

Modulation of Granulocyte Cells Development by VipAlbumin® Administration in BALB/C Mice with Diabetes Mellitus

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ABSTRACT

Diabetes mellitus is a metabolic disease that is caused either by the decrease of insulin secretion from pancreatic β cells or the insensitivity of target cells against insulin. High glucose levels (hyperglycemia condition) can trigger the formation of free radicals, the main cause of diabetes micro and macrovascular complications. The formation of free radicals and AGE (advanced glycation end-products) is assumed to become the key factor in the decline of granulocyte cell production as well as the disruption of these cells functional activity. The purpose of this research was to determine the role of VipAlbumin® in inhibiting the adverse effects of increased blood glucose levels, which highly influence the production of granulocyte. This study was divided into in vitro and in vivo stage. BALB/C mice were used as experimental animals at in vivo stage and induced to undergo diabetes through 100 mg/kg BW streptozotocin (STZ) injection at the age of 5 days. VipAlbumin® administered orally for 14 days, which began when mice reached the age of 14 weeks. The administration of VipAlbumin® divided into 3 doses i.e. 0,01664 mg/gr BW (1st dose), 0,416 mg/gr BW (2nd dose), and 10,4 mg/gr BW (3rd dose). The further step was a flowcytometric analysis to see the development of granulocyte cells relative amount, which were isolated from the bone marrow. The result of this analysis shows that VipAlbumin® administration, particularly at the 2nd and 3rd dose, were able to modulate granulocyte cells development in the bone marrow.

Keywords: *diabetes mellitus, free radicals, granulocyte cells, streptozotocin, VipAlbumin®*

INTRODUCTION

Diabetes mellitus, according to American Diabetes Association [1], is classified as a metabolic disease characterized by hyperglycemia or high blood sugar levels over a prolonged period. This disease is due to the defects in insulin secretion, insulin mechanism, or both. The significant influences in the pathogenesis of diabetes mellitus come from Reactive Oxygen Species (ROS). These free radicals are formed through Non-Enzymatic glycosylation, mitochondrial electron transport chain dysfunction, or activation of hexosamine induced by hyperglycemia [2]. In a case of type 2 diabetes, ROS is one of the main factors in the development of micro and macrovascular complications. It breaks transmission path between insulin receptor and glucose transport system, inducing the insulin resis-

tance and inactivation of two essential anti-atherosclerosis enzymes: endothelial nitrite oxide and prostacyclin synthase [3,4,5].

Besides playing an important role in the formation of advanced glycation end-products (AGE), ROS also mediates the effects of AGE upon target tissues. A connection between clinical complication of diabetes mellitus and oxidative stress is involved in high-dose AGE formation. The glycation product (Amadori), formed by a reaction between aldehyde and protein amino group, can be oxidized (i.e. by reactive oxygen species) into AGE formation. The biochemical process of this glycation is gradually increased in diabetic patients, affected by both hyperglycemia, oxidative stress and the increase of fatty acid [5].

In patients with diabetes mellitus, there is a decline in mobilization and chemotaxis abilities of mononuclear and polymorphonuclear/PMN (i.e. neutrophil) in which it decreases the phagocytosis capability. There is a negative correlation between phagocytosis and high levels of blood sugar in the diabetic patients [6].

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To determine the effect of hyperglycemia towards both developments and activities of granulocytes (especially neutrophil), mice model of diabetes mellitus are used. The mice are injected with streptozotocin or STZ (2-deoxy-2-(3-methyl-3-nitrosourea), which is a widely used antibiotic formed by *Streptomyces achromogenes*. According to Tesch & Allen [7] and Szkudelski [8], the toxicity mechanism of STZ starts when it infiltrates into β cells through GLUT 2 transporter and kills β cells due to DNA fragmentation associated with nitrosourea.

The decrease in insulin secretion by β cells as well as the increase of blood sugar level triggering both ROS and AGE formations are assumed as the key factor of granulocytes deficiency. This research used a supplement derived from albumin extract of snakehead murrel (*Channa striatus*) named VipAlbumin®, which allegedly has better antioxidants. Several researchers show that albumin has strong antioxidants, free radicals purification through redox cycle in thiol (Cys³⁴) [9], and metal ion binding [10,11,12]. VipAlbumin® administration is expected to repair β cells ability in excreting insulin and binding various ROS formed by hyperglycemia, which will later affect the production of granulocytes.

MATERIALS AND METHODS

This research was conducted from November 2014 to May 2015 in Animal Physiology Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang.

Research Design

This research was experimental research, which applies manipulative treatments and observes the emerged effects of the treatments by tightly controlling on polluters. In vitro procedure contains 5 treatments with 5 repetitions: negative control, positive control (with EPO/erythropoietin treatment), and VipAlbumin® 1st dose, 2nd dose, and 3rd dose. Moreover, in vivo experiment contains 5 treatments: negative control (healthy mice without any VipAlbumin® administration), positive control (diabetic mice without VipAlbumin® administration), 1st dose, 2nd dose, and 3rd dose of VipAlbumin®, in which each treatment consists of 5 repetitions.

In Vitro Procedures

Fabrication of VipAlbumin® Stock

The initial concentration was 33333.3 μ g/ml derived from dissolution of 33.3 mg VipAlbumin® in 1

ml medium. The third dose with the concentration of 3333.3 μ g/ml was derived from 10 times stock dissolution. This dose was diluted again by 100 times to form the second dose with the concentration of 33.3 μ g/ml. Last, the first dose with the concentration of 0.33 μ g/ml was derived from diluting the second dose by 100 times. The second dose is a standard treatment based on assumption that healthy man with average weight (\pm 60 kg) can consume VipAlbumin® 2 g per day.

Fabrication of Culture Medium

All procedures of culture medium manufacture were conducted in Laminar Air Flow (LAF) to prevent contamination. The medium used in this research was RPMI 1640 with additional Fetal Bovine Serum (FBS) 10%, 1% Penicillin and Streptomycin, and 1 μ g 2-Mercaptoethanol (2-ME) in 10 ml medium. The medium was also given LPS stimulant (lipopolysaccharide, to stimulate lymphocytes from bone marrow) 2 μ g in 1 ml medium. The usable medium was filtered by Millipore 0.20 μ m. The treatment of positive control by EPO/erythropoietin administration was done by mixing 1 μ l EPO and 99 μ l culture media. The suspension formed was then taken by 1 μ l to be mixed with 1 ml media and used in the culture process.

Cell Isolation from Bone Marrow

Mice was dislocated on the neck, cleaned with alcohol and dissected. The feet were taken and cleaned from fat. Afterward, the femur and tibia part were flushed with PBS at one end by using 1 ml syringe so that cells would come out from another end. The suspension of cells and PBS gained were transferred into propylene tube to be centrifuged at 2.500 rpm, temperature 10° C, for 5 minutes. Last, the supernatant was eliminated, and the pellet formed was resuspended in 1 ml medium.

Cells Calculation

The suspension of cells from bone marrow was taken 5 μ l, accommodated in a microtube and added 95 μ l of Evans blue (dilution of 20 times) which then was pipetted for homogenization. Afterward, cells were calculated by hemocytometer under the microscope. The number of cells was then formulated as follows:

$$\Sigma \text{ Cells} = \Sigma \text{ counted cells} \times 5 \times \text{diluent factor} \times 10^4 \text{ cells/ml}$$

Planting and harvesting the Cell Culture

3 million cells per ml (through the hemocytometer

calculation) were added to each medium with different treatments and homogenized. Next, cells were planted in plate culture 48 well, in which each well contained 1 ml cell suspension and medium. Cells were incubated in incubator CO₂ 5%, temperature 37°C for five days. After the incubation, cells were harvested from each well (based on treatment group), by pipetting cell suspension and medium and moved it into propylene tube. 1 ml PBS was added to the wells to take the rest of cell suspension remained. Moreover, it was centrifuged at speed 2.500 rpm temperature 10°C for 5 minutes. Formed pellet was resuspended in PBS and stained with antibodies.

Cells Staining with Antibodies

Bone marrow cell suspension gained from culture was accommodated in a micro tube and centrifuged at speed 2.500 rpm, temperature 10°C for 5 minutes. Formed pellet was then added with a 1µl antibody that has been diluted with 50 µl PBS and 10% FBS, and incubated in the icebox with temperature 4°C for 20 minutes. Staining combination used was FITC-conjugated rat anti-mouse Gr-1.

Running Flow cytometry

Incubated cells derived from antibody staining were added with 300-500 µl PBS, then transferred into a cuvette and processed in flow cytometry machine. The program was set as 'acquisition', and gating process was done based on cell types that would be identified and analyzed.

In Vivo Procedures

Induction of Diabetes Mellitus in Mice

Healthy neonatal mice aged five days was induced as the model of diabetes mellitus through STZ injection intraperitoneally, at a dose of 100 mg/kg body weight. Being assumed that each mouse has weight ± 2.5 g, the STZ injection should reach 50 mL, derived from stock made by mixing 0.005 grams of STZ powder with 1 ml citrate buffer. Mice that had been injected was allowed to grow in the chamber, being fed and drink. The replacement of the husk was routinely done. After four weeks, the blood glucose level of mice was measured by glucometer. The high blood glucose level, reaching more than 200 mg/dl showed that the mice had been successfully induced diabetes mellitus.

Oral Administration of VipAlbumin®

Mice that has positively diagnosed as diabetes mellitus was then given VipAlbumin® orally, divided into 4

dose treatments: 0 mg/gr BW (positive control); 0.01664 mg/gr BW (1st dose); 0.416 mg/g BW (2nd dose); 10.4 mg/g BW (3rd doses). Doses above were obtained from the conversion of albumin dose for 1-kg-weight man, which is assumed to have a normal weight 60 kg, i.e. 33.3 mg/kg. The VipAlbumin® administration was carried out for 14 days. The measurement of blood glucose level was regularly done once in every three days, to determine whether the administration of VipAlbumin® has influenced in reducing the blood glucose level.

Cells Isolation from Bone Marrow and Calculation

Mice was dislocated on the neck, cleaned with alcohol and dissected. Part of femur and tibia removed and cleaned from fat, then flushed with PBS at one end (using a 1 ml syringe). The suspension of cells was then transferred into propylene tube to be centrifuged at a speed of 2500 rpm, temperature 10°C, for 5 minutes. Formed pellet was resuspended in 1 ml PBS. The result of re-suspension was taken 5µl, accommodated in a microtube, and added with 95 µl of Evans blue (dilution of 20 times), which then was pipetted for homogenization. Cells were then calculated by hemocytometer under the microscope.

Cells staining with antibodies and Running Flowcytometry

The cell suspension of bone marrow (250 µl) was accommodated in a microtube and centrifuged at 2.500 rpm, temperature 10°C for 5 minutes. The formed pellet was then added with a 1µl antibody that has been diluted with 50 µl PBS and 10% FBS, and incubated in 4°C icebox for 20 minutes. Staining combination used was FITC-conjugated rat anti-mouse Gr-1. Incubated cells derived from antibodies staining were added with 300-500 µl PBS, then transferred into a cuvette and processed in flowcytometry machine. The program was set as 'acquisition', and gating process was done based on cell types that would be identified and analyzed.

Statistical Analysis

Cell profiles obtained from flowcytometry were analyzed using BD Cellquest Pro™ software. The data were collected and analyzed statistically with SPSS v16 for Windows. The analysis is one-way ANOVA parametric analysis with p = 0.05%, by applying Turkey advanced test.

RESULTS AND DISCUSSION

Flowcytometric Analysis of Granulocytes Relative

Amount In Vitro

From the data above, the highest granulocytes relative amount increase was after the third dose of VipAlbumin® (3333.3 µg/ml media). On the positive control with erythropoietin administration, despite being capable of stimulating the development of granulocytes which is better than the negative control, but the result was not significant. Meanwhile, the other two treatments, i.e. 1st dose (0.33 µg/ml) and 2nd dose (33.3 µg/ml) were not different in terms of triggering the development of granulocytes compared to the negative control.

The diagram above measures the ratio of blood glucose level in mice, which was done once in every three days and started when the mice were four-week-old (calculated from 5 days after birth). The graphic shows that mice with highest glucose level is a positive control, which was successfully induced to undergo diabetes mellitus through STZ without any VipAlbumin® administration. In the first dose of VipAlbumin® (0.01664 mg/gr BW), an increase of blood glucose level was directly proportional to age, although it was still significantly lower than the positive control. Blood glucose levels in VipAlbumin® treatments second dose (0.416 mg/g BW) and third dose (10.4 mg/g BW) seemed fluctuate, possibly decreased or increased while

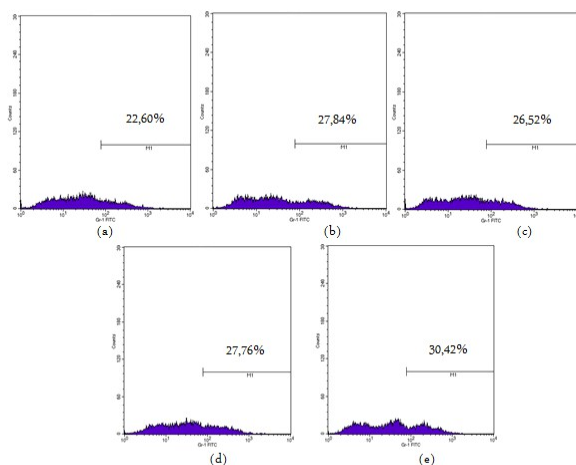


Figure 1. Flowcytometric analysis of Gr-1 profile in vitro; (a) negative control, (b) erythropoietin treatment, (c) VipAlbumin[®] 1st dose, (d) VipAlbumin[®] 2nd dose, (e) VipAlbumin[®] 3rd dose. Bone marrow cell suspension gained from culture process then added with a 1µl antibody that has been diluted with 50 µl PBS and 10% FBS. Staining combination used was FITC-conjugated rat anti-mouse Gr-1. The 3rd dose of VipAlbumin[®] showed the highest increase of granulocyte relative amount (%) compared with negative control.

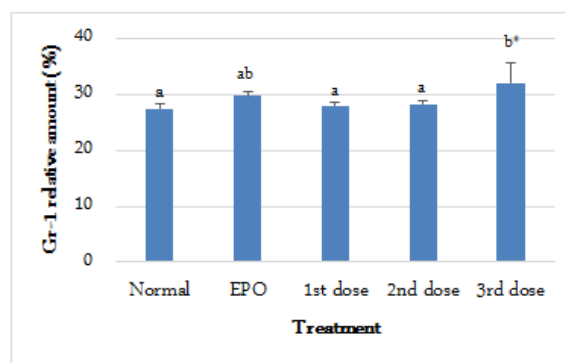


Figure 2. Gr-1 relative amount in culture condition (in vitro).

Cell profiles obtained from flow cytometry were analyzed using BD Cellquest Pro™ software. The data were collected and analyzed statistically with SPSS v16 for Windows. VipAlbumin[®] treatment third dose showed a significant increase in granulocyte relative amount compared to the positive control (*significantly different, $p < 0.05$).

being measured on different days. In the final days of measurement, the third dose of VipAlbumin[®] treatment showed a significant reduction in blood glucose level, although the decline had not reached the state of normal mice (negative control).

Flowcytometric Analysis of Granulocytes Relative Amount In Vivo

The results of flowcytometric analysis of the development of granulocytes at in vivo treatment showed a significant difference between normal mice (negative control) and diabetic mice (positive control). From three different VipAlbumin[®] treatments, 2nd dose (0.416 mg/g BW) and 3rd dose (10.4 mg/g BW) showed a significantly different increase in granulocytes growth compared to the positive control. The remaining dose of VipAlbumin[®] (1st dose, 0.01664 mg/gr BW) did not show any increase in the relative amount of granulocytes and was not considerably different from the negative control treatment.

From the graphic above, it can be seen the significant differences between normal mice and diabetic mice in terms of its ability to develop granulocytes. Hyperglycemia triggered by the action of STZ is estimated to be the leading cause of the decline in the relative number of granulocytes. Chronic diseases such as type 2 diabetes, insulin resistance caused by age, nutritional factors, and lifestyle have a significant effect on PMN function. Like the record, the risk of infectious diseases are two to four times higher in diabetic pa-

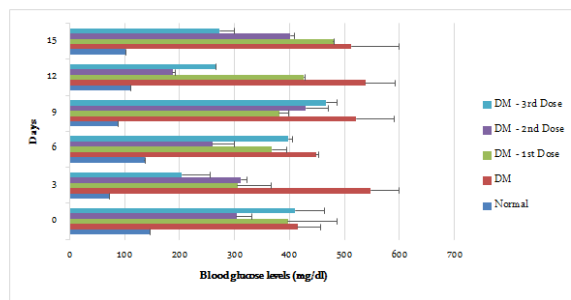


Figure 3. The comparison of blood glucose levels in each treatment. 100 mg/kg BW STZ was injected intraperitoneally to 5-day old healthy mice. After four weeks, the blood glucose level of mice was measured by glucometer and regularly done once in every three days after that, to determine whether the administration VipAlbumin® has influence in reducing the blood glucose level. Blood glucose level seemed fluctuate in the second and third dose of VipAlbumin® treatments. In the last six days of measurement, an only third dose of VipAlbumin® showed a significant blood glucose decrease.

tients [13]. ROS production by neutrophils in diabetic patients are increased; but on another hand, neutrophils also increases apoptosis induction and significantly decreases chemotactic response. It is known that the amount of circulated pro-inflammatory cytokines in blood flow has been increased in diabetic patients and was considerably suspected that dysfunction occurring in neutrophils contributes to the rise of susceptibility towards infections in these patients. Hyperglycemia or the presence of AGE can trigger the activation of neutrophils, and it can be proven through an increase in alkaline phosphatase produced by neutrophils [14]. Several studies have showed an increased release of TNF- α , IL-8, and IL-6 [14,15] and decreased secretion of granular proteins by neutrophils in diabetic patients [16,17].

According to Rifa'i and Widodo [18], regulatory T cells, especially in the CD4⁺CD25⁺ population, have an important role in maintaining normal homeostasis in many degenerative diseases including diabetes mellitus. Some research was suggesting that the activation of autoreactive T cells in the diabetic individual can be conducted from self-antigen recognition by cytotoxic T cells and Th1 cells. This activation later triggers the activation of other cells in the immune system to stimulate systemic homeostasis disorders [19,20,21,22]. Research conducted by Rifa'i [23] by using mouse model of autoimmune disease (CD122-deficient mice) shows

that the transfer of 2×10^6 CD4⁺CD25⁺ cells can enhance TER-119⁺ cells (erythroid-lineage) number increase while suppress the activity of granulocytic Gr-1⁺ cells to recognize self-antigen. This result supports the role of regulatory T cells as a principal regulator of body homeostasis, by normalizing leukocyte number in the peripheral blood and prevent any harmful consequences.

As we all know, neutrophils as the biggest fraction of granulocytic cells, play a significant role in the host inflammatory response towards infection. According to Mowatt & Baum [24], there is an important decrease in neutrophil chemotactic activity in diabetic patients compared to normal controls. Several studies have shown indications of a decline in bactericidal activity [25], interference in the phagocytosis ability and a reduction of lysosomal enzymes release [26], and decreased production of ROS [27] by neutrophils in diabetic patients. Moreover, a decline in the number of phagocytic leukocytes and bactericidal activity correlate with an increase in blood sugar levels [28]. Studies using diabetic mice have also showed a decrease in neutrophil migration [29,30,31,32], phagocytic activity [33]

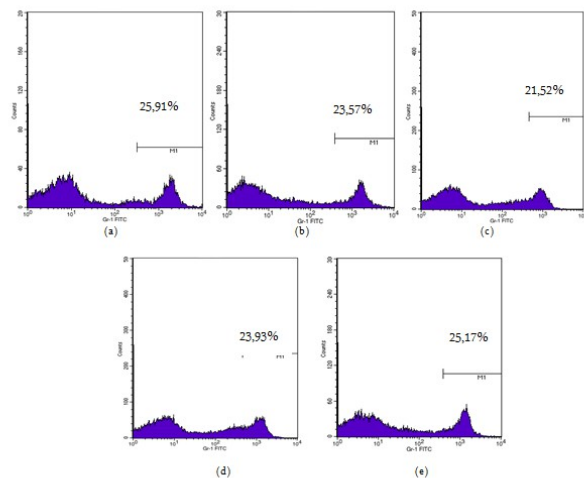


Figure 4. Flowcytometric analysis of Gr-1 profile in vivo; (a) negative control, (b) positive control, (c) VipAlbumin® 1st dose, (d) VipAlbumin® 2nd dose, (e) VipAlbumin® 3rd dose. Mice was dislocated, part of its femur and tibia were removed, then flushed with PBS to get the cell suspension. This suspension then centrifuged and processed in flow cytometry machine thereafter. Staining combination used was FITC-conjugated rat anti-mouse Gr-1. There was a significant decrease in granulocyte relative amount (%) of positive control compared to negative control.

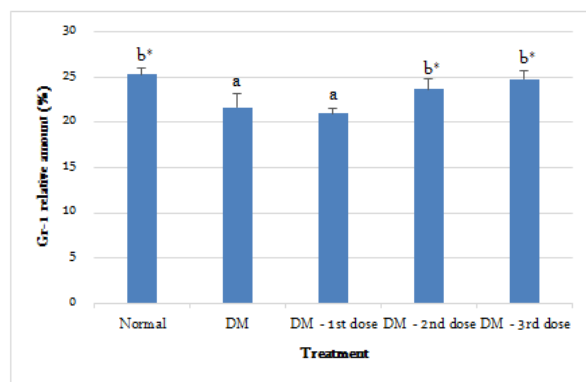


Figure 5. Gr-1 relative amount in vivo. Cell profiles obtained from flow cytometry were analyzed using BD Celquest Pro™ software. The data were obtained and analyzed statistically with SPSS v16 for Windows. VipAlbumin® treatment second and third dose showed a significant increase in granulocyte relative amount compared to the positive control (*significantly different, $p < 0.05$).

and the production of hydrogen peroxide [34]. Furthermore, the efforts to reduce blood sugar levels by administering insulin to diabetic rats [34] are reported to have a correlation with a significant increase in the capacity of phagocytosis by neutrophils. Some antimicrobial proteins produced by neutrophils such as lactoferrin, protein enhancer for permeability/bactericidal and α -defensins are proportionally decreased with respect to the development of insulin resistance and diabetes. The reduced concentration of these proteins is in line with the low capacity of antimicrobials by neutrophils in diabetic subjects. Furthermore, one of these proteins (lactoferrin) is known to have a direct influence on the metabolism, enhance the action of insulin, increase the activity of AMP kinase protein, and trigger weight loss [35,36].

The graphic above also shows a significant rise in the relative amount of granulocytes in the second dose and third dose treatment, compared to the positive control. This increase can even match the corresponding number of granulocytes in the negative control, which is normal mice. VipAlbumin® role in inhibiting the effect of free radicals/ROS caused by hyperglycemia condition is assumed to be an important factor of an increase in the relative amount of granulocytes. Albumin is an important extracellular source for reduced sulphydryl cluster, which is in the form of a single cysteine residue at the 31st position in the molecule. This sulphydryl group, which is referred as thiol, is the primary scavenger of reactive oxygen and nitrogen species, particularly superoxide and hydroxyl per-

oxynitrite. Albumin may also limit the production of reactive species through free copper Cu^{2+} bond, which is an ion that is known to play a significant role in accelerating the production of free radicals [37]. Albumin itself is the owner of the majority of total thiol in plasma so that albumin can affect the redox equilibrium and has an important implication in indicating various critical diseases such as capillary permeability, rheology change, cell-signaling processes, anti-hemostatic effects, drug metabolism and transport processes [38].

The decreased amount of free radicals and β cells repair will trigger the improvement of insulin production, which is very influential on the development of granulocytes, especially neutrophils. Some studies suggest that insulin has a strong influence on the regulation of the functional activity of immune cells [39,40]. The essential action of insulin on the PMN activity may be seen as the body provides global protection for the support of the primary immune response against foreign antigens entering through food intake [41]. Walrand et al. [42] states that the change in the PMN function in diabetic subjects can be improved through the hyperglycemia control by using insulin. Although PMN does not require insulin to take up glucose, both glucose and glycogen metabolism in PMN depends on insulin activity. Also, expression of the insulin receptor is known to have good correlation with PMN chemotaxis in young and elderly subjects, after insulin treatment. An antimicrobial protein produced by PMN is considerably impaired due to the presence of insulin resistance in older subjects [42].

CONCLUSIONS

In *in vitro* procedure, the third dose of VipAlbumin® administration can increase granulocyte relative amount and significantly different from negative control. The administration of VipAlbumin® at *in vivo* procedure, particularly on the 2nd dose (0.416 mg/g BW) and 3rd dose (10.4 mg/g BW), can also significantly increase granulocytes relative amount compared to the positive control.

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