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RESEARCH ARTICLE

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# Bioassay of Recombinant Human Granulocyte Colony Stimulating Factor (rhG-CSF) for Neutropenia Treatment in Male Sprague Dawley Rats

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**Background:** Recombinant human granulocyte colony stimulating factor (rhG-CSF) is a first line therapy for neutropenia. However, it is less affordable for most patients in developing and poor countries. Therefore, biosimilar products are developed to suppress the cost of treatment, namely with rhG-CSF. This study aimed to explore the establishment of an affordable rhG-CSF that has similar potential to induce neutrophils recovery as the positive control.

**Materials and Methods:** The rhG-CSF was expressed as inclusion body in *Escherichia coli* NiCo21(DE3). The inclusion body was then solubilized, refolded, purified and characterized prior to be used in the bioactivity assay. Cyclophosphamide-induced male Sprague Dawley rats were used as animal model and administered with rhG-CSF. Blood sample was collected at several points of time, before and after rhG-CSF treatments. Complete blood count and peripheral blood smear were conducted to investigate the activity of the rhG-CSF on each blood cells type, particularly neutrophil.

**Results:** Specific activity on neutrophil proliferation was shown after treatments with our rhG-CSF and positive control. Positive control dose 40 mg/kg BW was statistically similar with that of the rhG-CSF dose 80 and 120 mg/kg BW. However, in neutropenic condition, recovery of neutrophil counts could not be achieved within 4 days of treatments. Thus, a longer treatment is needed to observe the activity of the rhG-CSF as an antineutropenia agent.

**Conclusion:** The rhG-CSF has been proven having specific activity on neutrophil proliferation. However, improvement in the rhG-CSF preparation is still needed and longer administration of the rhG-CSF has to be applied in the future study.

**Keywords*:*** rhG-CSF, biosimilar, neutropenia, Sprague Dawley rats

## Introduction

Granulocyte colony stimulating factor (G-CSF) or colony stimulating factor 3 (CSF-3) is a glycoprotein-cytokine with around 20 kDa molecular weight that plays role in hematopoesis control, particularly in neutrophils maturation.[1] Lacking of G-CSF generates reduction of progenitor cells in bone marrow and spleen.[2] Chemotherapy administration is

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known as the main cause the progenitor cells suppression.[3-5] Neutropenia or condition with neutrophil less than 1500 cells/µL is generally found in patients with cancer who received chemotherapy regimen.[3] Lower neutrophils counts in peripheral blood attenuates immune response, thus patients with neutropenia are vulnerable to infection. In patients with cancer, infection leads to severe complication and increases death risk.[3] Therefore, synthetic G-CSF is then suggested for patients receiving such chemotherapy treatments. It induces proliferation and differentiation of neutrophils to regain normal counts of neutrophils in the peripheral blood (1500-8000 cells/µL).[6,7]

The synthetic G-CSF or filgrastim or Neupogen which was developed as a recombinant protein and produced in *Escherichia coli* was approved for clinical used in 1991.[8,9] It has been used as the first line therapy for chemotherapy-induced neutropenia and proven to be well tolerated by the patients.[10]

Following the patent expiration of Neupogen as the recombinant human G-CSF (rhG-CSF) originator, development of rhG-CSF biosimilar has become interesting for researchers and pharmaceutical industries. The biosimilar products are potential to reduce cost for development and manufacturing, thus they are potentially marketed with almost 50% lower price than the originator is.[11,12] A list of rhG-CSF biosimilars has been approved by drug regulator such as European Medicine Agency (EMA), namely Tevagrastim (2008); Ratiograstim (2008); Biograstim (2008); Filgrastim Hexal (2009); Zarzio (2009); Nivestim (2010); Grastofil (2013); and Accofil (2014).[13]

Our group has developed and characterized the synthetic soluble thioredoxin fused-rhG-CSF (Trx-rhG-CSF) in *E. coli* expression system. However, purification of rhG-CSF from Trx is high cost and low-yielding.[14-17] Therefore, in this study, rhG-CSF was expressed as insoluble protein without Trx-fusion and found abundantly in inclusion body agregates which are caused by misfolded protein. The inclusion body was then solubilized and purified to obtain active rhG-CSF.[18] The active rhG-CSF was evaluated and compared with a commercial rhG-CSF which has been marketed and clinically used in Indonesia.

The activity of our rhG-CSF as antineutropenia agent was investigated using cyclophosphamide induced animal model. Cyclophosphamide is an alkylating agent used as antineoplastic and immunosuppresants. It binds to a DNA strand resulting inhibition of cancer cells proliferation.[19,20] However, by reason of non-selective action, it is potential to cause a number of adverse effects, such as myelosuppresion, haemorrhagic cystitis, gastrointestinal toxicity, infection, and also includes neutropenia.[21,22] Therefore, cyclophosphamide was frequently used in preclinical study to develop neutropenic animal model.[23-25] In the recent study, the induced animal model was treated with the rhG-CSF in various doses and compared with positive control that had been tested in a pre-clinial study.[26] Complete blood cells analysis was performed to monitor the effect of rhG-CSF on each types of blood cell, particularly on neutrophils. This study aimed to explore whether we could established an affordable rhG-CSF that has similar potential to induce neutrophils recovery as positive control in treating neutropenia, particularly after chemoterapy regimen.

## Materials and methods

### *Expression, Purification, and Characterization of rhG-CSF*

The rhG-CSF was expressed as inclusion body from an expression vector patented in Patent No. P00201708906 by IPTG induction 0.1 mM (Thermo Scientific, Massachusetts, USA) in Luria Bertani medium which was incubated at 37°C, 150 rpm, overnight.[27] From 100 mL cells culture, cells pellet was obtained by centrifugating at 6,000 rpm for 6’ and then resuspended in phosphate buffer saline enriched with 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by sonification for 5’ on ice and washed in buffer pH 7.9 containing 20 mM Tris-Cl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton-x. Afterwards, the remaining pellet that was known as inclusion body was solubilized and refolded.[18] Refolded protein was purified using Amicon® Centrifugal filter 10 and 30 molecular weight cut-off (MWCO) (Merckmillipore, Massachusetts, USA). Profiles of solubilized and refolded protein were analyzed with Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and characterized with western blot using monoclonal anti-rhG-CSF antibody. All other chemical reagents were purchased from Merck (Darmstad, Germany) and Sigma (Santa Cruz, USA).

### *Preparation of Recombinant Human Granulocyte Colony Stimulating Factor Dosage Form*

The purified rhG-CSF was lyophilized in phosphate buffer saline using a freeze dryer (Alpha 1-4 LDPlus, Christ, Osterode am Harz, Germany). It was immediately

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reconstituted in sterile aquabidest (Ikapharmindo Putramas, Jakarta, Indonesia) before administered in animal model. The positive control, Leukokine (Novell Pharmaceutical Laboratories, Bogor, Indonesia) was reconstituted in 5% dextrose (Widatra Bhakti, Jakarta, Indonesia) following the manual instruction. Each reconstituted rhG-CSF with different doses, positive control (before reconstituted), and not lyophilized rhG-CSF were analyzed for absorbance and fluorescence spectrum using Varioskan (Thermo Scientific).

***Administration of rhG-CSF to Neutropenic Male Rats Sprague Dawley***

The following procedure was approved by Animal Care and Use Committee of Institut Pertanian Bogor – ACUC No. 84-2017. Healthy male Sprague Dawley rats aged 2-3 months with body weight 200-300 g (Biofarmaka, Institut Pertanian Bogor, Indonesia) were selected and grouped into six groups (six rats per group). Each groups acquired different treatments as follow: Group 1 as normal control which was not received either cyclophosphamide (Endoxan) induction or rhG-CSF treatment; Group 2 as negative control received 5% dextrose, subcutaneous (s.c); Group 3 as positive control (Leukokine 40µg/kg BB, s.c); Group 4; 5; and 6 administered with the rhG-CSF 40; 80; 120 µg/kg BW, s.c, respectively. Positive control dose was calculated with dose conversion from human to rat.[28]

The experiment design, specifically in time of induction, treatment, and blood sampling was referred to previous study.[23] Rats in Group 2-6 were induced with cyclophosphamide 100 mg/kg BW, intra peritoneally (i.p) at 24 h after baseline blood sampling (Day -1). The rhG-CSF treatments were started at 24 h after cyclophosphamide induction and continued for 4 days, once per day. Volume administration of cyclophosphamide, dextrose, and rhG-CSF was 2.5 mL/ kg BW. Blood sampling (approximately 0.5 mL) was carried out from lateral tail vein on Day 1 and Day 4, 6 h after treatments on the same day. The rats were sacrificed on the Day 5 by ketamine (Kepro) and xylazin (Interchemie, Netherlands) injection (75 mg/kg BW, 8 mg/kg BW, intramuscular (i.m), 2.5mg/kg BW). Subsequently, approximately 2-3 mL of blood was collected from left cardiac vein.

***Blood and Organs Analyzes***

Blood samples were analyzed by using hematology analyzer (VetScan HM5, Abaxis, California, USA) for complete blood count and peripheral blood smear with Giemsa staining (Merck). Comparison of each groups at the same sampling time were analyzed by using One Way ANOVA SPSS 16.0 (IBM Corporation, New York, USA), followed by PosHoc Multiple Comparison – Ducan Method with significance level 0.5.

## Results

***Expression, Purification, and Characterization of rhG-CSF***

A significant band was found at size approximately 17 kDa in supernatant after solubilization. As an addition data, remaining pellet after solubilization was also analyzed on SDS-PAGE and showed more abundance of rhG-CSF but containing endogenous contaminant proteins from expression host *E.coli* NiCo21(DE3) (Figure 1A). In order to obtain rhG-CSF in an active form, the solubilized rhG-CSF was refolded and purified. The rhG-CSF which had been prepared in native form was then analyzed with non-reducing PAGE as depicted in Figure 1B. The native form of rhG-CSF was found at approximately 14-15 kDa in size. This non-reducing PAGE was used to confirm that refolded process had been completed. It is shown in Figure 1B lane 2 and 4 in which migration of positive control and refolded protein was similar. As expected, migration of solubilized protein (Figure 1B, lane 3) was slighly faster compared than migration of positive control and refolded protein. However, smear migration was found in the refolded rhG-CSF which was not as clean as positive control (Figure 1B). Result of western blotting is shown in Figure 1C. Both of our rhG-CSF and positive control positively bound to the monoclonal anti-rhG-CSF antibody.

As supplementary data, graphs of absorbance and fluorescence spectrum were prepared to access physical similarity of our rhG-CSF and positive control. Those proteins, except not-lyophilized rhG-CSF, have similar absorbance spectrum with optimum wavelenght at approximately 276 nm (Figure 2A). Moreover, the profiles of fluorescence spectrum of positive control and rhG-CSF dose 40; 80; and 120 mg/kg BB were also similar, but spectrum of rhG-CSF dose 40 mg/kg BB had higher intensity (Figure 2B).

***Bioactivity Assay of rhG-CSF to Blood Cells Profile of Male Sprague Dawley Rats***

Numbers of each type of blood cell, *i.e.,* leukocytes, platelets, and erythrocytes are represented in the following graphs (Figure 3). Neutrophils, lymphocytes, and monocytes

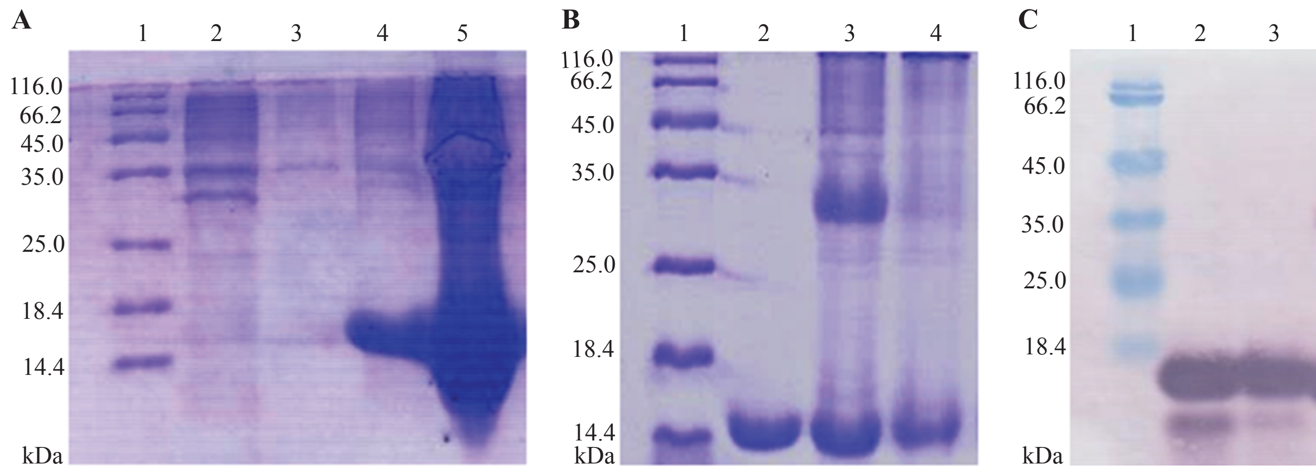


**Figure 1. Protein profiles of rhG-CSF.** A: SDS PAGE of isolation and solubilization of inclusion bodies, 1) protein marker, 2) supernatant after washing, 3) sitoplasmic protein, 4) supernatant after solubilization, 5) pellet excess of solubilization. B: Non reducing PAGE rhG-CSF, 1) protein marker, 2) Leukokine as positive control, 3) solubilized rhG-CSF or before refolding, 4) refolded rhG-CSF. C: Western blot of rhG-CSF against monoclonal anti rhG-CSF antibody, 1) protein marker, 2) concentrated and refolded rhG-CSF, 3) positive control.

were obtained as percentage in the leukocyte. In Figure 3, they are represented as cell counts by calculating the percentage towards leukocytes total count. Basophils and eosinophils were found extremely low in rat bloods, thus not detected by the hematology analyzer.

Significant differences were found in leukocytes and neutrophils count on Day 1 in groups treated with positive control; rhG-CSF dose 40 and 80 μg/kg BW compared to normal and negative control group. However, on Day 4 and 5, no differences of leukocytes and neutrophils count were found among groups. Except leukocytes and neutrophils count, rhG-CSF did not affect other types of blood cell (Figure 3).

Density of blood cells (erythrocytes) were manually observed and found similar among normal, rhG-CSF dose 80μg/kg BW, and positive control on Day 1. In addition, neutrophils were easily found in rhG-CSF dose 80 μg/kg BW and positive control group. On Day 5, all groups showed reduction in blood cells (erythrocytes) density (Figure 4).

## Discussion

The recent study was initiated by cyclophosphamide administration to gain neutropenic animal model. Likewise other chemotherapy agents, cyclophosphamide induces bone marrow suppression or myelosuppresion causing depletion of blood cells (erythrocytes, leucocytes and platelets) counts. It inhibits proliferation of hematopoetic progenitor cells (HPC), so the degeneration of new blood cells is neglected. Extensive cyclophosphamide exposure does not only impact the HPC, but also perturbs renewal of hematopoietic stem cells (HSC) that causes more severe effects such as bone marrow failure or even death.[29,30]

All types of blood cells (erythrocytes, leukocytes, and platelets) gradually deteriorated by cyclophosphamide induction (Figure 1). On the last day, all of the blood cells parameters were below normal range for male rats aged 2-4 months[31], whereas the blood cells counts in normal group were relatively steady. However, erythrocytes in normal group were slightly declined that probably due to blood sampling. From the same data, it is known that only neutrophils were affected by rhG-CSF administration. The graph of neutrophil count corresponded with that of leukocytes' one, even though lymphocytes are the most dominant cells in the leukocytes. It indubitably proves that the rhG-CSF specifically acts on neutrophils regeneration, not on other blood cells. This led by G-CSF receptors (G-CSFR) are expressed in neutrophils and their precursors including HSC and HPC.[32]

In our experiment, the administration of rhG-CSF and positive control significantly increased the neutrophils count on Day 2 only. However, at that time point, neutropenic condition was not achieved because the neutrophils count of negative control group was not significantly lower than

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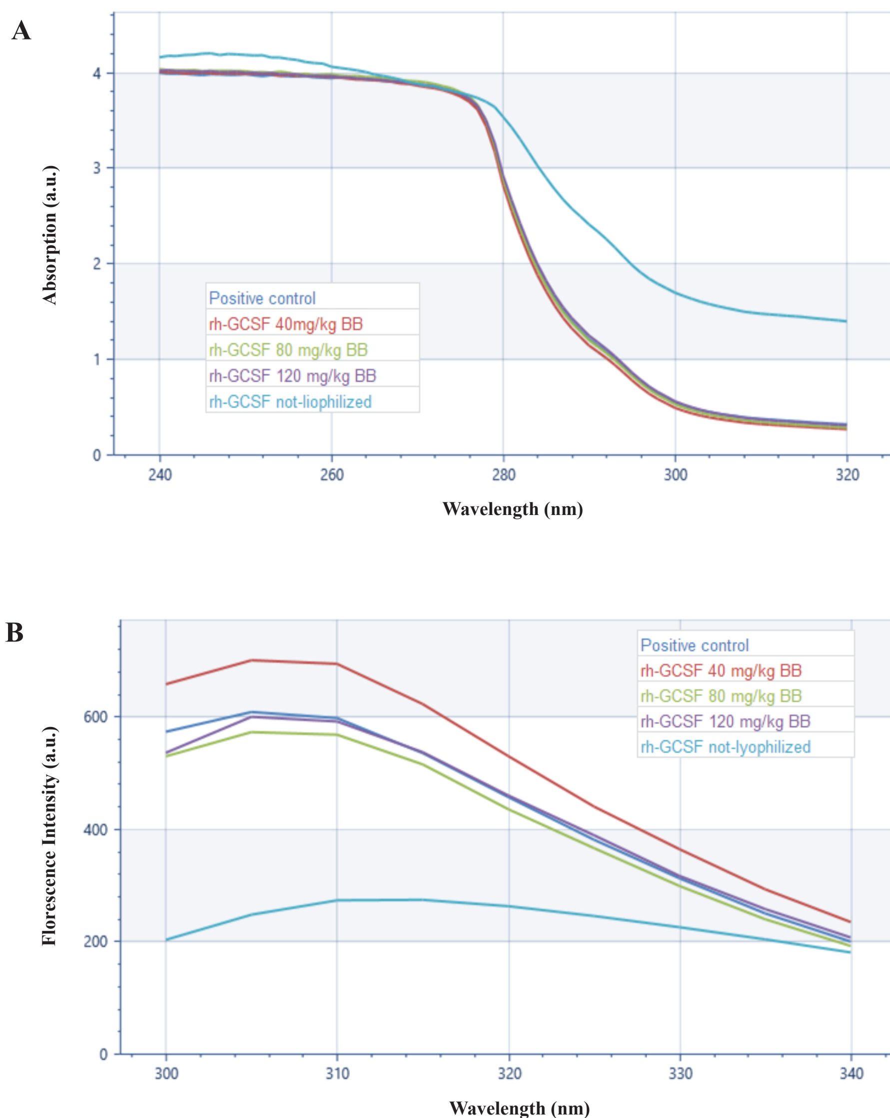



**Figure 2. Absorbance and flourescence spectrum of rhG-CSF and positive control.** A: asbsorbance spectrum; B: fluorescence spectrum of rh-GCSF and positive control.

that of normal group. It means that either the rhG-CSF or positive control did increase the neutrophils count, but not in neutropenic condition.

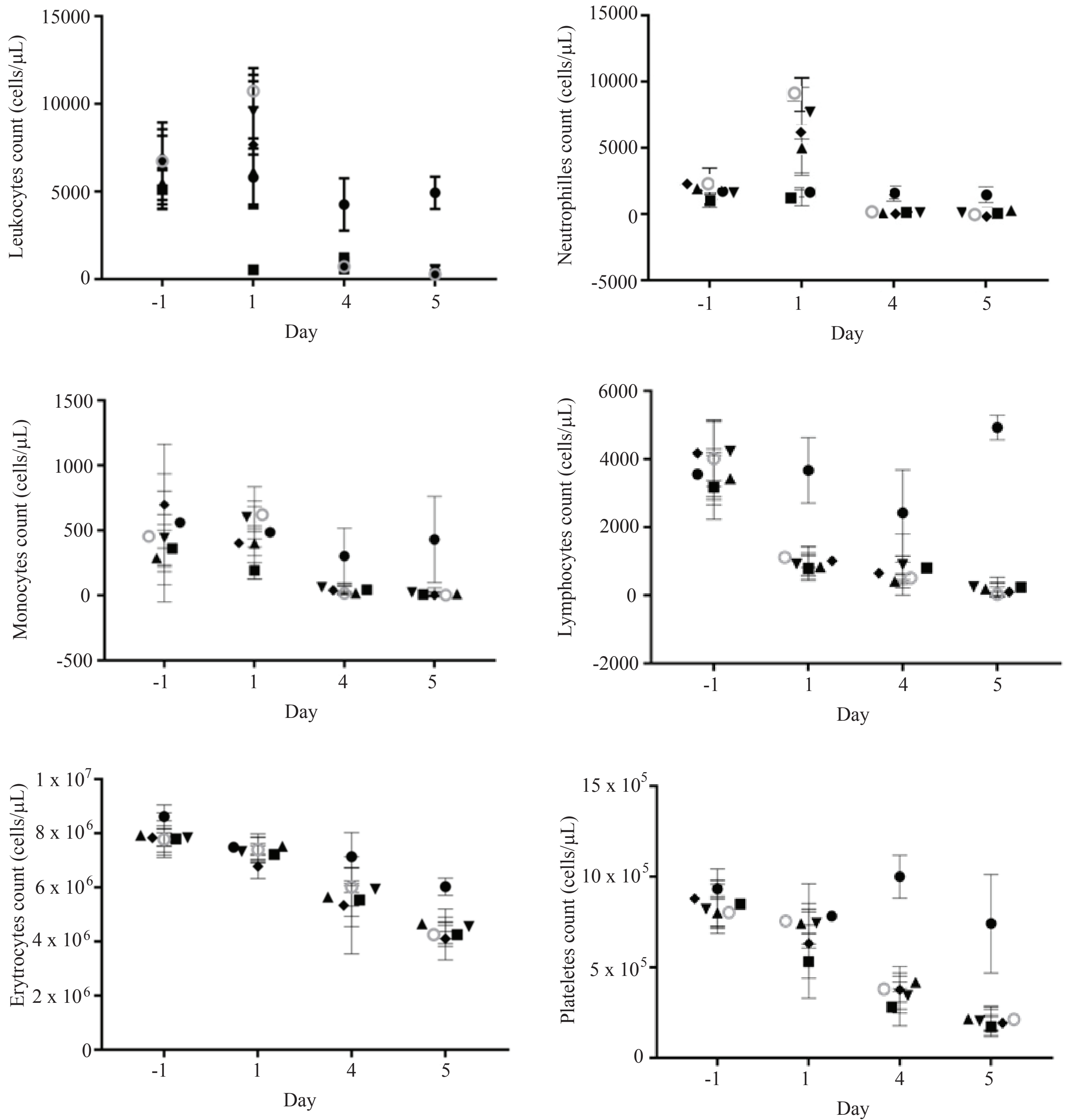
Similar effect was found in the rhG-CSF dose 80; 120 mg/kg; and the positive control 40 mg/kg. This indicates that in order to obtain similar activity in same dose with the positive control, preparation of the obtained rhG-CSF needs to be improved, including dispensing, purification, refolding, and lyophilization.

The refolded rhG-CSF was purified from other contaminants protein, which is dominantly found at approximately 33 kDa, by centrifugal filter with 30 kDa cut off and then concentrated by centrifugal filter with 10 kDa cut off (Figure 1B). In order to obtain clean purified rhG-CSF as the positive control, an advanced purification such as size exclusion or ion-exchange chromatrography need to be applied.

In addition, similarity of biological activity of positive control; rh-GCSF dose 80 mg/kg; and 120 mg/kg was supported by fluorescence spectrum. The fluorescence spectrum of those groups had the same profile and intensity.

Since neutropenia condition did not meet yet on Day 2, the adminitrastion of rhG-CSF and positive control led over-proliferation of neutrophils counts which reached >5000, and considered as neutrophilia (normal neutrophil counts on rat 2-4 months = 220-1570).[15] Moreover, Welte and Lim performed similar experiment by administrating G-CSF on healthy animal model, also resulting neutrophilia in 4 h and 24 h after injection, respectively.[26,33]

Cyclophosphamide started to suppress neutrophil counts on day 3. Therefore, on the day 3 rats underwent neutropenia (<220) and became severe on day 4 (<50).[24] However, in the experiment set, neutrophil counts were




- Normal
- Negative control
- Dose 40 µg/kg BW
- Dose 80 µg/kg BW
- Dose 120 µg/kg BW
- Positive control

**Figure 3. Complete blood count of blood sample at Day -1 (baseline or before cyclophosphamide induction), Day 1 (6 h after treatments on Day 1), Day 4(6 h after treatments on Day 4), and Day 5 (30 h after treatments on Day 4).**

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not boosted by the rhG-CSF and positive control in the neutropenic condition. In keeping with Welte and colleagues' report, a significant effect of rhG-CSF was observed after 8 days of treatment in cyclophosphamide-induced monkey.[33] The neutrophils that were counted until 34 days remained in normal range after receiving rhG-CSF for 20 days. Therefore, in regards to our experiment, it is assumed that the activity of the rhG-CSF in neutropenia condition needs be observed with longer regimen which is more than 4 days.

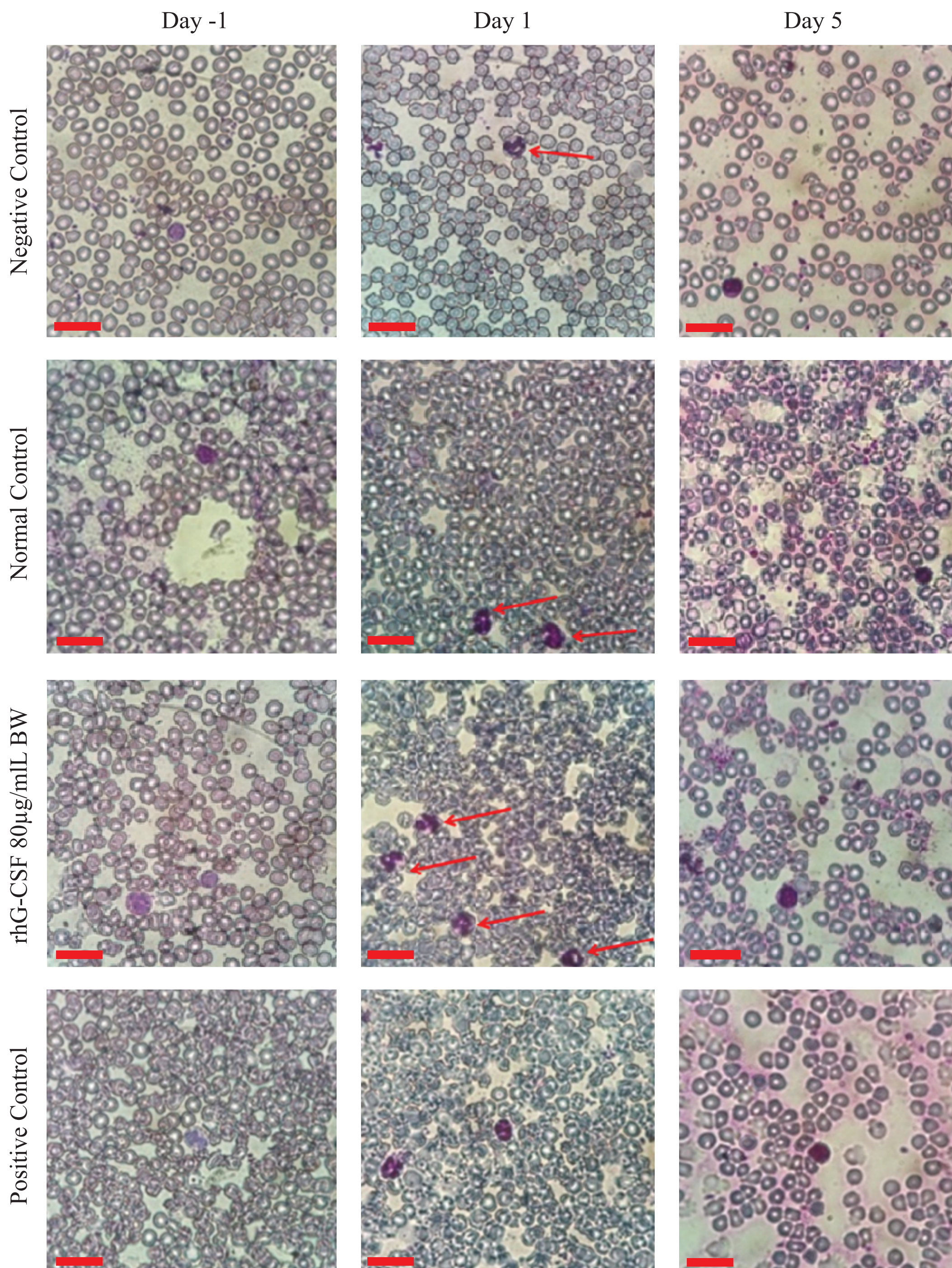


**Figure 4. Microscopic visualization of peripheral blood smear, with 100x magnification.** Red bar: 20 µm.

## Conclusion

The rhG-CSF that had been produced in our lab showed similar potency in increasing neutropils count in health animals compared with the positive control, but needed 2-3 time higher dose. The effects of both the obtained rhG-CSF and positive control to enhance neutrophils production in neutropenic animal models could not be evaluated in the current study due to short rhG-CSF administration and observation period. Hence, it is suggested that in the further study, the administration of rhG-CSF is requested in longer period of time. In addition, downstream processes of our rhG-CSF have to be improved and more characterzations are required.

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