Reduction of Oxidative Stress by Seluang Fish (*Rasbora spp.*) in Brain of Malnourished Rats (*Rattus norvegicus*)

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***Abstract*—Malnutrition children have lower intelligence due to brain cells damage caused by oxidative stress. Seluang (*Rasbora spp*.) has the potential te reduce this problem. This study aimed to analyze the correlation between hydrogen peroxide (H_2O_2) levels with malondialdehyde (MDA) levels in the brain of malnourished rats after administration of seluang. This study used a posttest only with control group design. 30 white rats (*Rattus norvegicus)* made into malnutrition and divided into three groups, M: malnutrition group, P1: malnourished rats fed standard diet, P2: malnourished rats fed seluang for 4 weeks. The results showed that malnourished rats had the highest levels of H_2O_2. ANOVA statistical tests showed a significant differences between H_2O_2 levels ($p = 0.001$) and MDA levels ($p = 0.001$) among malnutrition group with the other groups Pearson correlation test results concluded that there was a correlation H_2O_2 levels with MDA levels ($p = 0.001$, $r = 0.684$) as indicated by decrease of H_2O_2 levels followed by decrease in MDA levels. This study showed a correlation between H_2O_2 levels and MDA levels in the of malnourished rat brain fed seluang. Thus seluang can improve the condition of oxidative stress in malnourished rat brain.**

***Index Terms*—H_2O_2, malnutrition, MDA, oxidative stress, seluang**

I. Introduction

Malnutrition is still a serious problem in the health sector in both of developed and developing countries. In Indonesia, the prevalence of malnutrition remains high. Basic Health Research [Riskesdas] in 2013 reported that the prevalence of malnutrition in children under five at 5.7%, up from 5.4% in 2010. The prevalence of stunting increased to be 13.9% from 13.0% in 2010. In addition, of the 33 provinces in Indonesia, 20 provinces have prevalence of malnutrition above the national average. South Kalimantan was ranked 5th out of 18 provinces have toddlers weighing less than the national average [1].

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Children who have less or poor nutritional status and short or very short at risk of losing ntelligence quotient [IQ] by 10-15 points [2]. Nutrients, especially protein, minerals, vitamins and essential fatty acids are needed in the development of brain cells. Malnutrition due to protein deficiency would interfere with the synthesis of enzymes that act as antioxidants and reducing the concentration of antioxidants in the tissue, causing a state of deficiency of antioxidants and oxidative stress in the brain [3]. Excessive levels of oxidative stress [free radicals] will damage many cellular components, including cellular proteins, DNA, phospholipid membranes, and enzymes [4], [5]. Cellular component disruption have an impact on overall brain damage and intelligence [6].

Endogenous enzyme that works as antioxidant such as superoxide dismutase (SOD) which catalyze superoxide radicals to be H_2O_2. Furthermore, H_2O_2 will be catalyzed by peroxidase (GPx) and catalase into water (H_2O) and oxygen (O_2). Thus, H_2O_2 can be used as one of the parameters of oxidative stress [5]. in addition, one of the most stable markers of oxidative stress is malondialdehyde (MDA) [7], [8]. MDA levels may indicate oxidative reactions, thus MDA levels rise in various diseases accompanied by excessive oxygen free radicals, so the relationship with the MDA levels of free radical damage is quite often researched [9], [10]. In a previous study, new malnutrition protein leads to oxidative stress in the brain of rats [11], [12]. There was an increase in lipid peroxidation accompanied by increased of MDA levels in the group of malnourished rats when compared to control rats [11].

Free radicals or other reactive oxygen species increase should be counterbalanced by raising synthesis of enzymatic antioxidants. However, this may not be sufficiently resolved by endogenous antioxidants only, thus exogenous antioxidants are needed. Endogenous antioxidant defenses in the brain are not adequate, can be reduced by administering exogenous antioxidants, so the balance of reactive oxygen species and antioxidant will be corrected [3]. These exogenous antioxidants can be obtained from natural components in food stuffs. One of

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them can be obtained from fish. Fish is a good source of nutrients; rich in protein and essential fatty acids [13]. Saluang *[*Rasbora *spp.]* is a river fish, known and widely consumed by the people of South Kalimantan. Based on research conducted by Yunanto *et al* [14], saluang contains amino acids cysteine (0.32 mg) and Docosahexaenoic acid (DHA) (1.04 grams) per 100 grams. Cysteine and DHA has a role in lowering the binding of various types of free radicals. DHA has an ability to inhibit the oxidative stress and protect the brain from lipids and proteins peroxidative damage in the brain [15], [16]. This is an opportunity to do research on potency of Seluang in the response to nutritional problems in Indonesia.

II. Material and Methods

A. Material

30 white male rats weighing 80-100 grams with an age range of 4 weeks, rice crust, seluang, standard rat feed (protein 30%, fat 5%, 8% fiber, calcium 9%, phosphorus 1%, and 3.8% amino acids), ether, phosphate buffer pH 7,4, 200 mL TCA 100%, 100 mL Na-Tiobarbiturat 1%, 250 mL HCl 1 N, H_2O_2, dichromate, acetic acid glacial, adrenalin, Na_2CO_3, EDTA, and distilled water.

B. Methods

This study used male white rats *(Rattus norvegicus)* as the models. This work was approved by the Animal Care and Experimentation Committee (Ethical Committee], Faculty of Medicine Lambung Mangkurat University).

1) Making seluang fish meal

Fresh seluang fish was milled and obtained raw fish porridge. Then steamed with hot steam for 1 hour. Then steaming results were dried in an oven until the moisture content reached 8%. Next step, grinding back to destroy clots or bone particles. After becoming dry grain, seluang fish meal was made into pellets and used as rat feed.

2) Giving treatment

All rats fed a low protein and fat for 8 weeks. Low-protein feed such as nasi karak (dry spoiled rice) was used as the equivalent of a low-protein feed 4% [13]. After 8 weeks (after rats are malnourished), 10 rats from malnutrition group were sacrificed and brains were taken and then measured the levels of H_2O_2 and MDA in the rat of brain. The P1 group fed with standard diet and P2 group fed seluang formula for 4 weeks. After that, all groups were terminated for the measure H_2O_2 and MDA levels in the rat brain.

3) Making brain homogenization

Brains were crushed with a mortar at room temperature and added 1 mL of phosphate buffer at pH 7.4 until it turned into a liquid. Then take 5 mL and centrifuged at a speed of 8000 rpm for 20 minutes. Furthermore, the supernatant was taken to measure levels of SOD and H_2O_2.

4) Measurement of rat brain MDA

Brains had just taken were crushed with a mortar at room temperature and added 1 mL of phosphate buffer at pH 7.4 until it turned into a liquid. Take 5 mL and centrifuged at a speed of 8000 rpm for 20 minutes. Furthermore, 200 mL of the supernatant was taken to measure the levels of MDA. Making the MDA standard curve. A total of 0.05 µM MDA standard was added 1 mL aquadest, then accommodated in an Eppendorf tube. After that, successively added 100 mL TCA 100%, 100 mL Na-Tiobarbiturat 1%, and 250 mL of HCl 1 N. Then heated at 100 ℃ for 20 minutes, then centrifuged at 3500 rpm for 10 minutes. After that, 450 mL of the supernatant was taken and added to distilled water up to 3500 µL. Then read with a spectrophotometer with a wavelength of at most 540 nm. The same procedures were done to 0.025, 0.0125, 0.00625, 0.003125 and 1.56 x 10^{-5} µM MDA. Then graphed the relationship between the absorbance on the Y axis with MDA on the X axis to obtain a linear equation.

5) Examination of hydrogen peroxide

Examination of the samples to measure the levels of H_2O_2 using a spectrophotometer.

6) Making the standard curve

A total of 20 mol H_2O_2 was added 2 ml of a mixture of dichromate: glacial acetic acid (1:3). Then the mixture was heated in boiling water for 10 minutes. The mixture was cooled and then measured the absorbance at 570 nm wavelength. The same procedures were done to 40,60,80,100,120, 140.160 and 180 mol H_2O_2. Then graphed the relationship between the absorbance on the Y axis with the levels of H_2O_2 on the X axis to obtain a linear equation.

7) Making the test solution

1 ml of brain homogenates was added 5 ml phosphate buffer at pH 7.4. A total of 1 ml of the mixture was taken and added to a mixture of 2 ml of dichromate: acetate (1: 3) and then wrapped in aluminum foil for 30 minutes. The mixed solution is heated using a water bath for 10 minutes at 100^{o}C. The solution was cooled at room temperature. The solution was then transferred into a cuvette and its absorbance was measured using a UV-VIS at a wavelength of 570 nm.

C. Data Analysis

The data obtained were tested using normality and homogeneity test. Normal and homogenously distributed data were tested by one way Anova test followed by post hoc test (Tukey HSD) and Pearson correlation test at 95% confidence level.

III. Results

This study used malnourished white rats by providing low protein fat for 8 weeks. After 8 weeks, 10 rats were sacrificed to measure serum protein levels and brain were taken to measure the levels of H_2O_2 and MDA. Furthermore, on the other malnourished rats were divided into two groups and fed in accordance with their respective groups for 4 weeks. After 4 weeks, rats were terminated and brains were taken. The results of H_2O_2 and MDA measurements were presented respectively in Fig. 1 and Fig. 2.

Fig. 1 showed that group of malnourished rats without other feeding had the highest H_2O_2 levels. While groups of malnourished rats fed seluang had the lowest levels of H_2O_2. The statistical test result with *one-way ANOVA* on the data of H_2O_2 showed $p = 0.001$ ($p < 0.05$), which

showed a significant difference at a minimum of one group. These results indicated that the condition of malnutrition increased oxidative stress which marked the levels of H_2O_2 was high. Then after rats were fed standard diet (P1) and seluang (P2), levels of H_2O_2 tended to be lower. This proved that the feed able to reduce oxidative stress in the brain of malnourished rats.

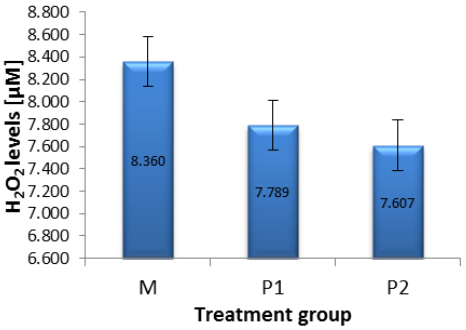

Figure 1. Average levels of H_2O_2 in the malnourished rat brain (M), standard diet (P1), and Seluang (P2) (p = 0.001).

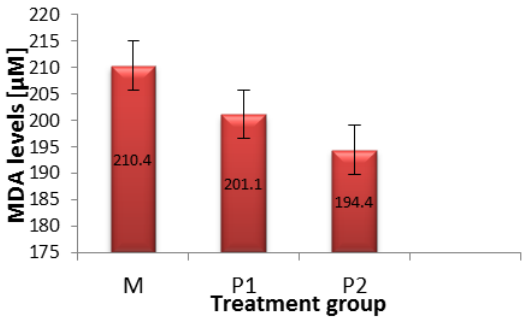

Figure 2. Average levels of MDA in malnourished rat brain (M), after being given a standard diet (P1), and Seluang (P2) [p = 0.001]

Fig. 2 showed that malnutrition group without any other diet had the highest MDA levels. After given the standard diet (P1) and Seluang (P2) there were MDA levels reduction. One way Anova test results concluded that there were differences between groups [p = 0.001]. These results proved that seluang could reduce MDA levels of malnourished rat brains.

Pearson correlation test with the 95% confidence level was done to determine a correlation between H_2O_2 levels and MDA levels in malnourished rat brain fed seluang. Fig. 3 showed the correlation between H_2O_2 levels and MDA levels of malnourished rat brain.

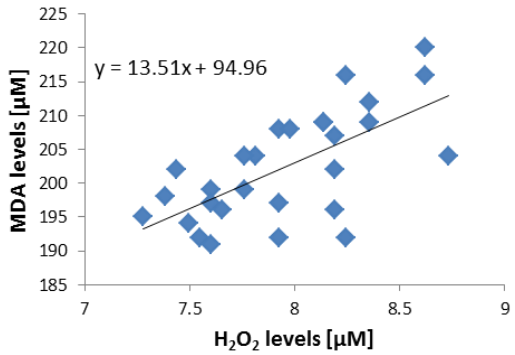

Figure 3. Correlation between H_2O_2 levels and MDA levels of malnourished rat brain after feeding seluang

Based on correlation test, there was significant relationship [p = 0.001] with moderate positive correlation [r = 0.684]. It meant increased H_2O_2 levels followed by elevated MDA levels and vice versa. This proved that seluang able to reduce H_2O_2 levels of malnourished rat brain followed by decreased MDA levels.

IV. Discussion

Malnutrition is a condition caused by an imbalance between the supply of energy and protein to fulfill body needs to function and grow optimally. Children who are malnourished have more oxidant damage products, antioxidant levels are lower, and decreased antioxidant defense system [3]. This is due to a deficiency of the protein in the diet interfere with the synthesis of antioxidant enzymes and reduce the concentration of antioxidants in the tissue, causing a state of antioxidants deficiency [3].

Brain is well protected from free radicals damage by the balance between prooxidant and antioxidant mechanisms, including antioxidant enzymes and free chemical radical *scavenger* [5], [9]. This balance tends to be impaired in protein energy malnutrition (PEM). Many clinical and pathologic manifestations in the PEM alleged to be the result of an imbalance between the defense of free radicals and free radical production [17], [18]. Pathological hallmark of the protein deficiency include shrinkage of brain size and an increase in lipid peroxidation in the brain [19].

Oxidative stress may occur when there is an imbalance between the reactive oxygen species [ROS], consisting of superoxide, hydrogen peroxide, and hydroxyl radicals, with defensive oxidative inadequate, including superoxide dismutase [SOD], catalase [CAT], and peroxidase [POX] [5], [11]. This condition is very harmful for cells. ROS is the result of normal aerobic metabolism that can not be avoided. However, ROS production can be increased also by the amount of stress that disturb cellular homeostasis [5]. ROS will react with lipids, proteins, pigments, and nucleic acids, causing lipid peroxidation [20], [21].

This study demonstrated that H_2O_2 levels of in the malnutrition group were very high. This indicated that under malnutrition conditions would increase the formation of H_2O_2. H_2O_2 is derived from superoxide radicals and converted by the SOD enzyme into H_2O_2. Furthermore, the H_2O_2 levels should be counterbalanced by an increase in the activity of GPx enzyme and catalase which converts H_2O_2 to H_2O and O_2. When this enzyme or its activity slightly lower then H_2O_2, it would be decomposed spontaneously into hydroxyl radicals (OH●) that more dangerous than H_2O_2 [5], [6].

This research showed that in the malnutrition group, levels of malondialdehyde (MDA) was higher. One of the markers of oxidative stress is the MDA. Malondialdehyde is a three-carbon component formed from peroxidation of polyunsaturated fatty acids, particularly arachidonic acid and is one of the most stable end product of lipid peroxidation. Oxidative stress can damage the central nervous system because it is very susceptible induced by a variety of biological agents. Due to the high metabolic

activity, brain requires molecular oxygen in large quantities, which is then followed by the formation of free radicals in high levels. The brain also contains polyunsaturated fatty acids and oxidized easily [22], [23]. In addition, the total capacity of antioxidants found in the central nervous system are relatively few [22], [24]. Increased oxidative stress may also be a result of the bad effects of calorie deficiency and intake of micronutrients [3].

Excessive levels of free radicals will damage many cellular components, including cellular proteins, DNA, phospholipids membrane and enzyme inactivation [4], [5]. It also causes a loss of function of specific proteins, clearance of abnormal proteins, cellular redox balance depletion and disruption of the cell cycle, and ultimately cause death of nerve [22], [23].

This study denoted the impact of protein malnutrition to levels of free radicals in experimental animals. Other study found malnourished children have more oxidant damage products, while lower antioxidant levels with decreased antioxidant defense system [3]. This was due to a deficiency of the protein in the diet interfere with the synthesis of antioxidant enzymes and reduce the concentration of antioxidants in the tissue, causing a state of antioxidants deficiency [3].

In this study, malnourished rats were given treatment in the form of standard diet [P1] and seluang [P2]. After the diet was given for 4 weeks, the rats were sacrificed and the measurement of the brain levels of H_2O_2 and MDA. The results showed all groups had decreased H_2O_2 levels and MDA levels significantly. The decline that occurred in the group fed seluang larger than the group fed standard. This proves that seluang was able to improve the condition of brain oxidative stress better due to malnutrition. The results of Pearson correlation test also proved that the decreased levels of H_2O_2 in the malnourished rat brain by seluang correlated with decreased MDA significantly. This proved that seluang able to improve the condition of the brain oxidative stress due to malnutrition better than regular feed.

Seluang had been shown to contain cysteine amino acids and DHA [14]. Both of these nutrients have some roles as antioxidant [16], [25]. Amino acids are known to have significant use as an antioxidant. The reaction between oxidized lipids with amino acids produce a lot of non-enzymatic reaction components, which have antioxidant effects [16], [25]. One of the 20 amino acids for basic human needs is cysteine. Cysteine is a natural amino acid that contains sulfur and unique because it contains thiol, and can be found in most of the protein, although only in very small amounts. The natural form of cysteine is L-cysteine. This amino acid is a precursor of glutathione, which its sulfhydryl group *(thiol)* acts as a proton donor and responsible for the biological activity of glutathione. Glutathione is an antioxidant tripeptide made of three amino acids, cysteine, glycine, and glutamate. Glutathione is an important antioxidant contained in vital organs and bone marrow of live animals, and often referred to as primary antioxidants [16]. Glutathione works on the peroxide substrate. Seluang contains cysteine which act as antioxidants with levels of about 0.32 mg/100g [14]. Cysteine plays a role in binding various types of free radicals *in vitro*, one of them is OH•, whereas *in vivo,* cysteine together with glycine and glutamate is a glutathione precursor enzyme, an enzyme which plays a role in the ROS oxidation reduction [16].

In addition, seluang also contains DHA levels of 1.04 mg/100 g. Docosahexaenoic acid [DHA, 22; 6n-3] is a polyunsaturated fatty acid chains [LCPs], which is an important substance for the development of the central nervous system of mammals [14]. DHA plays an important role in neurocognitive development [26] and suggested to have a relationship with malnutrition incident [27].

DHA has an important role in inhibiting oxidative stress and protect from damage lipid peroxidation and protein in the brains of children who are still developing and adult brain, thus avoiding the loss of neurons, cognitive deficits, and locomotor. In this study we demonstrated that DHA and amino acids in the seluang [*Rasbora spp*.] was better than standard diet against to oxidative stress on malnourished rat brain.

V. Conclusion

Based on the results, it can be concluded that there was a significant correlation between H_2O_2 levels and MDA levels in the brain of malnourished rats fed seluang. Seluang [*Rasbora spp*.] can improve oxidative stress profile as indicated by decrease of H_2O_2 levels and decrease in MDA levels. Therefore, seluang [*Rasbora spp*.] have potency to be a source of nutrient for malnutrition.

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