liver lipid peroxidation index by 430% and a high-fat diet accompanied by pioglitazone and PCAE can reduce the index by 56-88%. These results indicate that the administration of PCAE to rats fed a high-fat diet can improve conditions of oxidative stress in plasma and liver, and the effect of PCAE is affected by the dose. The best PCAE dose to correct oxidative stress is a medium dose PCAE.

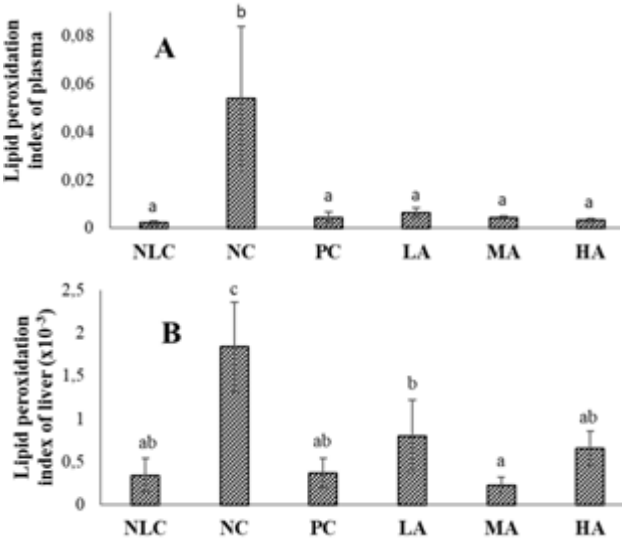

Figure 4: The effect of purple corn anthocyanin extract (PCAE) on plasma lipid peroxidation index (A) and liver lipid peroxidation index (B) in rats with high fat diet. See Fig. 2 for further explanation

3.4 Liver organ

Fig. 5 showed the effect of giving PCAE on liver weight and liver index in rats fed high-fat diet. Significant increase in liver weight by 54% occurred in rats in the high-fat diet group compared to the normal diet group, but at the end of the experiment, administration of pioglitazone or PCAE resulted in a marked decrease of 11-30% in liver weight (Figure 5A). The liver index in rats fed a high-fat diet increased significantly by 27%, but the ratio significantly decreased by 0.07-26% after the administration of pioglitazone or PCAE for six weeks. Among the three doses of PCAE, an intermediate dose of PCAE (12.5 mg CGE / kg bw) was the best treatment, evidenced by liver weight and liver index that was closest to the normal control group (Figure 5B). This result is in line with the research of Xu et al. who concluded that a high-fat diet with 30% of calories from fat causes an increase in the liver index starting at the 4th week of treatment, and a sharp increase occurred after treatment at 16 weeks compared to the normal feed rat group [23].

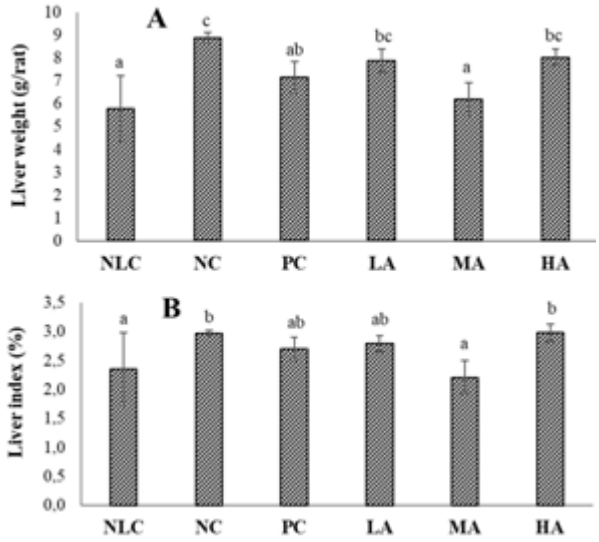

Figure 5: Effect of administration of purple corn anthocyanin extract (PCAE) on liver weight (A) and liver index (B) in rats high in fat diet, n=4. See Fig. 2 for further explanation

3.5 Superoxide Dismutase (SOD)

Figure 6 shows the effect of PCAE administration on liver mitochondrial SOD and cytoplasmic levels, as well as liver SOD activity in rats with high fat diets. Rats fed a high-fat diet showed elevated levels of liver mitochondrial SOD compared to normal diet rats, while cytoplasmic SOD levels did not differe. PCAE and pioglitazone supplementation reduced levels of these endogenous enzymes in the liver mitochondria.

The results of the analysis of SOD levels in the liver mitochondria were not in line with the results of measurements of liver SOD activity. High mitochondrial SOD levels in high-fat diet rats exhibited low activity. This showed that SOD in the mitochondria of high-fat diet rat liver overexpression so that it did not play a positive role but gave side effects. SOD is a mitochondrial antioxidant enzyme that converts and then reduces the level of superoxide by turning it into hydrogen peroxide. Hydrogen peroxide is then detoxified by a number of mitochondrial antioxidant enzymes [24]. Among the three PCAE doses, the intermediate dose is the best PCAE dose to increase liver SOD as evidenced by the highest increase in liver SOD activity.

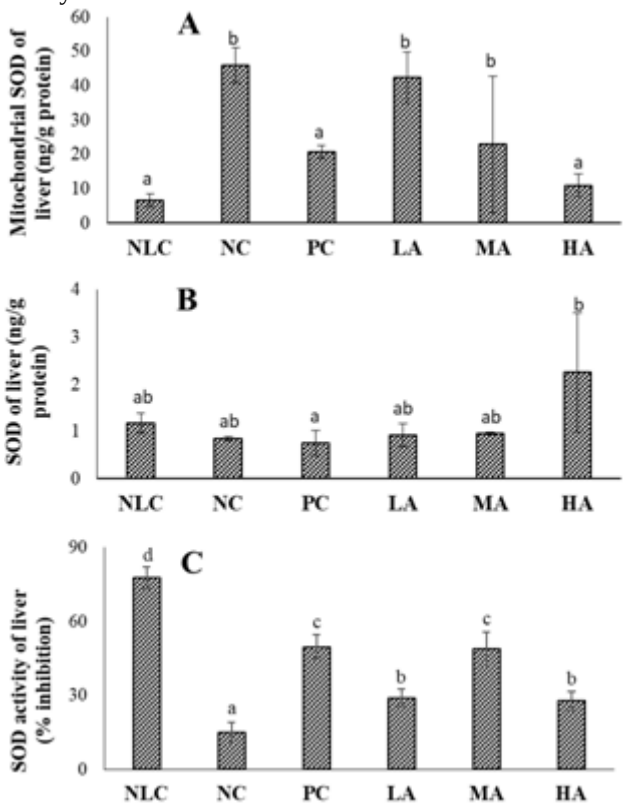

Figure 6: Effects of PCAE administration on liver mitochondrial SOD (A) levels, liver cytoplasmic SOD (B) levels and liver SOD (C) activity in high-fat diet mice. See Fig. 2 for further explanation

3.6 Liver histopathological image

Fig. 7 captures liver histopathology of rats fed HFD with or without PCAE treatment. Figures 7A show the histopathological capture of the liver of normal group rats fed AIN93M diet. The picture shows the liver cells (hepatocytes) look normal with the cell nucleus and sinusoid clearly visible. Sinusoid is a gap that exists between rows of hepatocytes [25]. Normal hepatocytes do not show fatty liver, or are present but in small amounts, which are characterized by empty vacuoles.

The histopathological picture of the liver of the Wistar rat in the negative control group that was given a high-fat diet for ten weeks of treatment can be seen in Figures 6B. The picture shows the presence of fatty liver as indicated by empty vacuoles of various sizes in the cytoplasm of hepatocytes. Fatty process is one type of reversible lesion. Lipids accumulate in liver cells as vacuoles which provide clear appearance with hematoxylin and eosin staining [26].

Qualitatively, the histopathological picture of the liver of the Wistar rat in the negative control group showed fatty liver in

mild to moderate degree. These results indicate that ten weeks of research is not enough to make fatty liver of Wistar rats at a moderate or high level. Therefore, in subsequent studies, additional treatment time is needed.

Research by Karacor et al. showed that a high-fat diet with 60% calories from fat causes fibrosis, inflammation and steatosis (fatty liver) after 16 weeks of treatment [25]. Likewise, the research of Xu et al. concluded that a high-fat diet with 30% calorie from fat (with the addition of 10% cow fat and 2% cholesterol, w/w) causes the appearance of level 1 fatty liver (hepatic steatosis) (that is, fatty acids less than 33% of liver parenchyma) in the 4th week of treatment, fatty liver (fatty liver), which is over 33% of the liver parenchyma occurs at week 8, at weeks 12 to 48, the development of steatohepatitis and liver function become abnormal. While mild liver fibrosis appeared at the 16th week of treatment [23].

Histopathological liver of the HA group Wistar rats fed a high-fat diet accompanied by PCAE 25 mg CGE / kg bw of rats is depicted in Figures 7. The picture shows the presence of empty vacuoles at mild to moderate levels, which indicates the presence of fatty liver, even though a high-fat diet has been accompanied by high-dose PCAE. Histopathological picture of the liver shows that the changes that occur in the blood have not yet affected the repair of Wistar rat liver cells. These results reinforce the previous histopathological picture, that the duration of treatment (ten weeks) in this study needs to be extended so that changes at the tissue and cell level can be seen.

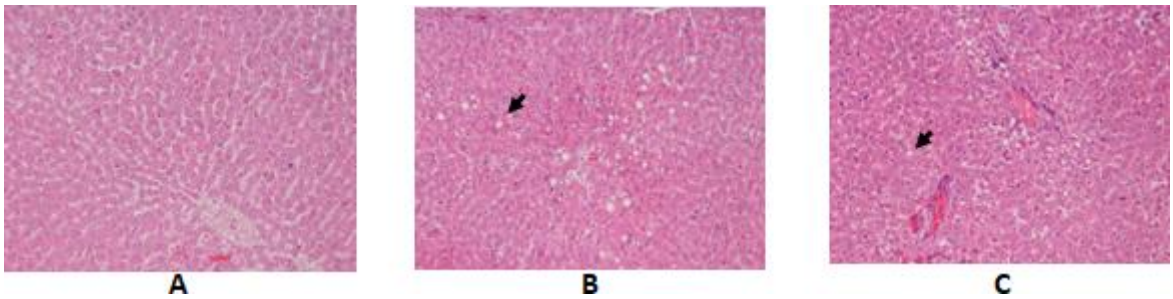

Figure 7: Image of liver histopathology of rats fed HFD with or without PCAE treatment of normal control group (A), negative control group (B) and high anthocyanin group (C); H&E-staining at 500x; Normal diet rat's liver showed no vacuole, while rats fed high-fat diet and PCAE administration in rats fed high-fat diet showed vacuoles (arrow) at mild to moderate degree

3.7 Correlation between parameters

Table 1 shows the correlation scores between lipid peroxidation index and other parameters.

Table 1: Correlation between lipid peroxidation index and other parameters

Parameters	*Pearson's Correlation Score with*	
	LPI plasma	*LPI liver*
Plasma LPI	1	0.720**
Liver LPI	0.720**	1
Plasma TBARS	0.643**	0.248
Liver TBARS	0.536**	0.752**
Plasma total antioxidant capacity	-0.613**	-0.672**
Liver total antioxidant capacity	-0.472*	-0.579*
Liver weight	0.480*	0.649**
Liver index	0.326	0.499*
Mitochondrial SOD of liver	0.450	0.460
Cytosolic SOD of liver	-0.220	-0.221
Liver SOD activity	-0.498*	-0.673**

**correlation is significant at the 0.01 level (2-tailed)
*correlation is significant at the 0.05 level (2-tailed)

Based on the Pearson correlation score, it was concluded that there were significant positive correlations between the plasma lipid peroxidation index with plasma TBARS, liver weight, and liver TBARS, and there were significant negative correlations with plasma total antioxidant capacity, liver total antioxidant capacity and liver SOD activity. On the other hand, Table 1 also concluded that there were significant positive correlations between liver lipid peroxidation index with liver weight, liver index, and liver TBARS, but significant negative correlations with plasma total antioxidant capacity, liver total antioxidant capacity, and liver SOD activity. There was a significant correlation (-0.682, $P<0.05$) between liver mitochondrial SOD levels and liver SOD activity. This showed that there was an over expression of liver mitochondrial SOD as a compensation for oxidative stress conditions in rats fed high fat diet [24].

4. Discussion

In healthy individuals, high fat consumption causes increased levels of free fatty acids in plasma and increases plasma free radicals, which indicates that oxidative stress occurs. Boden showed that the increase in free fatty acids induces the production of ROS and ROS causing oxidative stress that affects the production of proinflammatory cytokines from white adipose tissue, and results in insulin resistance [27].

The result showed that HFD increased lipid peroxidation in plasma and liver. High fat diet induces mitochondrial dysfunction and subsequently it may induce free radical formation [28]. A high-fat diet causes damage at the cellular and molecular levels, and it triggers an oxidative stress process and subsequently generates different responses such as peroxidation of lipids [2]. Long-term feeding of a high-saturated fat diet acts as an inducer of oxidative stress, since it significantly attenuates the hepatic enzyme antioxidant system and increases the levels of lipid peroxidation (LPO)

products in the liver and plasma [29].

The PCAE treatment reduced membrane damage through decreased lipid peroxidation compared to negative controls. According to Reis et al., anthocyanins act as antioxidants by different mechanisms, such as capturing free radicals and/or anions, inhibiting xanthine oxidase, chelating metal ions, targeting arachidonic acid and adhesion of molecules [30].

Fat accumulation in the liver could happen because the use of saturated fat (beef tallow) in the diet. When the fat is polyunsaturated fat, the greater oxidative rates compared to saturated fatty acids made polyunsaturated fat had an "anti-obesity" effect as an opposite of fat accumulation via PPARα mechanism [31]. High fat diet induced an increase in liver weight because of fat accumulation [32], [33]. If the liver index is increase, it means that there is an inflammation condition of the organ. While a decrease of organ-to-body ratio means that there is a cell constriction. This research showed that there was an increase in the rats' liver and the liver index in rats fed HFD, meant that there was an inflammation in the liver. It probably accounts for its antioxidant activity as reflected in plasma and liver TBARS, total antioxidant capacity, and lipid peroxidation index. These results are in line with other studies that high fat diet especially high saturated fat induce oxidative stress since it increases the levels of lipid peroxidation products and attenuates the hepatic enzyme antioxidant system in the liver and plasma [29],[34],[35].

Administration of PCAE at an intermediate dose can improve SOD activity equivalent to improvement by pioglitazone (Figure 6). These results are in line with previous studies which found that the administration of flavonoid extracts was able to improve oxidative stress [36]. On the other hand, high level of PCAE reduced liver SOD activity although increase liver SOD expression, indicated that high level anthocyanin could be a prooxidant. Anthocyanin acts as an antioxidant at the right level or dose. Too high anthocyanin levels cause that anthocyanin is more like a prooxidant rather than as an antioxidant. The catalytic activity of antioxidant enzymes is important for effectively cleaning oxygen radicals. The formation of high free radicals due to high fat can reduce antioxidant enzymes, for example SOD. Despite there was a decrease in liver SOD activity, the level of liver SOD was increase in rats fed HFD. It indicated that SOD is an inducible enzyme that the enzyme expression would be increase if there were induction factors. The expression of both of the two major sod genes, Cu/Zn-SOD and Mn-SOD, has been found to be up-regulated by oxidative stress. It is also known that massive up-regulation of Mn-SOD contributes very little or nothing to overall SOD activity [37].

A long-time consumption of high fat feeding provided positive energy balance resulted in excessive energy storage particularly in the form of triglyceride (TG) in the liver. TGs are ectopically deposited as microlipid droplets in the cytosol and near the cell membrane in the liver (35). This is why high fat diet provide a vacuole or a clear circle in the histopathological images of liver. Histopathological studies show that lipid accumulation occurs in the livers of rats fed a high-fat diet in the mild to moderate degree. Administration of PCAE seemed no change in lipid accumulation, meant that changes in the blood had no effect in tissue yet. It probably because short time of experiment and not enough to make changes in tissue, i.e. liver.

Fatty liver is caused by the accumulation of TG formed from the esterification of free fatty acids and glycerol in hepatocytes. High free fatty acids in the liver come from three sources: lipolysis (TG hydrolysis produces free fatty acids and glycerol) in adipose tissue, from food consumption, and de-novo lipogenesis (DNL). On the other hand, free fatty acid can be utilized either through β-oxidation, reesterification to TG and stored as fat vacuole (lipid droplet), or packaged and excreted as very low density lipoprotein (VLDL). Therefore, fat accumulation in the liver can occur as a result of increased fat synthesis, increased fat transport, decreased fat loss, and / or decreased fat oxidation [38].

Xu et al. carried out a research using Sprague Dawley rats with a high-fat diet with 30% calorie fat gave the results that at week 4 an increase in liver weight and liver index, at week 8 an increase in epididimal fat weight, free fatty acid levels, total cholesterol, and serum TNF-α, and mild fatty liver, at week 36 hyperglycemia and insulin resistance occur [23].

Anthocyanin extract of purple corn has a role in the improvement of oxidative stress through the activation of SOD in Wistar rats fed high-fat diets so that it has the potential as a functional food source for patients with related oxidative stress diseases. In conclusion, this study shows that PCAE is a repair agent for oxidative stress which works through an increase of endogenous enzymatic antioxidant activity (i.e. SOD) in the liver. One should be careful in using antioxidant because a lot of antioxidant could be a prooxidant. Antioxidant should give in a right time, a right place, a right person, and a right dose. It needs a longer study in order to observe liver histopathological changes of rats fed high fat diet and the improvement by purple corn anthocyanin extract.

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