Significance of EMSA Eritin Administration on Erythropoiesis and Complement Regulators in Irradiated Mice

Radyasyona Maruz Kalan Farelerde EMSA Eritin Uygulamasının Eritropoez ve Kompleman Regülatörler Üzerindeki Önemi

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ABSTRACT
Objectives: This study aims to examine the effect of polyherbal erythropoiesis modulatory and stimulatory agent (EMSA) Eritin administration in modulating the activity of erythropoiesis on TER-119/VLA-4+ cells and complement regulators TER-119/CD55+ and TER-119/CD59+ cells in Balb/c mice after total body irradiation (TBI) with a dose of 500 rad.

Materials and methods: EMSA Eritin was administered orally in mice after exposure to TBI with a dose of 500 rad daily for two weeks with three different doses including a low dose (1.04 mg/g BW), normal dose (3.125 mg/g BW), and high dose (9.375 mg/g BW). Mice were treated with HEMAPO Epoetin alfa twice a week as positive control with a dose of 0.21 mg/g BW. On day 15, the bone marrow was isolated and the number of TER-119/VLA-4+, TER-119/CD55+ and TER-119/CD59+ cells were analyzed using flow cytometry. Examination showed marked reduction in the number of TER-119/VLA-4+ cells in the bone marrow of mice after TBI with dose of 500 rad compared to those of healthy controls. The number of TER-119/VLA-4+ cells in the irradiated mice was markedly increased with EMSA Eritin from low dose to high dose compared to mice with TBI and mice treated with Epoetin alfa.

Results: The highest dose of EMSA Eritin was more effective to promote erythropoiesis than HEMAPO Epoetin alfa and the other dose of EMSA Eritin.

Conclusion: Our study findings suggest that EMSA Eritin is a powerful medicinal herbal supplement which may serve as a protective agent to normalize homeostasis and erythropoiesis after irradiation with a dose of 500 rad.

Keywords: Antioxidant; bone marrow; complement regulators; erythropoiesis; total body irradiation.

ÖZ

Gereç ve yönteler: Düşük doz (1.04 mg/g BW), normal doz (3.125 mg/g BW) ve yüksek doz (9.375 mg/g BW) içeren EMSA Eritin uygulandı. Fareler pozitif kontrol olarak haftada iki kere 0.21 mg/g BW dozunda HEMAPO Epoetin alfa ile tedavi edildi. On beşinci günde kemik iliği izole edildi ve akış sitometrisi kullanılarak TER-119/VLA-4+, TER-119/CD55+ ve TER-119/CD59+ hücrelerin sayısı analiz edildi. İncelene, 500 rad dozunda TVI sonrası farelerin kemik iliğindeki TER-119/VLA-4+ hücrelerin sayısında sağlıklı kontrollere göre anlamlı azalma olduğu gösterdi. Radyasyona maruz kalanan farelerde EMSA Eritin ile TER-119/VLA-4+ hücrelerinin sayısı TVI olan fareler ve Epoetin alfa ile tedavi edilen farelere göre düşük dozdan yüksek doza anlamlı şekilde arttı.

Bulgular: En yüksek dozda EMSA Eritin eritropoezi destekledi HEMAPO Epoetin alfa ve diğer dozdaki EMSA Eritin’e göre daha etkili idi.

Sonuç: Çalışma bulgularınıza göre, EMSA Eritin 500 rad doz ile irradasyon sonrası homeostaz ve eritropoezi normalleştiren koruyucu bir ajan olduğu gösterilib ve güçlü bir tedavi edici herbal destekleyicidir.

Anahtar sözcükler: Antioksidan; kemik iliği; kompleman regulatörler; eritropoez; total vücut irradasyonu.
Radiotherapy is a treatment used to kill cancer cells with high dose X-rays or to prepare the body for bone marrow transplant. Radiotherapy is also used to kill malignant cells in chemotherapy. Radiotherapy for bone marrow transplant preparation is called total body irradiation (TBI). For this purpose, radiotherapy makes space for the marrow to be transplanted, and suppresses the immune system to prevent rejection in transplantation.\[1\] In cancer treatment, radiotherapy is the primary modality. Eighty percent of cancer patients require radiotherapy for curative treatment. The balance between the total dose of radiotherapy and the threshold of normal tissue around the cancer cells is significant to obtain optimum results. To control the growth of cancer cells more effectively by using high-dose radiation, normal tissue should be given the protection against radiation injury. Therefore, radioprotective compounds are very important in clinical radiotherapy.\[2\]

An injury due to radiation exposure is bone marrow suppression. Bone marrow suppression is the leading cause of death after exposure to moderate doses or high doses of TBI.\[3\] In addition to triggering bone marrow damage, radiation also disrupts the hematopoiesis system in long periods. Damage may take place as defect in renewal of hematopoietic stem cells and loss of the ability of self renewal.\[3\] Furthermore, the radiation may lead to neutropenia and thrombocytopenia, thus increasing morbidity and complications, such as infection and bleeding.\[1,4\] Management of acute myeloid suppression covers several hematopoietic growth factors, such as granulocyte-colony stimulating factor or erythropoietin. Hematopoietic growth factors induce the repair of bone marrow hematopoietic function by stimulating the proliferation and differentiation of hematopoietic stem cells and hematopoietic progenitor cells.\[3,4\] For developing countries, the provision of growth factors in patients who received radiation is still considered expensive. Management of radiation-induced bone marrow suppression using Erythropoiesis Modulatory and Stimulatory Agent (EMSA) Eritin has not been established yet. EMSA Eritin is a polyherbal medicine which consists of soy, coconut water and red rice that contains antioxidants and have put a damper function of reactive oxygen compounds and radiation-induced oxidative stress. In this study, we examined the effect of EMSA Eritin administration in modulating the activity of erythropoiesis based on profile of TER-119\(^{-}\)VLA-4\(^{-}\) and complement regulators based on the profile of TER-119\(^{-}\)CD55\(^{-}\) and TER-119\(^{-}\)CD59\(^{-}\) in Balb/c mice after TBI with a dose of 500 rad.

**MATERIALS AND METHODS**

This study was conducted at Animal Physiology Laboratory, University of Brawijaya. The duration of the study ranged between January 2015 until April 2015. This experiments were conducted with three replications in six groups (3 mice in each group): the healthy control group, the irradiated group, the irradiated group followed by HEMAPO Epoetin alfa (EPO) administration, and the irradiated group followed by EMSA Eritin administration with three different doses including dose 1 (D1), dose 2 (D2), and dose 3 (D3).

**Mice**

A total of 18 normal Balb/c female mice, between the ages of 7-8 weeks (weighted at least 25 g) were used. Mice were obtained from Gadjah Mada University, Yogyakarta and maintained in pathogen-free facility. The experimental protocol was approved by Ethical Clearance from the Research Ethics Committee (Animal Care and Use Committee) of University of Brawijaya. No. 255-KEP-UB.

**Total body irradiation**

Total body irradiation was performed using Cobalt-60 teletherapy NPICEM with a dose of 500 rad. Mice were placed in a box sized 10x10 cm\(^2\) and each box contained five mice. The dose was measured at the middle (the position of box) within the field for 50x50 mm\(^2\) at 80 cm source to surface distance for machine GWXJ80 of NPICEM China installed at Dr. Saiful Anwar Hospital, Malang. The gantry and collimator angles of the Cobalt-60 teletherapy units were kept at 0 degrees for these measurements.

**EMSA eritin and HEMAPO Epoetin alfa treatment**

EMSA Eritin doses for in vivo experiments were determined based on the human consumption of 60 kg of body weight that consume as much as 15 g of EMSA Eritin. EMSA Eritin doses were grouped into three: D1 (1.04 mg/g body weight), D2 (3.125 mg/g body weight), and D3 (9.375 mg/g body weight). EMSA Eritin was dissolved in distilled water until 1 mL and administered in mice by oral gavage (force-feeding) in 24 hours after radiation exposure once a day for two weeks. We also used EPO or HEMAPO Epoetin alfa at 0.21 mg/gram body weight with intraperitoneal injection twice a week.

**Bone marrow isolation and flow cytometry analysis**

The isolation of bone marrow was done by flushing out the femur and tibia of mice into the 50 mL Falcon tube by inserting a 26-gauge needle attached to the 20 mL syringe filled with phosphate buffered saline at the knee side of both types of bone. Phosphate buffered saline was passed through the bone until the color of the bone turned from red to white, indicating that all...
the marrow had been expelled and then collected into microtubes. The filtrate was centrifuged at 2500 rpm 4 °C for 10 minutes. The supernatant was discarded, washed once, and then centrifuged again to obtain a pellet of bone marrow cells, which was co-incubated with monoclonal antibodies: phycoerythrin (PE)/Cy5 anti-mouse TER-119/Erythroid Cells (clone TER-119), PE anti-mouse CD55 (DAF) (clone RIKO-3), PE anti-mouse CD59a (clone mCD59.3), and PE anti-mouse CD49d (VLA-4) for 15 minutes. Antibodies were purchased from BioLegend Inc. (San Diego, CA, USA). Next, the pellet was resuspended in 500 μl phosphate buffered saline and assessed via a BD FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data was then processed using the BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

**Statistical analysis**

One-way analysis of variance was used to analyze the data. The differences between groups were considered significant at p<0.05. All results were presented as the mean of ± standard deviation (SD) values of three mice in each group.

**RESULTS**

EMSA Eritin promoted erythropoiesis in irradiated mice. In examination of erythroid-lineage cells expression of TER-119 and VLA-4 antigen, an erythroid specific marker showed marked reduction in the number of TER-119+VLA-4+ cells in the bone marrow of mice after TBI with dose of 500 rad compared to healthy controls (18.06% vs. 51.4%). Furthermore, the number of TER-119+VLA-4+ cells in the irradiated mice was markedly increased with EMSA Eritin from low dose to high dose (65.61%, 72.15%, 79.70%, respectively) compared to mice with TBI and mice treated with HEMAPO Epoetin alfa (46.11%). The highest dose of EMSA Eritin was significantly more effective to promote erythropoiesis than HEMAPO Epoetin alfa and the other dose of EMSA Eritin (p<0.05) (Figure 1). Representative results of flow cytometric analysis was obtained from two weeks old mice after TBI.

**CD55 and CD59 expression levels in mice after total body irradiation and EMSA eritin treatment**

We further examined the effect of TBI and EMSA Eritin treatment on CD55+ and CD59+ expression on bone marrow cell. We showed depletion of CD55+ and CD59+ cells with dose of 500 rad in the bone marrow of mice after TBI compared to healthy controls (40.23% vs. 56.1% on CD55+ cells and 5.82% vs. 38.82% on CD59+ cells, p<0.05). CD55+ and CD59+ cells were significantly higher in HEMAPO Epoetin injected mice (CD55+ cells were 61.1%, whereas CD59+ cells were 18.3%, p<0.05), However, no significant difference was found in CD55+ and CD59+ cells counts in response to EMSA Eritin administration. (Figure 2 and 3). Irradiation dose of 500 rad affected hematopoiesis in bone marrow. We demonstrated that TER-119+ cells expressing CD55 (TER-119+CD55+) and CD59 (TER-119+CD59+) molecules were depleted after irradiation. The depletion of CD59 molecules on TER-119 cell was much greater than CD55 molecules. EPO injection elicited the expression of both molecules which almost reached to normal level. Interestingly, an oral

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**Figure 1.** EMSA Eritin accelerates growth and differentiation of erythroid lineages. The relative number of TER-119+VLA-4+ cells in bone marrow of mice after two weeks of treatment and analyzed by flow cytometry. HC: Healthy Control (un-irradiated mice); C+: Positive control (irradiated mice); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW); D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW). On day 15, mice were terminated and analyzed using flow cytometry (left) and tabulated into Microsoft Excel (right). Data are mean of ± standard deviation values of three mice in each group with p-value <0.05. Different letters indicate significant difference based on Tukey’s high significant differences test at a 95% significance level.
gavage of EMSA Eritin increased the expression of both CD55 and CD59 molecules much higher when compared to those of normal mice or EPO treated mice.

**DISCUSSION**

Irradiation may cause to impairment of bone marrow hematopoietic function and may lead to the leucopenia, erythropenia, and thrombocytopenia which in turn may develop predisposition to infection, hemorrhage, and death. EMSA Eritin has a wide range of important components such as genistein, cytokinin, nicotinic acid, pantothenic acid, biotin, riboflavin, folic acid, thiamine B1, vitamin C, pyridoxine, daidzein, glycitein, phenolic acids, and anthocyanins. The content of EMSA Eritin might contribute to the repair of hematopoietic process in mice after irradiation. EMSA Eritin normalized the hematopoietic system and increased the survival rate of mice after irradiation although the mechanism remains unknown. The largest composition in EMSA Eritin is soybean which contains some isoflavones including genistein, daidzein and glycitein. Soybean contains more genistein than other isoflavones which is about 0.2-1 mg/g.

**Figure 2.** EMSA Eritin prevents red blood cell damage by increasing CD55 expression in vivo. The relative number of TER-119+CD55+ cells in bone marrow of mice after two weeks of treatment and analyzed by flow cytometry. HC: Healthy control (un-irradiated mice); C+: Positive control (irradiated mice); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW); D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW). On day 15, mice were terminated and analyzed using flow cytometry (left) and tabulated into Microsoft Excel (right). Data are mean of ± standard deviation values of three mice in each group with p-value <0.05. Different letters indicate significant difference based on Tukey’s high significant differences test at a 95% significance level.

**Figure 3.** EMSA Eritin prevents red blood cell damage by increasing CD59 expression in vivo. The relative number of TER-119+CD59+ cells in bone marrow of mice after two weeks of treatment and analyzed by flow cytometry. HC: Healthy control (un-irradiated mice); C+: Positive control (irradiated mice); EPO: Irradiated mice followed by intraperitoneal injection of HEMAPO Epoetin alfa treatment; D1: Irradiated mice followed by oral administration of low dose of EMSA Eritin (1.04 mg/g BW); D2: Irradiated mice followed by oral administration of normal dose of EMSA Eritin (3.125 mg/g BW); D3: Irradiated mice followed by oral administration of high dose of EMSA Eritin (9.375 mg/g BW). On day 15, mice were terminated and analyzed using flow cytometry (left) and tabulated into Microsoft Excel (right). Data are mean of ± standard deviation values of three mice in each group with p-value <0.05. Different letters indicate significant difference based on Tukey’s high significant differences test at a 95% significance level.
although this ratio varies in different soy products. Genistein in soybean has structural similarity to estradiol but rather weaker estrogenic activities (10-2 to 10-3 fold). Many studies have demonstrated that genistein is one of the most important phytoestrogens which has an ability in inhibition of tyrosine kinase activities and helps to improve the immune system. Consequently, it has strong beneficial effects on persons with breast cancer, prostate cancer, cardiovascular disease, high cholesterol levels, and osteoporosis. As a radioprotective agent, genistein plays a significant role in reducing the acute cellular effects in normal cells caused by radiation exposure from environment or cancer treatment. The other content of EMSA Eritin is red rice. Red rice has colored pigmentation or grains which are distinguished by red or dark purple pigment enveloping outer layer of rice. Pigments located in the aleurone layer of grains have been reported as a mixture of components of anthocyanin, which belong to the flavonoid family. Anthocyanin in red rice is known to be a radioprotector. Meanwhile, coconut water contains cytokinin consisting of kinetin and riboside. Kinetin contained in coconut water can reduce the formation of reactive oxygen species and acts as an antioxidant and indirectly acts as a regulator of antioxidant. The content of genistein and other substrates in EMSA Eritin might contribute to ameliorate the hematopoietic process in mice after irradiation. EMSA Eritin repaired the hematopoietic system and improved recovery process and survival of mice after irradiation. Although the mechanism of action by which EMSA Eritin increases erythrocyte production is unknown, some researches elucidated that genistein contained in soybean is useful to prevent blood cell damage and to increase hematopoiesis.

It has been demonstrated that VLA-4-mediated signaling led to cytokine gene expression and autocrine growth in mature T lymphocytes and eosinophiles. Cells committed in early hematopoiesis process will express VLA-4 that plays a role in directing the path towards erythropoiesis than lymphopoiesis. TER-119 is a cell surface marker specific for erythroid subsets in the early stages of pro-erythroblast development into mature erythrocytes, so the TER-119 is a marker of mature erythrocytes. The existence of cells expressing TER-119+VLA-4 indicates that cells are in a reticulocyte phase.

The present study revealed that administration of EMSA Eritin into irradiated mice increases the survival and stimulate recovery of hematopoietic system by increasing number of TER-119+VLA-4 after falls of bone marrow induced by irradiation. Interestingly, the efficacy of EMSA Eritin on enhancement of the survival and promoting recovery of erythrocyte was stronger in high dose than low and normal doses in our experiments. Floersheim, et al. has concluded that genistein affords hematological protection by both preventing the destruction blood cells and enhancing hematopoietic recovery.

CD55 and CD59 are glycosylphosphatidylinositol-anchored proteins expressed on hematopoietic and non-hematopoietic cells and very essential for erythrocyte survival. Both CD55 and CD59 are named as decay accelerating factors which are responsible for protecting the cell from damage by complement activation and polymerization of membrane attack complex. The abnormality in expression of these proteins is a leading cause of paroxysmal nocturnal hemoglobinuria which in turn augments sensitivity to complement mediated cytolysis and results in hemolytic anemia. A significantly reduced anchorage of CD55 and CD59 to erythrocytes was detected from mice after TBI when compared to controls. It was tempting to speculate that the depleted expression of CD55 and CD59 by erythrocytes from mice is due to a decreased synthesis of the glycosylphosphatidylinositol anchor or abnormal coupling of the protein to its membrane cohering by the reticulocyte or red blood cell progenitors.

The administration of EMSA Eritin to the irradiated mice increased and stimulated the complement regulators through the expression of TER-119+CD55+ and TER-119+CD59+ after TBI. Interestingly, the numbers of TER-119+CD55+ cells and TER-119+CD59+ cells were increased in mice who had low dose EMSA Eritin compared to those who had high dose EMSA Eritin. We may speculate that this was due to an increased cleavage of CD55 and CD59 proteins from the erythrocyte surface by enzymatic activity like the phosphatidylinositol-specific phospholipase C (and even D) which yielded increased serum levels of soluble CD55 and CD59, although such an enzyme is specific for phosphatidylinositol and not for CD55 and CD59. To our knowledge, erythropoietin is the most known drug to drive hematopoietic stem cells to differentiate rapidly and become mature blood cells. In our study, erythropoietin increased the number of reticulocyte and complement regulators but EMSA Eritin had a much better effect in promoting the erythropoiesis and complement regulators through the higher relative number of TER-119+VLA-4+, TER-119+CD55+ cells, and TER-119+CD59+ cells compared to EPO.

In summary, we demonstrated that EMSA Eritin administration after TBI with dose of 500 rad plays a role in increasing the survival rate and promoting the erythropoiesis and complement regulators. Furthermore EMSA Eritin may be a protective agent to minimize or prevent the damage from irradiation. Although our results might provide some basis for the possible use
of EMSA Eritin as a radioprotector of hematopoietic system, further studies are necessary to determine the mechanism of its radioprotective action.

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REFERENCES