Radioprotective Effect of Ethanolic Roselle Extract (*Hibiscus sabdariffa* L.) in Recurrent Radiodiagnostic Ionizing Radiation: The Study of Red Blood Cells Peripheral Blood in Mice

Deni Noviana*, Shatilla Stifana Griv, Mokhamad Fakhrul Ulum, Sri Estuningsih

Departement of Veterinary Clinic, Reproduction and Pathology
Faculty of Veterinary Medicine, Bogor Agricultural University
Indonesia, 16680

**Corresponding author, email: d_noviana@hotmail.com; deni@ipb.ac.id*

Abstract

The aim of this study was to determine the radioprotective effect of ethanolic roselle extract in recurrent radiodiagnostic ionizing radiation on peripheral red blood cells of mice. Forty eight male mice were used in this study and divided into 4 group; K-(physiological saline without radiation), K+ (physiological saline with radiation), R- (roselle extract without radiation), R+ (roselle extract with radiation). The mice were treated with 50 mg per kg body weight roselle extract prior to radiation exposure. Following these treatment, the mice were exposed with ionizing radiation at dose 0.2 mSv every two days for 8 weeks. Bloods sample were collected from each group at week 0 before treatment, 2, 4, 6, and 8 week after treatment. The bloods sample were taken at 4 and 8 week, which previously without radiation exposure for 30 days as recovery phase. The result showed that radiation exposure caused an increasing number of Mean Corpuscular Volume, Mean Corpuscular Hemoglobin, Mean Corpuscular Hemoglobin Concentration and Howell Jolly Bodies in the peripheral blood tissue. However, roselle extract caused a decreasing number of the parameters. This result indicated that ethanolic roselle extract has radioprotective effect on red blood cells repair process from ionizing radiation destruction.

Keywords: *radioprotective, roselle, mice, red blood cells.*

INTRODUCTION

X-rays are electromagnetic waves with a shorter wavelength and used in nuclear medicine but its use can affect the red blood cells and causing damage to cells in the body tissues because of the ionizing radiation energy (Suyatno 2008). Large amounts of ionizing radiation can cause illness even death (Wrixon 2008). Chemicals radioprotector in the preparation form is usually consumed in order to neutralize the free radicals that formed by radiation exposure (Beliga & Rao 2010). An ideal radioprotector has to be cheap, nontoxic, easy to use, quickly absorbed, has a wide dose range, and can work through several mechanisms. Plants and natureal products have all the ideal properties as a radioprotector. Herbal as natural product are usually non-toxic, relatively inexpensive, and they can be used orally (Jagetia 2007). One of these natural products are roselle (*Hibiscus sabdariffa* L.).

Roselle contains high antioxidant (Mardiah & Rahayu 2009), which are very effective in preventing the effects of X-ray radiation and also helpful for the recovery of hematopoietic cells from damage caused by radiation (Wambi *et al.* 2008).

Table 1. Groups and research design

Groups	n	Accumulation of radiation 2.9 mSv		Accumulation of radiation 5.3 mSv	
		Ra week-4	Ro week-8	Ra week-8	Ro week-12
K-	12	3	3	3	3
K+	12	3	3	3	3
R-	12	3	3	3	3
R+	12	3	3	3	3
Total	48	12	12	12	12

Description: K-(physiological NaCl 0.9% without radiation exposure); K + (physiological NaCl 0.9% with radiation exposure); R-(roselle extract without radiation exposure); R + (roselle extract with radiation exposure), n (number of mice); Ra (radiation); Ro (30 days after exposure recovery, without radiation).

Therefore, this study aimed at determining the potential of roselle's ethanol extract (*Hibiscus sabdariffa* L.) in recurring low doses radiodiagnostic ionizing radiation against red blood cells of mice.

MATERIALS AND METHODS

Time and Place

Radiation exposure and blood sampling were carried out in the Division of Surgery and Radiology, while the maintenance of mice was conducted in the Division of Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University (IPB). The study was conducted from February to June 2011.

Mice Preparations and Care

Materials and tools used in this research were 48 male mice, 6-8 months old and 20-25 grams in weight, commercial mice food, wood dust, *ad libitum* water, plastic cages with wire lid (35 x 25 x 15 cms), food bowl, marker and stomach tube.

Research Design

Twelve mice of each groups were caged and acclimatized for 2 weeks before the research was started. Anthelmintics, antibiotics and antifungals were given to the mice. Furthermore, these mice were divided into 4 groups (Table 1).

Roselle Ethanol Extract Preparation

Roselle ethanol extract was obtained from maceration and evaporation of dried roselle petals, 96% ethanol and distilled water. Maceration process was performed at Research Institute for Medicinal and Aromatic, Bogor and evaporation process was performed in the Laboratory of Biotechnology, Faculty of Fisheries and Marine Science, IPB. For the maceration, 1.5 grams of dried roselle extract was dissolved in 200 ml distilled water so that its concentration were 7.5 mg / ml.

Administration of Roselle Extract

Roselle extract were given to the R- and R+ groups, while the K- and K+ groups were given physiological saline. Mice were restrained from behind the ear to the dorsal part of their back. Roselle extract solution was administered orally by using stomach tube carefully in order to prevent the solution from entering the respiratory tract. Roselle extract solution was given at a dose of 50 mg/kg body weight (Ali et al. 2005). Roselle extract and physiological saline were given every two days before being irradiated with X-rays.

X-rays Radiation Exposure

Mice in K+ and R+ groups were irradiated by using portable radiodiagnostic machine (VR-1020, Medical corp, Japan), Pb apron, eye protector, thyroid protector and dosimeter (MyDose™ ALOKA CO, LTD Tokyo Japan). Irradiations were done for ± 1 minute, at a dose of 0.2 mSv/2 days, 80 kVp, and 12 mAs. Each cage with the mice inside were exposed to X-rays, Focal Film Distance 100 cm. X-rays exposure were done every 2 days after the mice were given roselle extract or physiological saline.

Peripheral Blood Sampling and Examination

Peripheral blood sampling was done for three mice, randomly in each group at weeks-0, 2, 4, 6, 8 and 12. Mice were anesthetized with a combination of ketamine (dose of 30 mg/kg body weight) and xylazin (5 mg/kg body weight) intraperitoneally. Blood was drawn through the venous sinus retro-orbital by using microcapiler hematocrit (Hrapkiewicz and Medina 2007), and then collected in the Eppendorf tubes which poured with 0.01 ml EDTA. The volume of blood taken was 0.5 ml. Blood examination were comprised of total number of erythrocyte (RBC count), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Howell Jolly Bodies. Howell Jolly bodies was calculated by using Giemsa stained blood smear. Blood in the microhematocrit were dripped onto a glass object. Blood were smeared using the other glass object which was pulled way slowly toward the back and then dried in the air. The dried preparations were fixed with methanol for 3 to 5 minutes and then dried in the air again. Furthermore, the preparations were stained with Giemsa 10% for 30 minutes. Then, the preparations cleared from excessed Giemsa by using flowing water for 30 seconds, and then dried in the air (Thrall 2004). Howell Jolly bodies examination were performed under a light microscope with objective lens magnification was 100x and ocular lens magnification was 10x and also using a square lens 0.225 mm of total length and 0.050625 mm2 of total area. Howell Jolly bodies that present in this area was calculated on a 10 field of view with the zigzag method, and used a formula (a / b) x (1/9) x (100%) (Noviana et al. 2004) as shown in Figure 1.

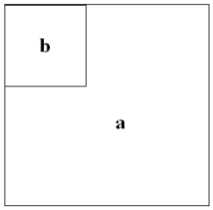

Figure 1. Counter lens. a: a total number of Howell Jolly bodies that presents in the large square. B: a total number of cells that presents in the small square.

Calculation of Howell Jolly bodies percentage changes in mice's peripheral blood caused by radiation exposure and roselle extract treatment was as follows:

- The percentage of Howell Jolly bodies due to radiation exposure:
  (b-a) / (b + a) x 100%

- Changes in the percentage of Howell Jolly bodies after 30 days of recovery:
  (d-c) / (d + c) x 100%

a = percentage of Howell Jolly bodies before treatment; b = percentage of Howell Jolly bodies on a given radiation dose; c = the percentage of Howell Jolly bodies on the dose x; d = percentage of Howell Jolly bodies after 30 days of recovery dose x; x = the amount of radiation exposure dose (mSv)

This formula was also used for the calculation of the MCV, MCH and MCHC percentage post-treatment and 30 days post-recovery.

The data obtained were statistically analyzed on a one way ANOVA posthoc with Duncan test using SPSS® version 16 for Microsoft® Windows® software to see

whether the data differences was significant or not ($p < 0.05$).

RESULTS AND DISCUSSION

The average value of MCV, MCH, MCHC, examination and Howell Jolly Bodies based on the exposure of the total radiation and the recovery phase can be seen in Table 2.

MCV (Mean Corpuscular Volume)

Mean Corpuscular Volume values show the average volume and size of erythrocytes. Normal values were called normocytic, below normal values were called microcytic and values above the normal were called macrocytic (Thrall 2004). MCV value of mice's blood based on treatment group and total radiation can be seen in Table 1. MCV values in all treatment groups at week-0 was in the normal range, but the R + group after receiving 5.3 mSv total radiation at week-6 exceeded 61.5 fL as the normal threshold for MCV (Thrall 2004; Raskin & Wadrop 2010).

Total radiation at week-4 was 2.9 mSv, it led to an increasing MCV values in the group of K + as much as 53.94 fL and 57.62 fL in group R +. Greater radiation at week-8 with 5.3 mSv total of radiation was causing an increase in the K+ group as much as 57.76 fL and 66.10 fL in R+ group as seen in Table 1 and Figure 2 A.

MCV value of R- group with roselle extract had an increase of 61.10 fL, while the K- group had an increase of 60.98 fL at week-4 compared with MCV values before treatment. MCV values at week-8 in R- group increased to 66.10 fL, while the K- group had only 63.04 fL of increase number.

MCV values after recovery from the total radiation 2.9 mSv was causing a decrease as much as 1.28% in the K+ group and 2.06% increase number in group R+. After recovery, 5.3 mSv total radiation caused a decrease in MCV value as much as 1.50% in K+ group and a decrease as much as 9.33% in group R +. The results of statistical analysis showed MCV values for treatment group and time were significantly different ($p < 0.05$).

Radiation exposure with total dose of 2.9 mSv led to a decrease of MCV value in K+ group from before the treatment, but this value was still within the normal range, while the R+ group increased from before treatment and this exceeded the normal range of MCV values (Figure 5A).

Total radiation as much as 5.3 mSv, MCV value in group R + and K + were increased, but the MCV value of R+ group were exceeded the K+ group, these values were exceeded the normal range (42.3-55.15 fL).

MCV values above normal range in the K+ and R+ groups were also called macrocytic anemia. This happened because of DNA synthesis inhibition result in the production of red blood cells. When DNA synthesis was interrupted, then the cell cycle could not be grown from the growth phase (G2) to mitosis phase (M) (Rumsey et al. 2007). This led to continued cell growth without division and seen as a macrocytic anemia. In this study the factor that was suspected of causing interference with DNA synthesis was ionizing radiation. Defects in DNA synthesis of red blood cells were most often caused by hypovitaminosis, particularly deficiencies of vitamin B12 and folic acid (Aslinia et al. 2006; Burgess 2012). In the anemia macrocytic, usually immature red blood cells were released by the bone marrow into the circulation to meet an increasing need (Rizwi 2010).

MCH (Mean Corpuscular Hemoglobin)

Mean corpuscular hemoglobin (MCH) values described the average amount of hemoglobin in the erythrocytes. MCH values in all groups during the recovery phase and different radiation treatments showed a fluctuating result and was still in the normal range values, i.e. 13.7-18.1 pg (Thrall 2004).

After 2.9 mSv of radiation, it caused an increase as much as 6.82% in the R + group, while in the K+ group that value

increased by 7.36%. After a higher radiation as much as 5.3 mSv at week-8, it led to an increase in MCH values as much as 7.75% in the K+ group and 15.50% in R+ group. MCH value of the K+ decreased as much as 1.60% and MCH value of R+ group increased by 3.30% after recovery of 2.9 mSv total radiation. But after 30 days recovery phase 5.3 mSv total radiation, the MCH values increased as much as 1.22 mSv in the K+ group, while the MCH value of R+ group was decreased as much as 8.21%.

After the week-4, it caused a decrease in MCH value of 6.67% in the K- group, and 9.91% in the R- group. At week-8 MCH value of K- group decreased 7.77% and R- group was decreasing 16.45% as seen in Table 1 and Figure 3C.

MCH values associated with the concen-tration of hemoglobin and red blood cell count. Hemoglobin concentration in the blood were influenced by blood volume. If volume decreases, it will lead to reduced hemoglobin. In addition, disruption of iron absorption in the digestive system might also lead to reduced hemoglobin. MCH value of R+ and K+ group after 2.9 mSv total radiation looked almost the same, but after 5.3 mSv total radiation MCH value of R+ group was higher than K+ group. This was caused by the antioxidants from roselle ethanol extract that can neutralize the damage caused by ionizing radiation. Research conducted by Noviana et al. (2010) showed that lower radiation of 0.2 mSv from reccurent radiodiagnostic in mice had no effect on red blood cell parameters. Doses indicated that blood components might be damaged after the exposure to X-rays.

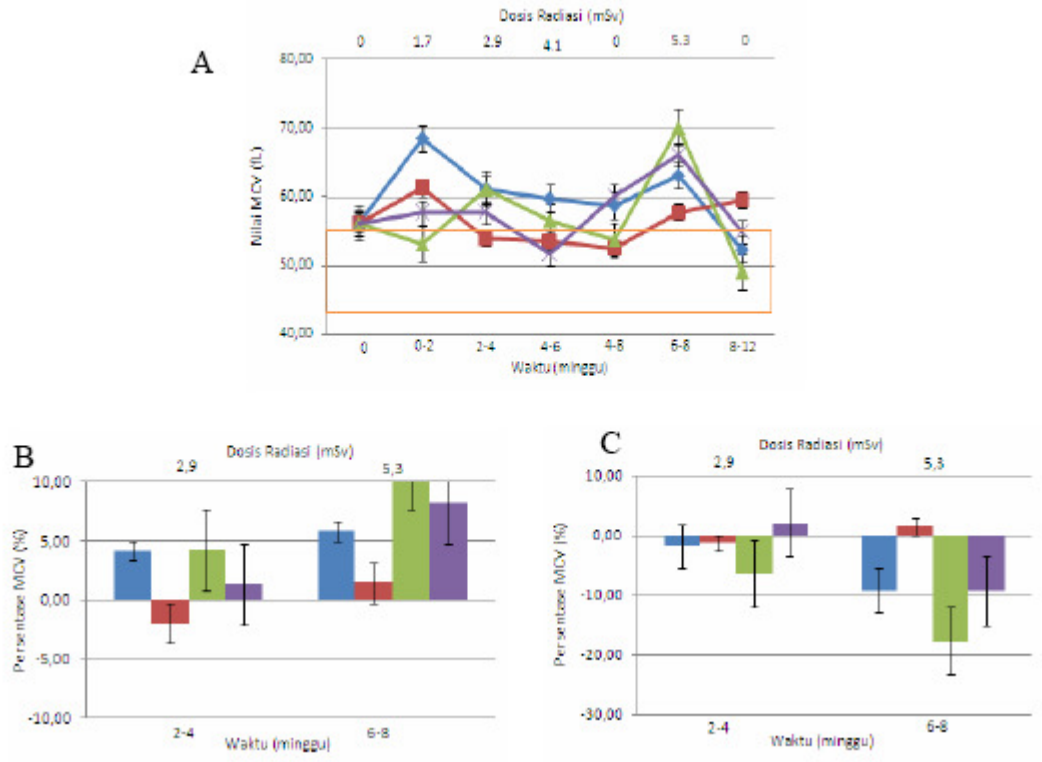

Figure 2. A. MCV percentage of mice's peripheral blood against recurrent radiodiagnostic; B. The percentage of MCV after treatment; C. The percentage of MCV after 30 days of recovery. ■(K-) = physiological saline without radiation exposure; ■(K +) = physiological saline with radiation exposure; ■ (R-) = roselle extract without radiation exposure; ■ (R +) = roselle extract with radiation exposure; □= normal value of MCV 42.3-55.15 fL (Thrall 2004).

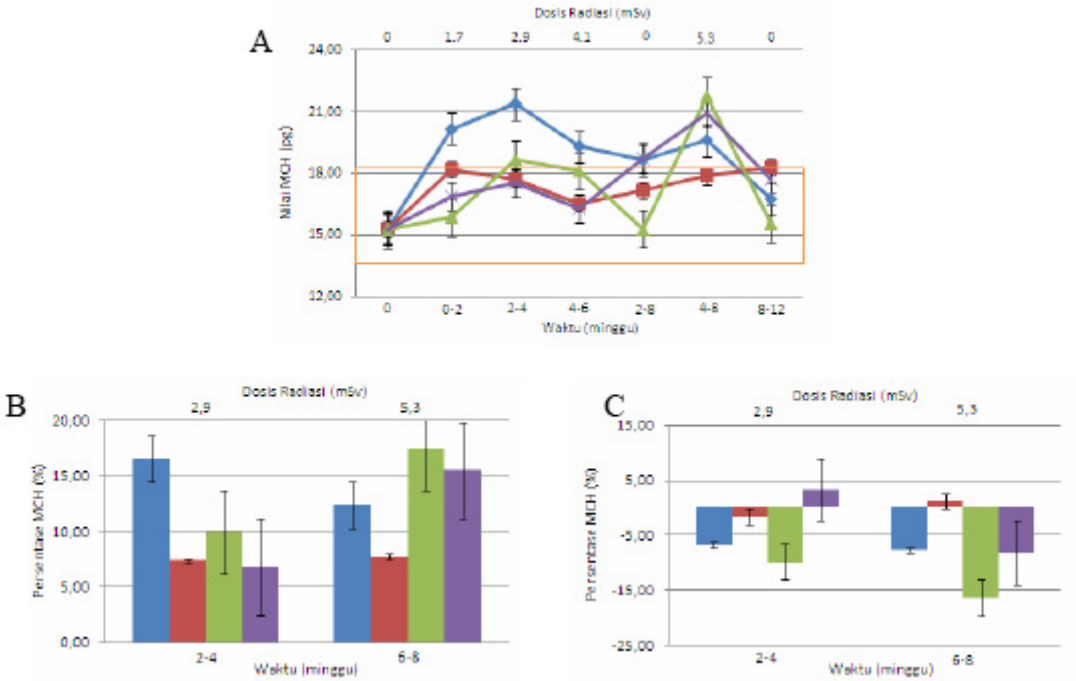

Figure 3. A. MCH percentage of mice's peripheral blood against recurrent radiodiagnostic; B. MCH percentage after treatment; C. MCH percentage after 30 days of recovery. ■ (K-) = physiological saline without radiation exposure; ■ (K+) = physiological saline with radiation exposure; ■ (R-) = roselle extract without radiation exposure; ■ (R+) = roselle extract with radiation exposure; □= normal values MCH 13.7-18.1 pg (Thrall 2004).

MCHC (Mean Corpuscular Hemoglobin Concentration)

Mean Corpuscular Hemoglobin Concentration (MCHC) values was the value obtained from the concentration of hemoglobin and erythrocytes. MCHC values indicate the concentration of hemoglobin in 100 ml of erythrocytes. Hemoglobin's role is maintaining hemoglobin oxygen transport function of the lungs to body tissues. Iron is a substance needed to heme formation that develop the hemoglobin. Interference in the absorption of elemental iron results in a lack of iron in the blood circulation, so that is reducing the amount of hemoglobin (Kemuning 2010). MCHC values of mice based on treatment group against the total radiation and recovery phase groups can be seen in Table 2.

MCHC values of the treated group can be seen in Figure 3A. MCHC values after treatment at week-4, K- 32.29%, K+ 30.91%, R- 32.09% and R+ 31.59%. These values were within the normal range (Thrall 2004). Week-8 as well, MCHC value in the K- 32.40%, K + 30.82%, R- 31.73% and R+ 32.48%.

2.9 mSv radiation at week-4 caused an increase in MCHC value as much as 9.28% in the K+ and an increase as much as 6.28% in R+ group from the value before treatment. Greater radiation at week-8, 5.3 mSv total radiation caused an increase in MCHC value as much as 6.35% in the K+ group and an increase 7.45% in the R+ group as seen in Table 1 and Figure 4 B.

MCHC value of R- group with roselle extract has increased as much as 5.62% and the K- group increased by 12.16% at week-4 from the value before treatment. MCHC values of the week-8 increased as much as 6.64% in the K- group and 6.41% increased number in the R- group.

MCHC values after recovery of 2.9 mSv total radiation caused a decrease as much as 0.28% in the K+ group and increased 0.44% in R+ group. Recovery after higher radiation total 5.3 mSv of

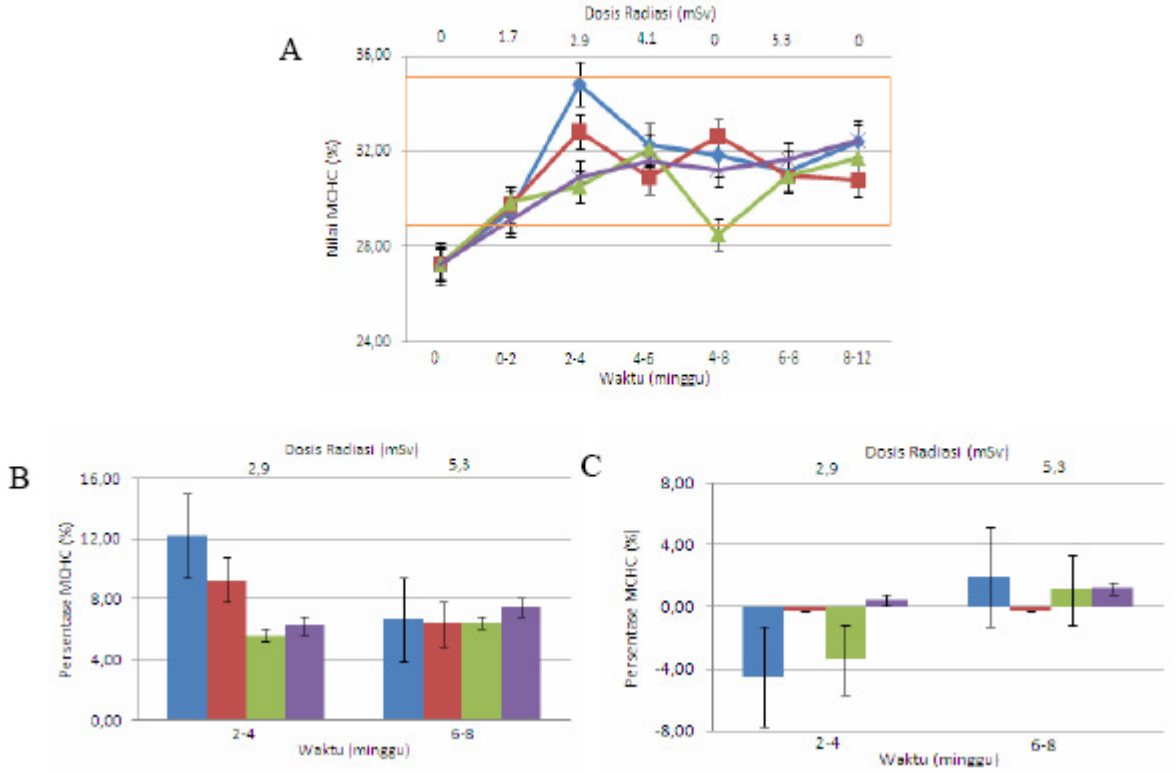

Figure 4. A MCHC percentage of mice's peripheral blood against recurrent radiodiagnostic; B. MCHC percentage after radiation; C. MCHC percentage after 30 days of recovery. ■ (K-) = physiological saline without radiation exposure; ■ (K+) = physiological saline with radiation exposure; ■ (R-) = roselle extract without radiation exposure; ■ (R+) = roselle extract with radiation exposure; □ = normal value of MCHC 29.5-35.6% (Thrall 2004).

radiation caused a decrease in MCHC value as much as 0.26% in the K+ group and increase 1.23% in R+ group.

MCHC values after week-4 decreased as much as 3.39% in the R- group, while the K- group decreased by 4.50% from the value before treatment. After recovery at week-8, MCHC value was increased by 1.95% in the K- group and MCHC value of R- group increased by 1.14% as seen in Table 1 and Figure 3 C. MCHC values of the treatment group and time were significantly different ($p < 0.05$).

MCHC values during treatment were in the normal range. Decrease in MCHC values were evident in the R- group that was treated with roselle extract at week-8 (Figure 4A). The cause of the decline in MCHC value after the recovery phase was uncertain. It was probably because there was a disrupted iron absorption in mice or there was a substance in roselle that caused disrupted absorption, so that the MCHC value decreased. Nonheme iron in plants is not easily absorbed by the intestines because plants contain oxalate, phytate, tannins and other phenolic compounds, which form chelates or precipitates with insoluble iron, preventing the absorption of nutrients. Tannin content in roselle can bind to iron so that iron absorption was disturbed (Besral et al. 2007). Vitamin C (ascorbic acid) can increase the amount of nonheme iron absorption from the gastrointestinal tract (Kasiyati 2007).

Howell Jolly Bodies

Average value of Howell Jolly bodies percentage of mice's peripheral red blood cells treatment groups were shown in Table 1. 2.9 mSv of total radiation at week-4 led to the emergence of Howell Jolly bodies by 0.10% in the K+ group, while in R+ group Howell Jolly bodies did not appears. It was also seen in the 5.3 mSv total radiation at week-8 in the K + as much as 0.13%, but in the R+ group

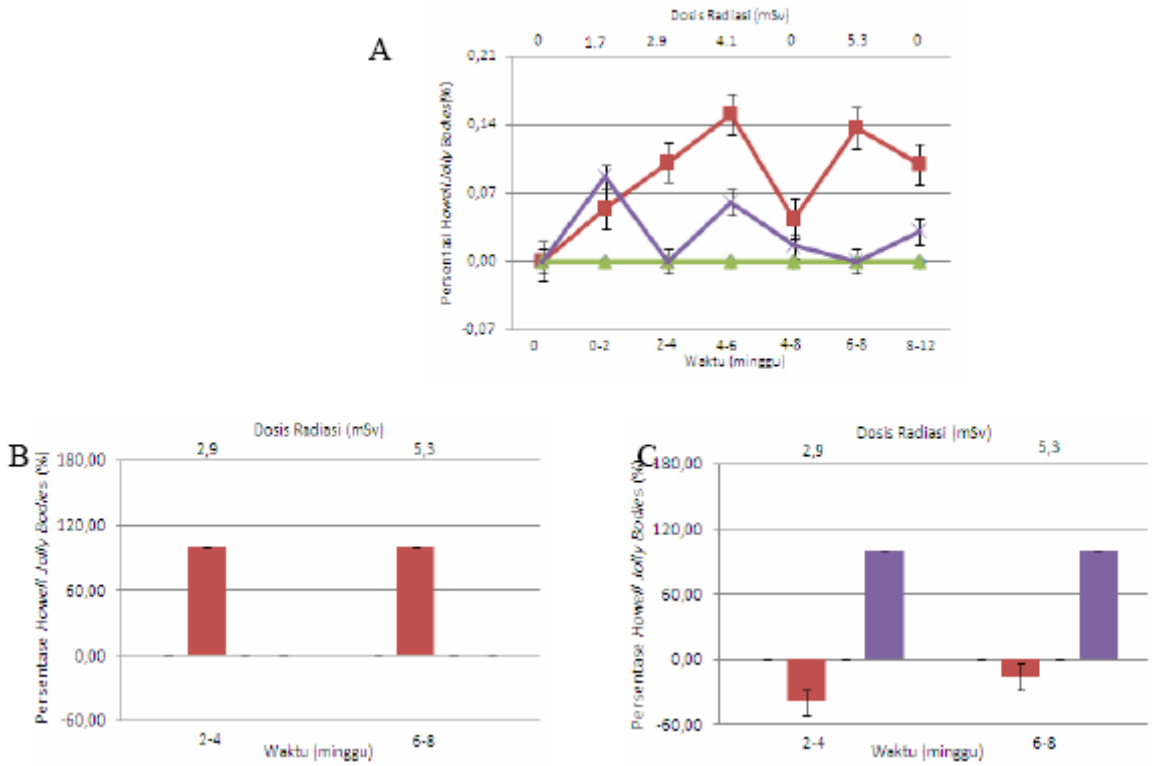

Figure 5. A. Howell Jolly bodies percentage on mice's peripheral blood reccurent radiodiagnostic; B. Howell Jolly bodies percentage after treatment; C. Percentage after 30 days of recovery. ■ (K-) = physiological saline without radiation exposure; ■ (K +) = physiological saline with radiation exposure; ■ (R-) = roselle extract without radiation exposure; ■ (R+) = roselle extract with radiation exposure.

Howell Jolly bodies were not also seen (Table 1 and Figure 5A). Howell Jolly bodies were not found in the K- and R- group. 2.9 mSv total radiation at week-4 caused an increase in the Howell Jolly bodies percentage as much as 100% from before the treatment in the K+ group. An increase up to 100% also occurred in 5.3 mSv radiation at week-8 in the K+ as seen in Table 1 and Figure 4B. Howell Jolly bodies percentage at week-4 and week-8 neither decreased nor increased in the treated group R-, K- and K + from the percentage before treatment as seen in Table 1 and Figure 5A.

After a recovery phase from 2.9 mSv radiation, it caused a decrease in the Howell Jolly bodies percentage as much as 39.60% in the K+ group, while the R+ group increased by 100%. Recovery after higher radiation (5.3 mSv) caused a decrease in the Howell Jolly bodies percentage as much as 15.82% in the K+ and increased 100% in R+ group. Howell Jolly bodies percentage after recovery phase at week-4 and week-8 neither increased nor decreased in both K- and R- groups as seen in Table 1 and Figure 4C. Howell Jolly bodies value of the treatment group and time were significantly different ($p < 0.05$).

Roselle ethanol extract can reduce the appearance of Howell Jolly bodies in red blood cells with a total radiation dose of 2.9 mSv, as well as the total radiation dose of 5.3 mSv. Number of free radicals formed by ionizing radiation influenced the antioxidant that found in the roselle petals ethanol extract. If the number of free radicals that are formed are too many, the ability of antioxidants to neutralize free radicals decreases (Evans et al. 2004) as occurs in the 5.3 mSv total radiation in R+ group (Figure 5A, 5B, and 5C). Antioxidant deficiency caused oxidative damage that led to cell damage such as the appearance of Howell Jolly bodies in red blood cells.

The presence of Howell Jolly bodies on peripheral red blood cells indicates red blood cells had leave the bone marrow in an immature state because of the high needs of the tissue and the increased erythropoiesis (Meyer & Harvey 2004). Release of immature erythrocytes into the tissue is a normal response of bone marrow to increase red blood cell production (Thrall 2004). Howell Jolly bodies indicating the presence of non-functioning spleen or spleen atrophy as Howell Jolly bodies should be eliminated in the spleen (Kovtunovych et al. 2010). In many splenectomy cases there were seen an increase in the presence of Howell Jolly bodies (Smith et al.1990). Howell Jolly bodies associated with regenerative anemia. Regenerative anemia caused by blood loss or destruction of erythrocytes (hemolysis) and can be seen in the recovery phase of bone marrow dysfunction. Hemolysis may occur in the blood vessels (intravascular) and outside the blood vessels (extravascular). Extravascular hemolysis occurs when abnormal erythrocytes were phagocyted by macrophages in the spleen or liver (Thrall 2004).

Hematopoietic cells are the cells that are sensitive to ionizing radiation of X-rays. Cells that are damaged by natural radiation have improved adaptability and responsiveness to become normal again, but the dose of radiation affects the biological damage that it cause. The greater radiation dose, the greater the damage as well (Mitchell et al. 2009).

Howell Jolly bodies that were formed due to radiation was much higher compared to the group that given the roselle extract. Adaptation response were seen in the 2.9 mSv and 5.3 mSv of radiation. Roselle can maintain the value of MCV, MCH and MCHC of red blood cells because they contain antioxidants, i.e. vitamin C (ascorbic acid) and phenolic compounds (Maryani & Kristiana 2009).

In general, MCV, MCH, and MCHC were in the normal range values, but most of MCV values were higher than normal value and lower than normal range. Oxidative damage caused by ionizing radiation was clearly visible in the MCV, MCH, and MCHC values decrease in the 5.3 mSv radiation dose. Increased and normal MCHC value were also called macrocytic normochromic anemia characterized by large size, but the concentration of erythrocyte hemoglobin within the normal range. This type of anemia is usually caused by disturbances of erythrocyte metabolism, deficiency of vitamin B12, folic acid and radiation. Normal MCV and MCHC values normally indicate the presence of anemia normochromic normocytic. This type of anemia is usually caused by blood loss, impaired kidney and bone marrow disorders (Thrall, 2004; Mayer & Harvey 2004).

According to Jagetia (2007) roselle in this study belongs to radioprotector agent. Radioprotector is administered prior to radiation and has the ability to prevent and minimize the damage of normal tissue. Roselle contains compounds called polyphenols, mainly flavonoids that can protect cells from damage by neutralizing free radical production that occurs during exposure to ionizing radiation (Jagetia 2007; Josiah et al. 2010).

Flavonoids are compounds that can protect the body from oxidative damage (Phipps et al.2007). The results showed that roselle was able to protect red blood cells from damage. Roselle antioxidants can suppress the formation of free radicals (Ologundudu et al. 2009). Chronic radiation dose was the dose of radiation in small doses with long exposure times. According to DeMasters et al. (2006), the body has the ability to replace damages or dead cells. Short-term recovery occurs every 24 hours after radiation. Long-term recovery occurs after 30 days of radiation exposure. Long-term and short-term recovery provide sufficient time to repair the radiation damage (Wambi et al.2008).

CONCLUSION

Roselle calyx extract has the radioprotective effect against damage caused by ionizing radiation of X-rays on the peripheral red blood cells of mice.

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