ORIGINAL ARTICLE

In Vivo Neuroprotective Activity of Erythropoietin-Alginate Microspheres at Different Polymer Concentrations

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Abstract

Aim: The aim of this study was to evaluate *in vivo* neuroprotectant activity of erythropoietin-alginate microspheres with different concentrations to modify release to reduce the administration frequency of erythropoietin. **Materials and Methods:** Sodium alginate and $CaCl_2$ were used to produce microspheres using aerosolization technique and erythropoietin as model. Balb/c-strain mice (*Mus musculus*) were used to study *in vivo* activity in terms of locomotor activity and glutathione peroxidase (GSHPx) activity as well as percentage of reticulocytes. **Results and Discussion:** Erythropoietin-alginate microspheres demonstrated neuroprotectant activity such as locomotor activity and GSHPx activity compared to erythropoietin alone and blank microspheres. Increased polymer concentrations (1–3%), however, did not have a significant effect on changes of the effectiveness of the activity of microspheres. Interestingly, these results also showed that erythropoietin-alginate microspheres which contain only 5000 units produced a high percentage of reticulocytes. **Conclusion:** The study indicates that erythropoietin-alginate microspheres showed the potential activity of erythropoietin-alginate microspheres although in low concentration. Increased concentrations of polymers showed similar effects in locomotors activity and GSHPx enzyme activity as parameters of neuroprotectant activity.

Key words: Alginate microspheres, erythropoietin, glutathione peroxidase, *in vivo* activity, neuroprotectant

INTRODUCTION

Erythropoietin is a glycoprotein hormone that is primarily secreted by the kidney in adults and by the liver in fetuses. This hormone acts on bone marrow cells to stimulate the production of red blood cells, also called hematopoietin and hemopoietin.[1] In recent years, erythropoietin has been extensively studied and it has been shown that erythropoietin and its receptor are also present in other tissues, including the brain, reproductive tract, lungs, spleen, and heart.[2] This study opens the possibility that EPO not only works as an erythropoietic hormone but it can also act as a neuroprotectant in other tissues.

Erythropoietin has several disadvantages, such as being difficult to absorb in the body because of its large molecular weight, short half-life, unstable within biological fluid, and easily degraded by enzymes,[3,4] and is sensitive to temperature. In addition, erythropoietin at 60°C indicated aggregation.[5] Therefore, there should be an appropriate delivery system to improve the weakness of erythropoietin, one of which using a microsphere delivery system.

Microspheres are the result of a microencapsulation process. Microencapsulation is a laminating or coating process using a polymer to obtain a small particle size.[6] The microspheres have many uses, such as making sustained release and controlled release preparations, protecting the drug from the environment (light, moisture, temperature, and oxidation), and covering taste and smell.[7] There are several methods that are often used for the manufacture of microspheres, one of which is the ionotropic gelation method. The manufacture of microspheres by this method is done by dripping the polymers into a crosslinking solution. In the preparation of

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microspheres by this ionotropic gelation method, there are several factors affecting the resulting microsphere, such as the ratio of the polymer concentration, the crosslinking solution level, and the crossover time, which will affect the particle size and distribution, entrapment efficiency, and drug release profile.[8] Benefits include encapsulation of drugs for stable, uniform, and spherical particle size, easy and fast process, safe drug, and relatively low costs.[9]

Polymers that are often used in the manufacture of microspheres are alginates. Alginate is a natural polymer extracted from brown algae and has properties that enable it to be a matrix in drug entrapment. Alginate is composed of a (1-4)-β-D-mannuronic acid (M) unit and a (1-4)-α-L-guluronic acid (G) unit arranged in homopolymer form (MM- or GG-block) and heteropolymer circuit (MG- or GM-block).[10] The frequently used crosslinking agents are Ca^{2+} and Ba^{2+} ions.[9] The addition of Ca^{2+}, Ba^{2+}, or other two-valency cations will form gelation through specific ionic bonds and may cause conformational changes to the sodium alginate structure.[11] Ca^{2+} crosslinker microspheres have greater trickle efficiency and more sustained and controlled drug release rates than microspheres with Ba^{2+} crosslinker.[9]

This study aimed to study the effect of alginate polymer (1%, 2%, and 3%) on the effectiveness of *in vivo* erythropoietin alginate microspheres as a neuroprotectant or neuroprotective agent in experimental animals induced by 1-methyl-4 phenyl-1,2,5,6-tetrahydropyridine (MPTP).

MATERIALS AND METHODS

Materials

Recombinant human erythropoietin (PT Daewoong Infion); pharmaceutical grade sodium alginate; pharmaceutical grade $CaCl_2.2H_2O$; pharmaceutical grade maltodextrin; pharmaceutical grade Sodium citrate; bicinchoninic acid reagent; saline phosphate buffer; pharmaceutical grade MPTP; purified water; Balb/c-strain mice (*Mus musculus*); NADPH; H_2O_2; glutathione (GSH); and GSH reductase were used.

Microsphere Formula

Erythropoietin-Alginate Microsphere Production

Sodium alginate (according to the formula) was dissolved in 100 ml of demineralized water. Erythropoietin was dispersed into alginate solution and was stirred until homogeneous. The resulting erythropoietin-alginate solution was sprayed using aerosol spray with a hole size of 35 μm, a constant pressure of 40 psi, and a spraying distance of 8 cm into 100 ml of $CaCl_2$ and was stirred constantly for 30 min at a speed of 1000 rpm. Microspheres were centrifuged at 2500 rpm for 6 min and then washed using distilled water 2–3 times. The washed microspheres were suspended in a 5% maltodextrin solution and then dried using freeze-drying at −26°C for 30 h.

Experimental animals

Mice (*M. musculus*) of BaIb/c strain obtained from Pusat Veterinary Farma, Surabaya, were used with the criteria as follows:

a. Inclusion
   - Male sex
   - Weight 20 g–30 g or 2.5–3 months old
   - Healthy mice condition
   - No defects or injuries to the body.

b. Exclusion
   - Injured during the study
   - Death due to squeezing, fighting, and cannibalism or
   - Other causes.

Mice were adapted for 1 week in a room with a certain temperature in a previously partitioned cage. The partition was done so that the mice do not fight with each other. This is because adult male mice tend to fight with other adult mice when placed in the same cage.

Number of experimental animals

The determination of the number of replications was done using the following formula:[12]

$$n=(Z\alpha - Z\beta)^2 \frac{S^2}{d^2}$$

$$n=[1.96-(-1.645)]\frac{1.5^2}{2.5^2}$$

$$n=5$$

From this calculation, it was determined that the minimum number of experimental animals required was five mice for each group. In this study, six mice were used in each group. A total of 42 mice were selected for seven treatment groups.

Dose determination

Based on research conducted,[4] the dose of erythropoietin for neuroprotectant was 5000 units/kg in total for mice body weight. Therefore, the dose of erythropoietin administered to the experimental animals weighing 20–30 g is 100–150 units. This study used the dose for *in vivo* effectiveness test on erythropoietin-alginate microspheres. The dose of animals was done by determining the content of erythropoietin in alginate microspheres. Then, from the content of erythropoietin, the number of erythropoietin alginate microspheres administered to the experimental animals was calculated:

$$\text{EPO content} = \text{Amount of EPO in formula} \times \% \text{ Protein loading}$$

$$\text{Dry microsphere dose} = \frac{\text{Average weight of dry microspheres} \times \text{Mice dose}}{\text{EPO content}}$$

Independent Variables

An independent variable was variable that became the main cause of the subject matter study, such as:

- Formula
- Treatment of each group.

Controlled variables

Control variables were factors that need to be controlled to avoid the interfere with the experimental process.

- Age, sex, weight, and species of mice used.

Dependent variables

Dependent variable or dependent variable was variable that showed results of the caused variables, such as:

- Locomotors activities
- Number of reticulocytes
- GSH peroxidase (GSHPx) activity.

Treatments of experimental animals

Observation of Locomotors Activities

Observation of animal locomotors activities was attempted to determine motor functions, which was done using the open field method. The measurement was done by observing experimental animal crossings from one region to another. Experimental animals were habituated in the observation area for 20 min, and then, the number of crossings in the past 10 min was calculated. The crossing measurements were then compared with the normal group. The data were then analyzed using statistics through two-way ANOVA.

Measurement of blood reticulocytes

The measurement of reticulocyte amount was performed using fluorescence flow cytometry method with Sysmex XT-1800 i. The number of reticulocytes between treatment groups was compared. The data were then analyzed using statistics through one-way ANOVA.

GSHPx activity determination

GSHPx activity was analyzed using colorimetric measurement by measuring the oxidation of NADPH at a 340 nm wavelength in the presence of hydrogen peroxide, GSH, and GSH reductase. The standard used consisted of 0.1 M phosphate buffer (pH 7.0); 1 mM GSH; 0.2 mM NADPH; I, 4 IU GSH reductase; 0.25 mM H_2O_2, and 0.04 ml of supernatant fluid from the sample.[13] The activity of GSHPx can be calculated through the absorbance changes that occurred to the blanks which did not contain homogenate. The result is nmol value of NADPH per mg of protein.[13] Data analysis was done using statistics through one-way ANOVA.

RESULTS

Infrared spectra examination

The observation of spectra of F1-F3 showed interactions between drug, polymer, and crosslinking $CaCl_2$ solution [Table 1, Figures 1 and 2]. Such interactions were marked by shifting wavelength numbers, the loss of guluronic fingerprint absorption, and one absorption of the carboxylate salt group (1614 cm^{-1}) from natrium alginate due to crosslinking reaction with $CaCl_2$.

Observation of locomotor activities

The observation of locomotor activity of animals was attempted 3 times during the trial, namely before the trial, after the last MPTP administration (4^{th} day of trial), and after the last erythropoietin administration (7^{th} day of trial). Observations were done using the open field. The amount crossings of experimental animals in the past 10 min were counted with 30 min of observation time. The observation results are shown in Figure 3.

The locomotors activity of the experimental animals was conducted using a two-way ANOVA analysis to determine whether there was a difference in the number of crossings at different times, namely pre-treatment, after the last MPTP administration, and after 7 days of erythropoietin administration in each group. Results of the analysis showed that there was no significant difference between the number of crossings before treatment, after the last MPTP administration, and after 7 days of erythropoietin administration in each group. However, there was a significant difference in the number of crossings after the last MPTP administrations.

Measurement of blood reticulocytes

Reticulocyte measurements were performed to determine the effects of erythropoietin that play a role in the process

Table 1: Erythropoietin-alginate microsphere formulas

Materials	Function	F1	F2	F3
Erythropoietin (unit/kg BW)	Active agent	5000	5000	5000
Na alginate (%)	Polymer	1	2	3
$CaCl_2$ (M)	Crosslinker	1	1	1
Crosslinking time (min)	-	30	30	30
Maltodextrin (%)	Lyoprotectant	5	5	5

BW: Body weight

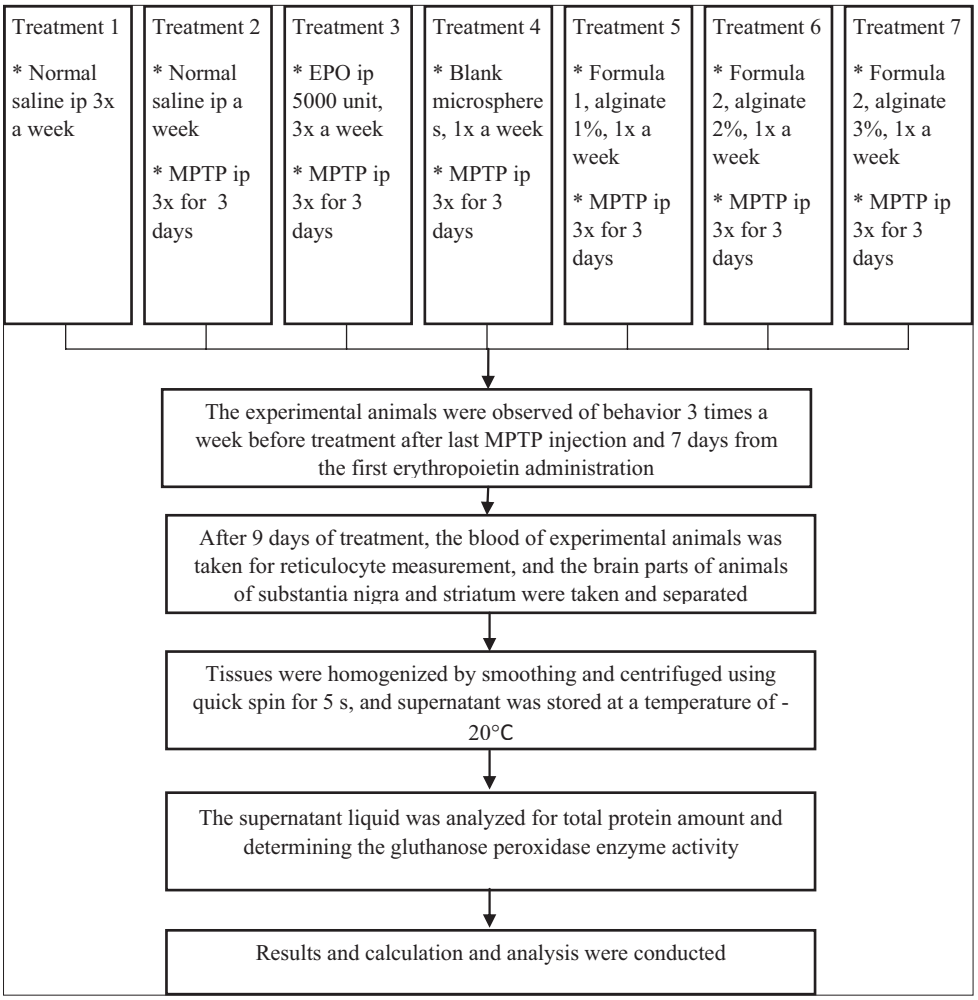

Figure 1: Experimental animal treatment scheme

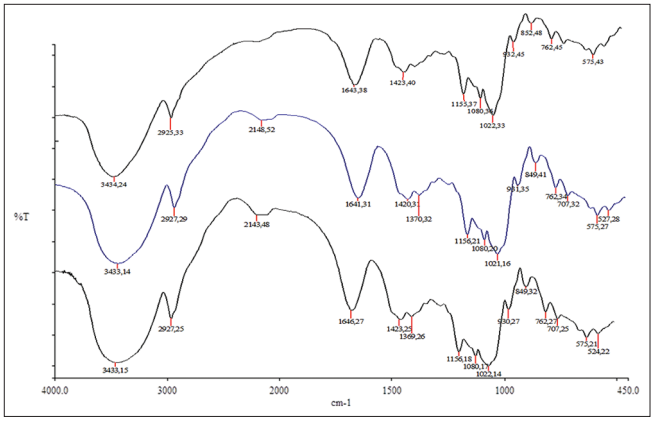

Figure 2: Spectra of erythropoietin-alginate microspheres of F1, F2, and F3

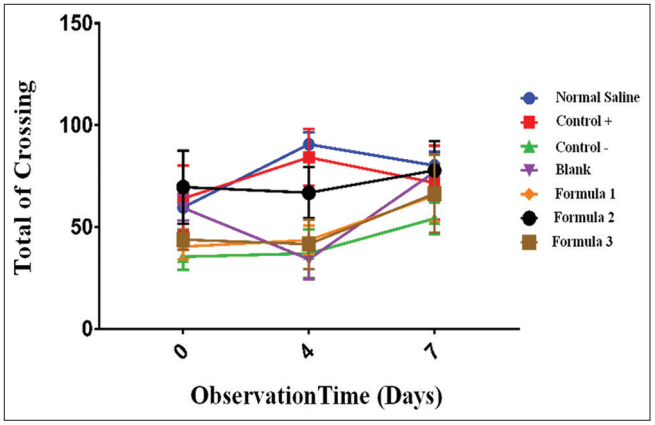

Figure 3: Number of crossings by experimental animals in each treatment group

of the formation of red blood cells. The following results of reticulocyte measurements in experimental animals' blood after 7 days of erythropoietin administration are shown in Figure 4.

In the analysis of the effect of treatment group on reticulocyte count, it was found that there was a significant comparison in P1:P6 with a $P = 0.016 < 0.05$, P2:P5 with a $P = 0.036 < 0.05$, P2:P6 with a $P = 0.005 < 0.05$, and P4:P6 with a $P = 0.012 < 0.05$. The results showed that the percentage of reticulocytes in the animal group of Formula 1 gave significant differences to the positive control group of animals and the group of Formula 2 gave significant differences to the normal saline, positive, and blank groups.

Thus, formula 1 and formula 2 with alginate concentrations of 1% and 2% and $CaCl_2$ 1M with 5000 units of erythropoietin increased the reticulocyte level greater than the positive control group but did not give significant differences to the negative group. Formula 3 in the analysis did not make a significant difference with the other groups. However, in Figure 4, the resulting reticulocyte percentage was higher than all control groups, except for Formula 1, Formula 2 and negative groups.

GSHPx activity determination

Activity determination of GSHPx used the previous method.[13,14] The activity of GSHPx is shown in Figure 5.

On the measurement of GSHPx enzyme activity, one-way ANOVA analysis was used to determine the difference of GSHPx enzyme activity between treatment groups in the substantia nigra and striatum. Figure 5 shows the results of the analysis which showed no significant differences in all groups of animals in the substantia nigra and striatum.

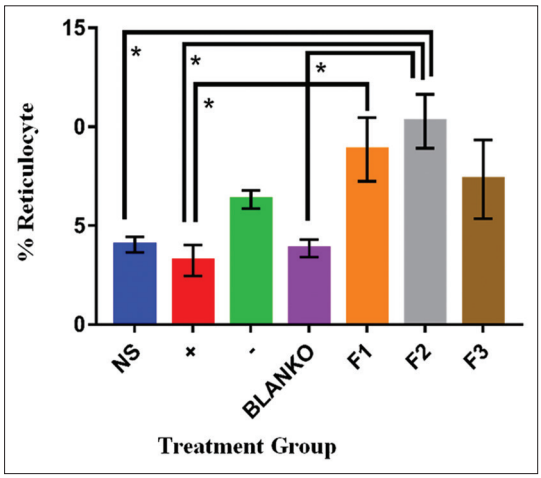

Figure 4: Reticulocyte percentage retrieved in each treatment group

DISCUSSION

The observation of neuroprotectant activities was determined as locomotor activity and GSHPx activity. Based on the results, there was a decrease in locomotor activity after the last MPTP administration to the experimental animals of the negative control group, blank group, F1 group, F2 group, and F3 group, but not for normal saline and positive control groups. An increase in locomotor activity after 7 days of erythropoietin administration was showed. The decrease in locomotor activity was due to the induction of administered MPTP. Locomotor activity increased again in 7 days after erythropoietin administration due to a lack of MPTP administered. This was also because the experimental animals were recovering within a few days of MPTP administration, causing the locomotor activity of experimental animals to increase on the last day of observation.[15] According to Meredith and Rademacher,[16] MPTP for 3 days is included in subacute regimen, where the experimental animals tried to survive well on the subacute regimen and with only a slight decrease in motor cells.

Reticulocyte percentage measurements depend on the red blood cell cycle,[17] where it is known that mice have a faster rate of blood regeneration compared to rats,[18] so this retrieval time greatly affected reticulocyte percentage measurements. If the blood-taking time has passed the peak period of reticulocyte formation, then the percentage of reticulocytes acquired was not maximum. In this study, no reticulocyte percentage was measured before treatment, so the percentage of reticulocytes beginning in each experimental animal could not be known in detail. However, based on the research,[19] erythropoietin-alginate microspheres increased a number of blood cells and hemoglobin, and this was in accordance with the results of the study. The administration of 5000 units of erythropoietin in alginate microspheres significantly increased reticulocyte percentage compared to normal saline, positive, and blank groups.

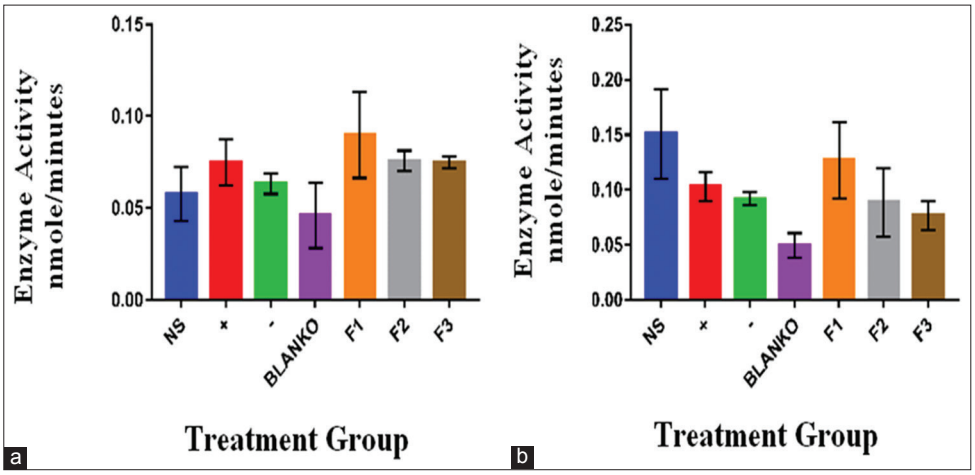

Figure 5: Glutathione peroxidase enzyme activity in experimental animals in substantia nigra (a) and striatum (b) after 1-methyl-4 phenyl-1,2,5,6-tetrahydropyridine and erythropoietin formula administrations; $n = 3$

Dopaminergic nerve damage in substantia nigra and striatum of the neuron parts was experienced by MPTP-induced animals in this research. The dopaminergic nerve damage in MPTP-induced experimental animals can be seen from GSHPx enzyme activity. GSHPx is an enzyme that plays a role in protecting cells from damage caused by free radicals.[20] The results of the statistical analysis in the study showed no significant differences between treatment groups. This can be due to the provision of MPTP for 3 days administered in subacute regimen; the experimental animals survive from MPTP; therefore, there is no major damage at the dopaminergic nerves. Low doses also affected the toxic effects of MPTP, thus rendering the toxic effects of MPTP temporary. The MPTP toxic effect can be long-lasting if the MPTP given was in the right regimen. Subacute regimen with MPTP was performed at an initial dose of 10 mg/kg BW for 1 day at 1-h intervals, whereas in this study, the given dose was 4 mg/kg BW for 3 days at 24-h intervals.

The overall performance of the erythropoietin-alginate microspheres yielded an increase in reticulocyte levels in microspheres formula compared to the positive control group (erythropoietin only). These showed the potential activity of erythropoietin-alginate microspheres although in low concentration. Moreover, increased concentrations of alginate polymers in erythropoietin-alginate microspheres showed similar effects in locomotors activity and GSHPx enzyme activity as parameters of neuroprotectant activity.

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