

Effect of Squid (*Sepiotheutis lessoniana*) Ink on Hematological Profile of Rats *Rattus norvegicus*

Abstract

Iron deficiency anemia includes the highest incidence nutritional deficiency diseases in the world. Red cell indices on full blood counts is regular used to diagnose early anemia. Currently strategies medicines of iron deficiency anemia are belong to preventif medicine as well as fortification of food and iron supplementation directly to the body. Recent studies show the effectiveness of treatment of iron deficiency anemia in rats with squid ink. This study aim is to analyse the iron content of of squid ink (*Sepioteuthis lessoniana*) and squid ink effects on hematological profile of male wistar rat *Rattus norvegicus*. The meassured iron (Fe) content of squid ink (*Sepioteuthis lessoniana*) is $2,14 \pm 0,46$ mg/g. Rats were used in this study, are induced NaNO_2 (50 mg/kgbw (1 ml dosing volume)) at first, then divided into 4 groups experiment for 14 days experimental. The 1stgroup is negatif control (without any treatment), 2ndgroup is positive control (with Sangobion®), 3rdgroup is given low dosage of squid ink (10mg/kgbw/day) and the last 4thgroup is given high dosage of squid ink (100mg/kgbw/day). Based on the result, there was an effect of given squid ink to the hemoglobin (Hb), hematocrit (Hct), MCV, MCHC, MCH, RDW and RBC of rats that showed by increasing numbers to the end of experiment compared to the negatif control (1stgroup). The most significant effect is in hemoglobin, especially Hb of rats which appeared on the low dosage of squid ink group and positif control group to the negatif group ($p < 0,05$).

Keywords: Fe, Hct, Hb, Red Blood Cell, MCV, MCHC, RDW-CV, Squid Ink. , *S. lessoniana*

Introduction

Anemia is a public health issue with a detrimental effect on the mortality rate of mother and child. The population which are at risk the most from anemia are children aged 0-5 years old, women during fertility and pregnant women (Lopez *et al.*, 2016). Iron deficiency has been attributed as the cause if anemia, yet this condition is among the most prevalent health issue all around the world (Wang *et al.*, 2014). Malnutrition can lower resistance to infection, exacerbate inflammation and increase the risk of anemia. A balanced diet complemented by mineral and vitamin supplements, especially those with antioxidant properties, is necessary for the hemopoietic functions of tissues and in the production of erythropoietin to regulate the formation of red blood cells (Bianchi, 2016). Several iron-rich compound has been applied in preventive solution to the issue of iron deficiency. Among them is iron sulfate with its high levels iron content and good absorption rate up to a certain level, which can help alleviate symptoms of iron deficiency and anemia. However supplement containing this compound can also cause undesirable side-effects including diarrhea, discomfort, constipation and epigastric tenderness (Souza *et al.*, 2009). Diagnosis for anemia is made after sharp decrease in red blood cells count is confirmed, indicated such by lab tests such as complete blood count test. However, it is considerably more difficult to diagnose iron deficiency, which may involve several status indicators for iron in the process. First, red blood cell index determined in complete blood count test can indicate mean hemoglobin cell value. Hemoglobin and mean corpuscular volume (MCV) tests are

affordable, readily available and sensitive procedures. However, these tests will show abnormal results in people with chronic iron deficiency and in cases where the person tested has been suffering from certain chronic illnesses, including hemoglobinopathy (such as thalassemia) or sideroblastic anemia. Results of MCV may be used integrated into indicators which determine nutritional deficiency (Lopez, 2016).

A recent study found that the supplementation of squid ink on rats can reduce the risk of anemia due to iron deficiency, which is attributed to high bioavailability of iron in squid ink (Wang *et al.*, 2014). Therefore, this research aims to determine the content of iron in squid ink and the impact of squid ink dietary supplement on the hematological profile of *Rattus norvegicus*.

Materials and Methods

Preparing the Squid Ink

The ink used in this study was collected from the species *Sepiotheutis lessoniana*. Fresh specimens were obtained from Fish Auction of Tambak Lorok Market, Semarang. Samples were laid in ventral position before being dissected. Afterwards, the ink duct was separated from the rest of the digestive organs using a pair of sterilized tweezers. The ink sac was then extracted from the body of the squid, and the ink duct was cut using a pair of sterilized scissors. Ink from all the extracted sacs were collected inside a brown, sterilized vial, after which it was stored in a freezer in -20°C of temperature. The squid ink preparation was made by putting 20 ml of stored ink into a 1 L beaker glass, and then 100 ml of distilled water and 1.2% commercial vinegar acid was added to a volume of 1 L. The solution was stored for 24 hours. After storage, the solution was coagulated using a rotary evaporator into dry, paste-like lumps. The content of iron per milligram of the preparation was determined using Atomic Absorption Spectrophotometry (AAS) at 248.3 nm of wavelength.

In Vivo Test of Squid Ink

Dry paste squid ink was introduced into 24 male rat subjects, all of which were made anemic by the induction of NaNO_2 at 50 mg/kg of body weight (Ghluhceva *et al.*, 2012). The subjects were grouped into four treatment groups, as presented in the image below. The same diet was administered across the treatment groups *ad libitum*, and each group were given different treatment for 14 consecutive days. The dose of Sangobion[®] used for the positive control group was 4.5 mg/kg of body weight. The description of each control group is presented in Figure 1. below.

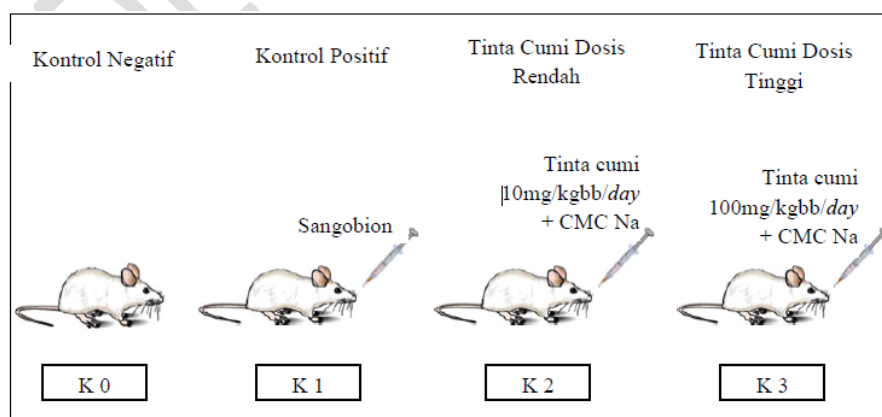


Figure 1. Treatment Scheme on Each Subject Group

Complete Blood Count Test and Data Analysis

After the treatment period ended, blood sample from all subjects was extracted from vein on the eye using capillary tubes. The blood sampling was performed twice on each subject, once after the induction of NaNO_2 compound prior to intervention and one more

time after the treatment period had expired to determine any change in the blood. The complete blood count test was performed using an Automated Hematology Analyzer device. This test can determine Hb count, Red Blood Cells count, MCV, MCH, MCHC, and RDW-CV. The data obtained were then statistically analyzed using one-way analysis of variance (ANOVA) which was ran on SPSS 17.0 for Windows.

Results and Discussions

AAS at 248.3 nm of wavelength determined that the ink of *Sepioteuthis lessoniana* contained 2.14 ± 0.46 mg/g of iron. The existence of iron in squid ink is attributed to its eumelanin, which has a very positive affinity to capture ferrite ion (Di-Mauro *et al.*, 2017). Acetic acid was used as a solvent in this research to create an acidic environment. The necessity of having an acidic environment was due to the fact that the extracted salt (containing iron) is soluble in acid, whereas a base solution would result in the hydrolysis of metallic contents into suspension, making measurements of iron content in the final result difficult to determine. Acetic acid or vinegar was used as a solvent in the extraction process because it is acidic, non-volatile, non-corrosive and non-toxic in nature (Rahmawati *et al.*, 2013).

Post intervention blood analysis determined that the highest mean of hemoglobin count was found in low dose squid ink treatment, at 15.150 ± 0.550 g/dL. This result was followed by positive control group at 14.833 ± 0.446 g/dL, high dose squid ink treatment group at 14.433 ± 0.807 g/dL. The lowest mean hemoglobin count was found in negative control group at 12.417 ± 1.599 g/dL. A pattern of condition improvement after intervention was observed among the treatment groups and positive control groups, whereas the negative control group showed no betterment in condition after the intervention period had expired.

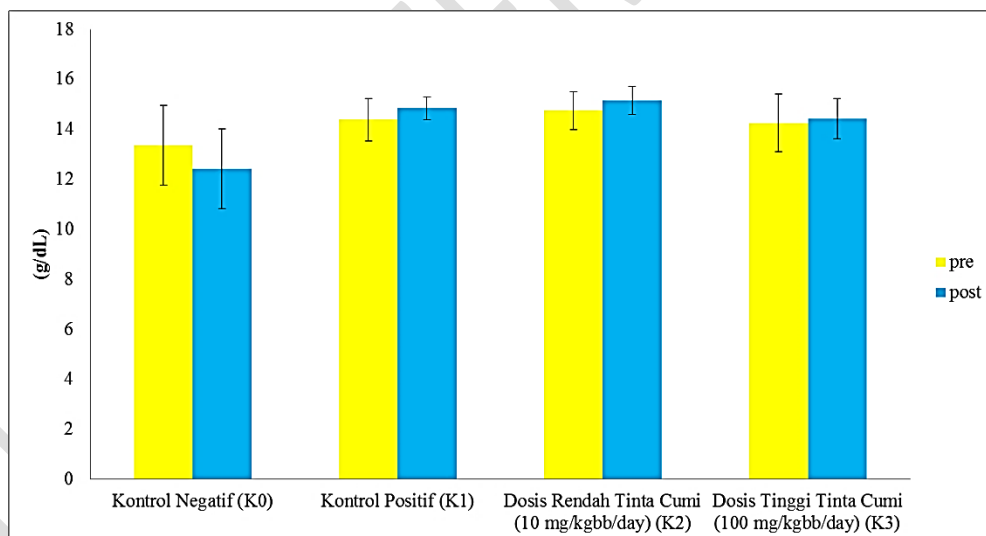


Figure 2. MHV Pattern Diagram Before and After Treatment

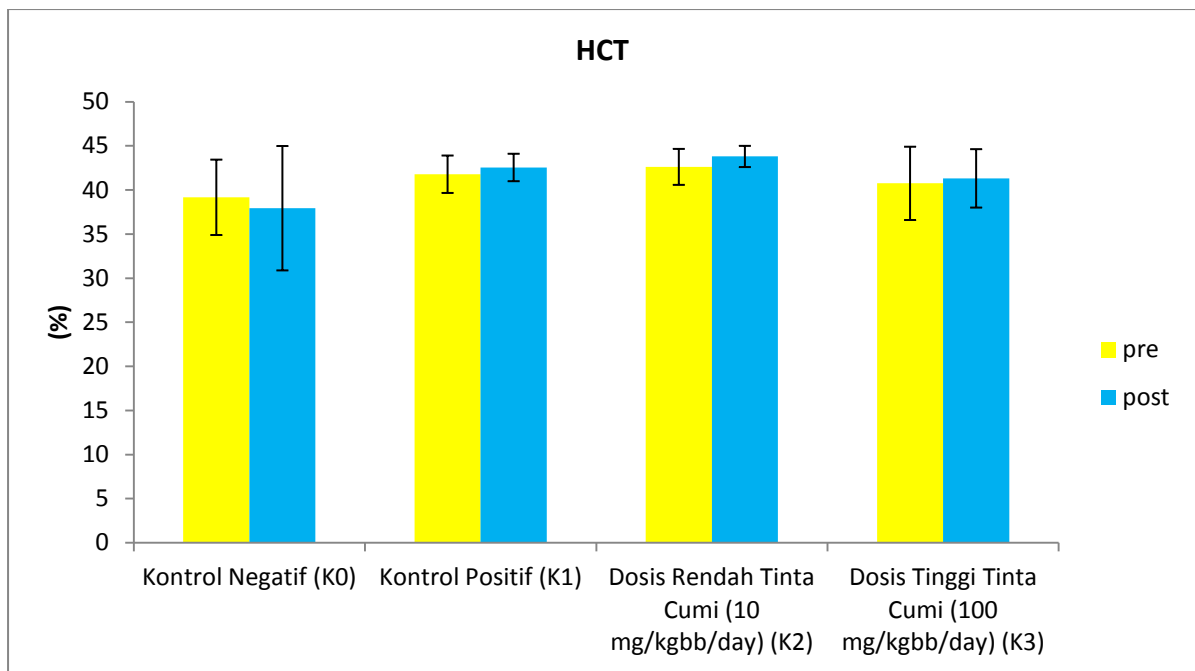


Figure 3. MHC Pattern Diagram Before and After Treatment

Post intervention blood analysis determined that the highest mean of hematocrit was found in low dose squid ink treatment, at 43.8 ± 1.202 %. This result was followed by positive control group at 42.55 ± 1.555 %, high dose squid ink treatment group at 41.317 ± 3.312 %. The lowest mean hemoglobin count was found in negative control group at 37.933 ± 7.053 g/dL. A pattern of condition improvement after intervention was observed among the treatment groups and positive control groups, albeit with almost negligible gain. The negative control did not show improvement in condition.

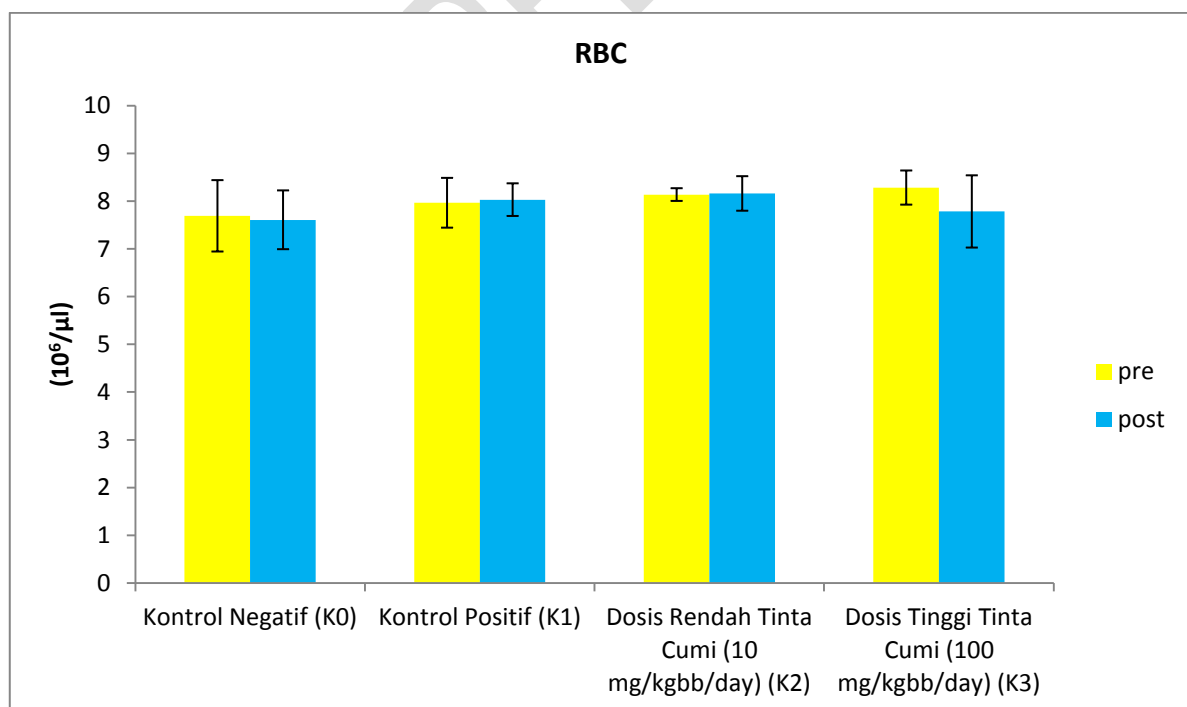


Figure 4. Mean RBC Pattern Diagram Before and After Treatment

The highest mean RBC after 14 days of treatment period was found in low dose squid ink treatment at 8.158 ± 0.362 ($10^6/\mu\text{l}$), followed by the positive control group at

8.028±0.342 ($10^6/\mu\text{l}$) and high dose squid ink treatment group at 7.782±0.757 ($10^6/\mu\text{l}$). The lowest mean RBC was found in negative control group at 7.607±0.616 ($10^6/\mu\text{l}$). The trend which loss of RBC occurring with high dose squid ink treatment group was considered high, even when compared to that of the negative control group. However, the mean value across all treatment group and positive control were still higher than that of the negative control group. Several pattern indicated gain from the condition observed prior to the treatment, albeit very slightly, except for the values observed in subjects from negative control group and high dose squid ink treatment group.

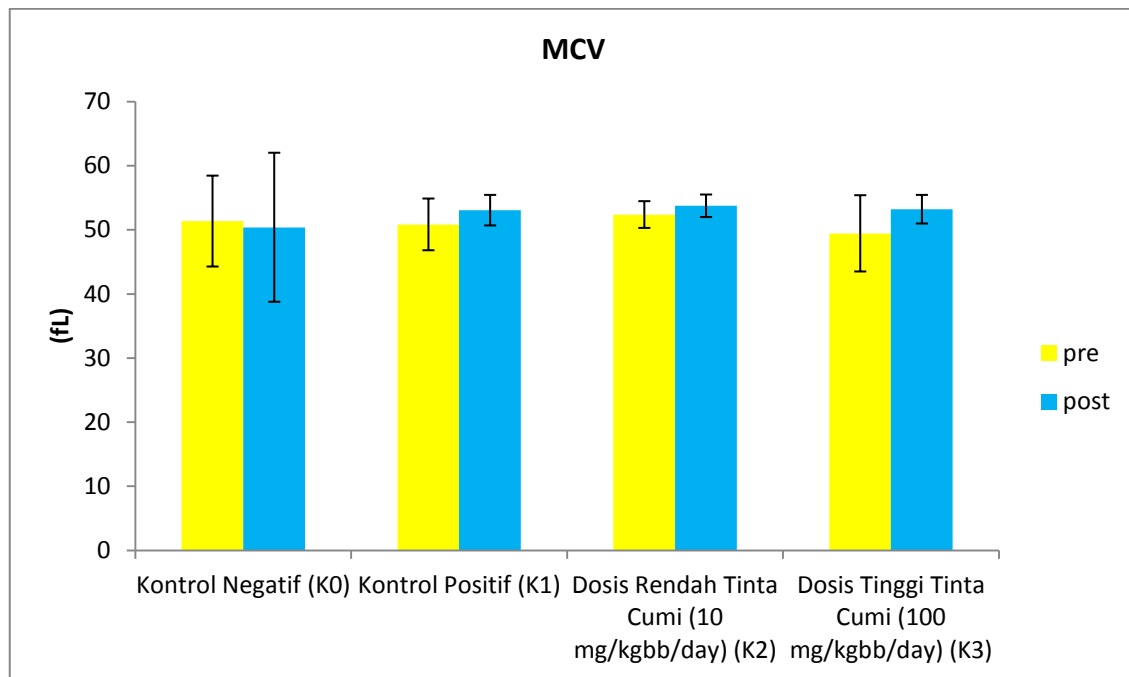


Figure 5. MCV Pattern Diagram Before and After Treatment

The highest mean MCV after 14 days of treatment period was found in low dose squid ink treatment at 53.733±1.758 fL, followed by the high dose squid ink treatment group at 53.2±2.231 fL and positive control group at 53.05±2.381fL. The lowest mean MCV was found in negative control group at 50.383±11.62 fL. A pattern of condition improvement after intervention was observed among the treatment groups and positive control groups, albeit with almost negligible gain. The negative control did not show improvement in condition.

The highest MCH count was found in the low dose squid ink treatment at 18.583±0.387 pg. This result was followed by positive control group at 18.483±0.755 pg, high dose squid ink treatment group at 18.35± 0.675 pg. The lowest mean hemoglobin count was found in negative control group at 16.400±2.356 pg. A pattern of condition improvement after intervention was observed among the treatment groups and positive control groups, albeit with almost negligible gain. The negative control did not show improvement in condition.

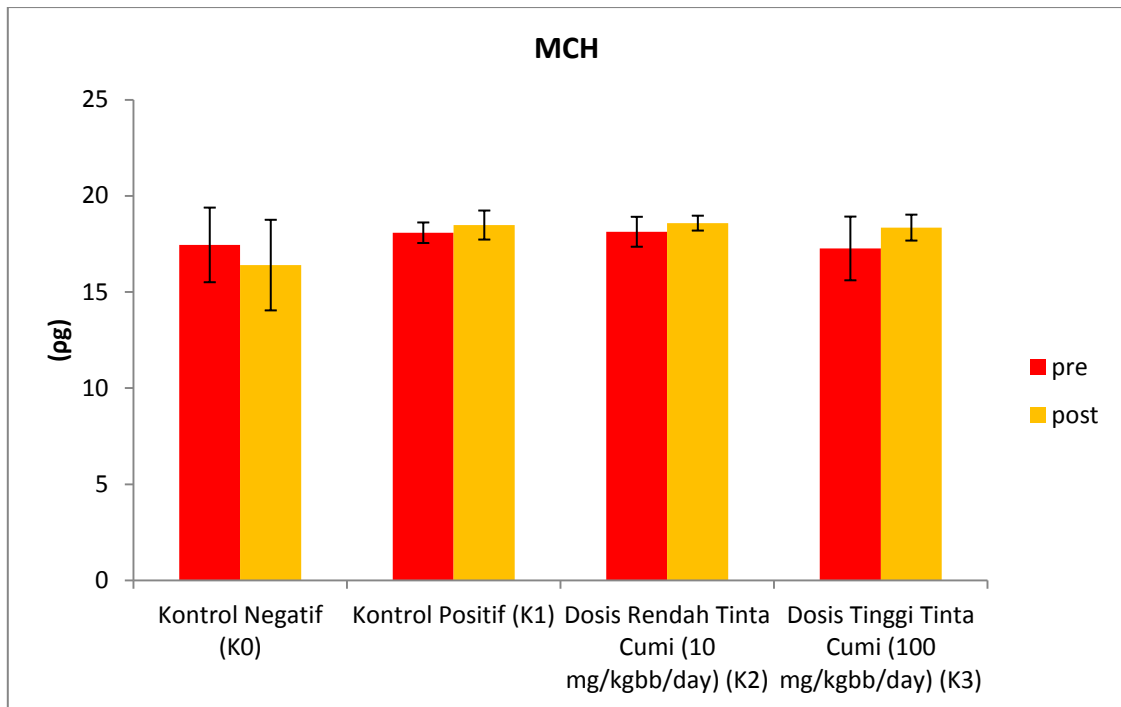


Figure 6. MCH Pattern Diagram Before and After Treatment

The highest mean MCH-C after 14 days of treatment period was found in the positive control group at 34.867 ± 0.383 g/dL, followed by the low dose squid ink treatment group at 34.6 ± 0.629 g/dL and the low dose squid ink treatment group at 34.517 ± 0.293 g/dL. The lowest mean MCH-C was found in negative control group at 33.167 ± 3.469 g/dL. Several pattern indicated gain from the condition observed prior to the treatment, albeit very slightly, except for the values observed in subjects from negative control group and high dose squid ink treatment group.

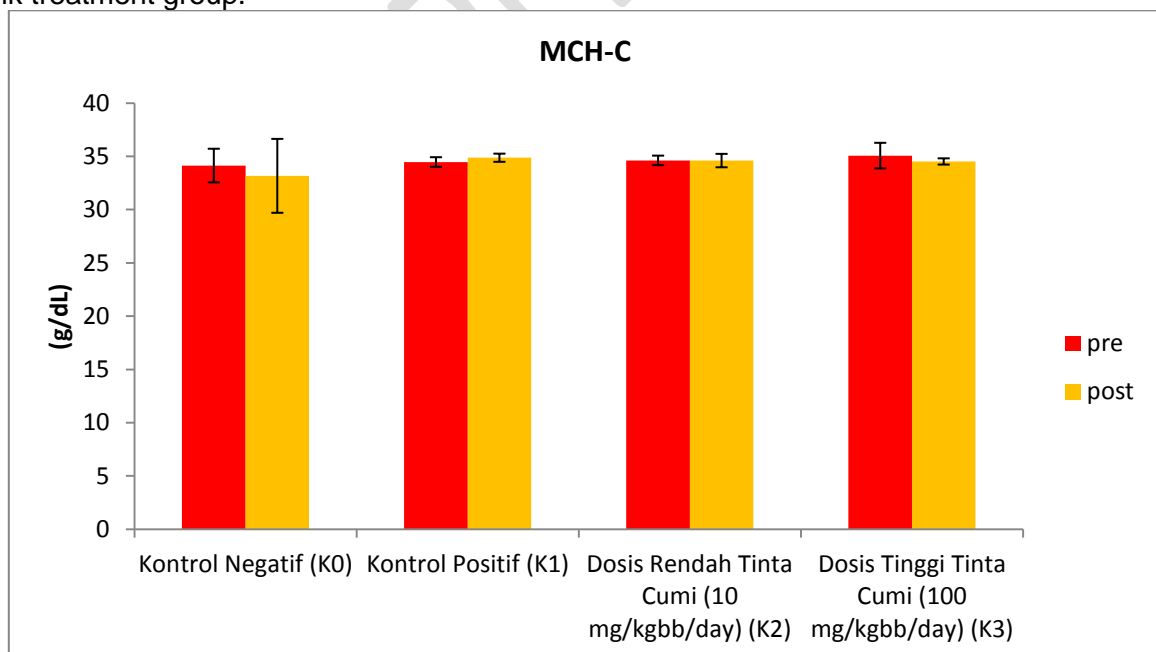


Figure 7. MCH-C Pattern Diagram Before and After Treatment

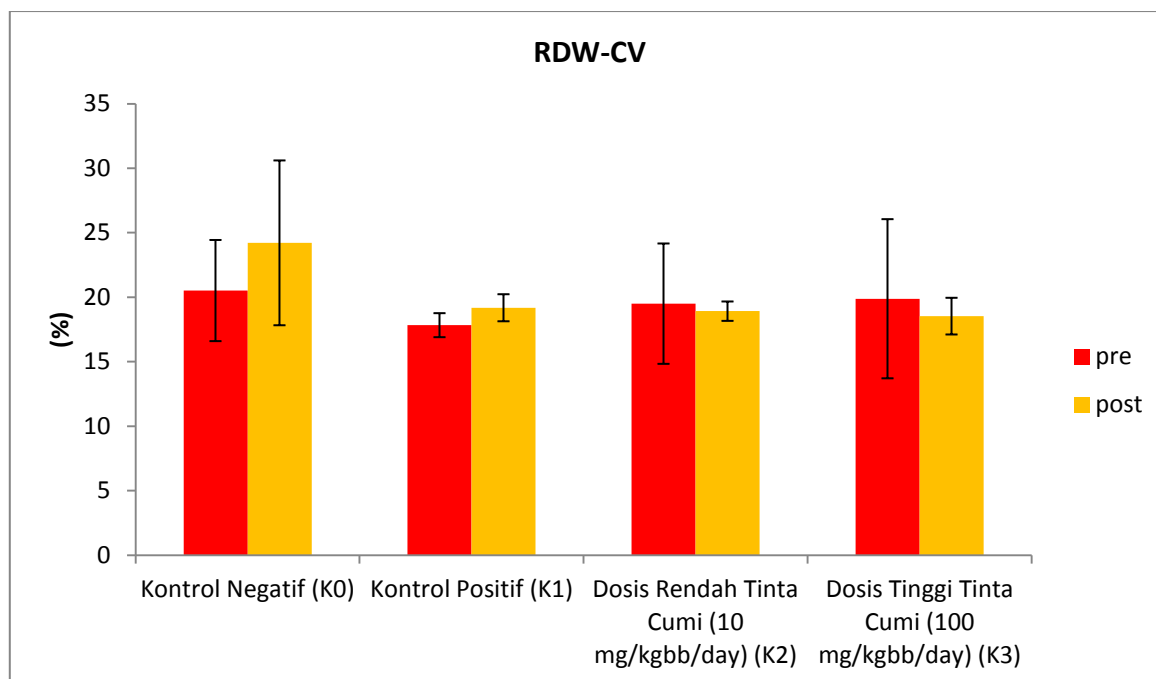


Figure 8. Mean RDW-C Pattern Diagram Before and After Treatment

The highest mean RDW-C after 14 days of treatment period was found in the negative control group at 18.583 ± 0.387 %. This result was followed by positive control group at 18.483 ± 0.755 % and the low dose squid ink treatment group at 18.35 ± 0.675 %. The lowest mean RDW-C was found in negative control group at 16.400 ± 2.356 %. A pattern of condition improvement after intervention was observed among the treatment groups and positive control groups, albeit with almost negligible gain. The negative control did not show improvement in condition. Several pattern indicated gain from the condition observed prior to the treatment, albeit very slightly, except for the values observed in subjects from low dose squid ink treatment group, and with high dose squid ink treatment group actually showed loss.

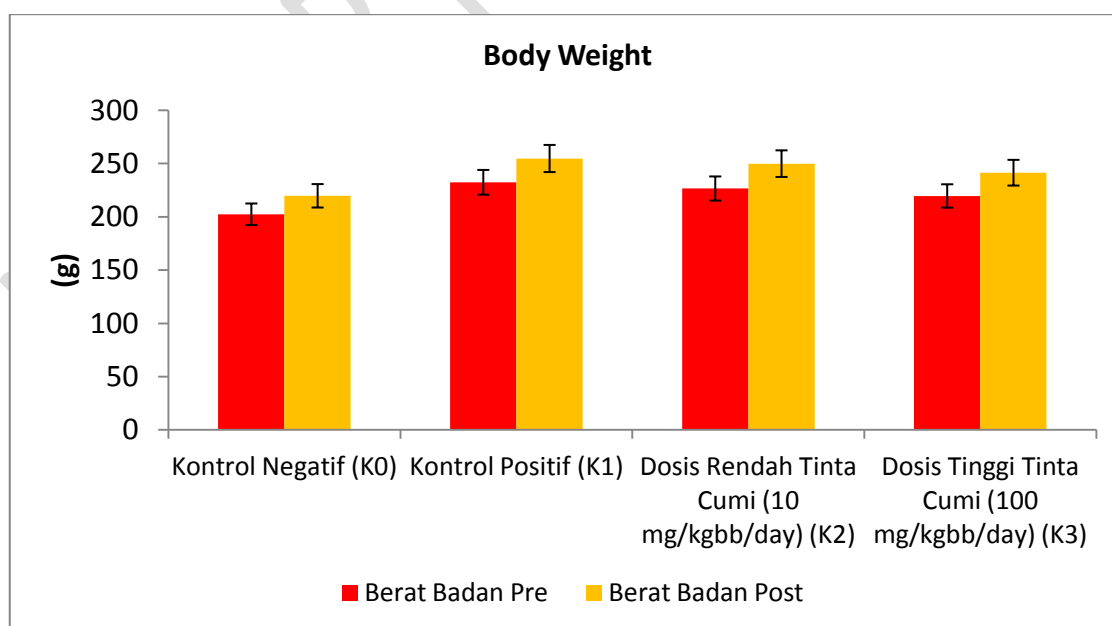


Figure 9. Mean Body Weight Pattern Diagram Before and After Treatment

Figure 9 indicated that the highest bodyweight gain was found in low dose squid ink treatment group at 23.33 grams. The second highest bodyweight gain was observed in the

positive control group, at a mean gain of 22,33 grams, or only 1 gram short of the highest gain. These results were followed by weight gain in high dose squid ink treatment group at 21.83 grams and in negative control group at 17.33 grams. All subjects generally gained more than 15 grams of bodyweight after the intervention.

This body weight gain in all the subjects were not considered to be significant. There was no specific, body weight-based grouping of the subjects during this study, although the lowest mean bodyweight increase was found in negative control group. The body weight of the subjects at the time of the study indicated insignificant correlation with Hb value ($p > 0.05$), with the more significant correlation with bodyweight increase found in nutritional intake. High body weight is commonly attributed to high fat. However, a study found significantly negative correlation between Hb count and Body Mass Index (BMI) along with body fat percentage. The gain in body fat is considered to be an indicator for low Hb count (Acharya *et al.*, 2018). Previous study did not find any significant difference in the concentration of hemoglobin, MCV, serum iron, TIBC, transferrin saturation index, and ferritin among individuals with normal, overweight and obese individuals (Anari *et al.*, 2014).

Table 1, below presents further data on the results presented in the diagrams above.

Table 1. Complete Blood Count Test Results, Pre-Intervention

Group	Hb (g/dL)	HCT (%)	RBC ($10^6/\mu\text{L}$)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW- CV (%)
Negative Control (K0)	13.367 ± 1.587	39.167 ± 4.273	7.69 \pm 0.747	51.35 \pm 7.087	17.45 \pm 1.941	34.133 ± 1.577	20.512 ± 3.922
Positive Control (K1)	14.383 ± 0.854	41.783 ± 2.123	7.963 \pm 0.521	50.833 ± 4.033	18.083 ± 0.534	34.467 ± 0.45	17.833 ± 0.93
Squid Ink Treatment, Low Dose (10 mg/kgbw/day) (K2)	14.75 \pm 0.756	42.612 ± 2.038	8.135 \pm 0.134	52.367 ± 2.086	18.133 ± 0.779	34.612 ± 0.449	19.5 \pm 4,667
Squid Ink Treatment, High Dose (100 mg/kgbw/day) (K3)	14.25 \pm 1.157	40.75 \pm 4,154	8.282 \pm 0.357	49.433 ± 5.947	17.267 ± 1.657	35.067 ± 1.2	19.883 ± 6.171

Table 2. Complete Blood Count Test Results, Post-Intervention

Group	Hb *(g/dL)	HCT (%)	RBC ($10^6/\mu\text{L}$)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW- CV (%)
Negative Control (K0)	13.367 \pm 1.587	37.933 ± 7.053	7.607 \pm 0.616	50.383 ± 11.62	16.400 \pm 2.356	33.167 ± 3.469	24.217 ± 6.390
Positive Control (K1)	14.383 \pm 0.854 ^a	42.55 \pm 1.555	8.028 \pm 0.342	53.05 \pm 2.381	18.483 \pm 0.755	34.867 ± 0.383	19.183 ± 1.050
Squid Ink Treatment, Low Dose (10 mg/kgbw/day) (K2)	14.75 \pm 0.756 ^a	43.8 \pm 1,202	8.158 \pm 0.362	53.733 ± 1.758	18,583 \pm 0,387	34.6 \pm 0,629	18.917 ± 0.752
Squid Ink Treatment, High Dose (100 mg/kgbw/day) (K3)	14.25 \pm 1.157	41.317 ± 3.312	7.782 \pm 0.757	53.2 \pm 2,231	18.35 \pm 0,675	34.517 ± 0.293	18.533 ± 1.422

* statistically significant $p < 0,05$

^a indication of significant difference form negative control group (*non-treatment*) $p < 0,05$

Erythrocyte index count is included in hematological test, which consists of hemoglobin concentration count (MCH-C), RBC, Hematocrit (Hct), Mean corpuscular volume (MCV), Mean hemoglobin content (MCH), Mean corpuscular hemoglobin concentration (MCHC) and Red blood cell

distribution width (RDW). These tests provide the base for clarification for anemia, and they are used in several combinations to distinguish between iron deficiency and thalassemia (Briggs and Bain, 2017).

NaNO₂ can induce anemia due to its ability to bind Hb (by nitric reaction with oxyhemoglobin), turning hemoglobin into methemoglobin, which in turn increases oxygen and erythropoiesis (Keszler *et al.*, 2008). Therefore, the introduction NaNO₂ allows the decrease in hematological profile of the subjects such as Hb count. The effect went further to affect several erythrocyte index parameters such as Hct, MCV, MCHC, MCH, RDW and RBC. The induction of NaNO₂ produced consistent results across all the subjects since the dose was adjusted to be proportional to the bodyweight of the recipients. All subjects were also given the same diet to ensure the validity of the experiment. The foodstuff given to the subjects was low in iron contents.

Post intervention observation indicated that there were different results in negative control group (without any treatment post induction of NaNO₂) and treatment groups (K1, K2, K3), both using squid ink extract and Sangobion®. The most significant differences was found in Hb indicator, since it was the most immediate target after induction. This result indicated that the administration treatment, both using squid ink and iron supplement, impacted the hematological profile of subjects, particularly the treatment using low dose squid ink. However, it was also found that there was no significant difference in the results from treatments with Sangobion® and low dose squid ink did. Thus it was concluded that treatment with Sangobion® and low dose squid ink did produced similar results.

One of the indicator of nutritional balance of the subjects in this research were the correlation between RDW and MCV. RDW is a mathematical count which describes the number of anisocytosis (cellular size variation) and in certain stages may indicate poikilocytosis (abnormal shape and size) of red blood cells on fringe blood test. RDW reflects the variation coefficient of red blood cell volume distribution. Both MCV and RDW are determined from the results of erythrocyte histogram (RBC) (Constantino, 2011; Turgeon, 2012).

This study found that the negative control group had high RDW-CV and low MCV, which indicated that this control group suffered from iron deficiency. High RDW and low MCV value can be interpreted as a condition of iron deficiency or Sickle β-thalassemia, whereas high RDW with normal MCV value can indicate early iron deficiency, early b12 deficiency, early folate deficiency, sickle cell anemia, sc disease, chronic liver disease, and myelodysplasia (Marks, 2012). Positive control group (K1), low dose squid ink treatment group (K2) and high dose squid ink treatment group (K3) indicated high RDW with normal MCV values. Therefore, K1, K2 and K3 were all in good condition, on an 'early iron deficiency' note.

Before and after intervention in this study, the negative control group showed decreasing trend of measured values such as Hb, RBC, Hct, MCV, MCH, MCHC and an increase in RDW. The opposite phenomenon was found in positive control and treatment groups, which showed trend of stable or increasing erythrocyte index value. Generally, the cause of high RDW value is attributed to iron, vitamin B12 or folic acid deficiency, making normal-sized erythrocyte mixed with smaller or bigger-sized ones (Hammarsten *et al.*, 2010).

RDW gain was also observed in positive control group, which was given supplement rich in iron and vitamin B12. This supplement increase or maintain erythrocyte index value after the induction of NaNO₂. This result confirms the findings in Hammarsten *et al.* (2010) in which RDW gain was also observed after administration of supplements rich in iron, vitamin B12 and folate during the proliferation of reticulocyte. RDW value also generally increase post blood transfusion, as found in individuals with hemolytic and thrombotic anemia, where the erythrocyte gets fragmented within circulation.

Squid ink treatment groups also showed significant difference pre and post intervention. The iron contents of the squid ink was attributed to the improvement, since its optimum absorption was enabled by the use of acetic acid during the preparatory process. However, it is also possible that ink from other squid species may produce similar or better

results compared to that which was determined in this study. Other compounds that may produce better results are squid ink with melanin containing tyrosine (Derby, 2014). This tyrosine content is believed to be able to maintain and, over time, recover Hb. The formation of MetHb by the induction of NaNO_2 is due to its reaction with ROS (reactive oxygen species) such as hydrogen peroxide H_2O_2 which took place during the auto oxidation of OxyHb in Hb vesicle, which may cause the deterioration of O_2 gaining capability. A mixture of MetHb and L-Tyrosine can effectively eliminate the capability of H_2O_2 by utilizing reverse peroxidase activity in MetHb, with L-Tyrosinase as its donor. This process will gradually turn MetHb into Hb (Atoji *et al.*, 2006). Residue of Tyrosine can act as a redox center which facilitate iron oxidation mitigation which is higher than Hb, and this process is often found in nature by reducing oxidative reactivity (Reeder *et al.*, 2008; Silkstone *et al.*, 2016). This finding is in line with the results of a study by Lei *et al.* (2008) which stated that squid ink enhances the internal environment of the subjects through free-radical scavenging of oxygen and by being antioxidant in nature, protecting erythrocyte from hemolysis and improving the therapy for iron deficiency anemia. However, inorganic iron in commercial food supplements do not seem to give this effect.

The statistical analysis of K2 and K3 showed that the differences in the dose administered had no significant correlation on the level of measured Hb, since $p > 0.05$. It was found that the lower dose of squid ink treatment had better Hb value. Further study is required to determine the exact cause of this phenomenon. Although there was no issue on the absorption of iron in the ink, the authors suspected that this was caused by intoxication from extremely high dose of squid ink in K3, exactly 10 times of that which was administered to K2 or exceeding 96 ppm. One study by Pringgenies *et al.* (2013) also found that at 95.951 ppm, 100% of *Artemia salina* population was exterminated. This indication was also reflected by the MCHC and RBC count, which was measured lower on pre-treatment of high dose squid ink (see RBC diagram). Another suspected cause of this phenomenon was that the content of hepcidin in K3 subjects might be lower than that of K2 subjects, or it might be that the iron reserve (in the form of ferritin or hemosiderin) in K2 subjects was higher than that of K3 subjects. However, these postulation were merely educated guess since the cause was not exactly studied during the experiment. This result was particularly apparent during serum iron (SI), free erythrocyte protoporphyrin (FEP), total iron binding capacity (TIBC) and unsaturated iron binding capacity (UIBC) tests.

Conclusion

The research found that the ink of *Sepioteuthis lessoniana* squid contained 2.14 ± 0.46 mg/g of iron. After 14 days of treatment, data analyses indicated that the squid ink affected the hematological profile of the subjects, particularly on erythrocyte index value of hemoglobin, red blood cell count (RBC), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW) without observable body weight loss on the subjects of treatment and positive control groups. The subjects of negative control groups showed loss in all values but RDW. The statistical analysis of data showed that the administration of low dose squid ink and positive control indicated significantly positive correlation ($p < 0.05$) compared to the negative control group.

The most significant correlation was found on the Hb count between the treatment group of low dose squid ink and positive control group compared to the negative control group ($p < 0.05$).

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