UNIVERSITI PUTRA MALAYSIA

EFFECT OF SYSTEMIC ADMINISTRATION OF GRANULOCYTE-COLONY STIMULATING FACTOR ON WHITE BLOOD CELL COUNT IN RABBITS

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EFFECT OF SYSTEMIC ADMINISTRATION OF GRANULOCYTE-COLONY STIMULATING FACTOR ON WHITE BLOOD CELL COUNT IN RABBITS

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A project paper submitted to the

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CERTIFICATION

It is hereby certified that I have read this project entitled "Effect of Systemic Administration of Granulocyte-Colony Stimulating Factor on White Blood Cell Count in Rabbits", by Zul Khidir Mohd Robai and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project.

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DEDICATIONS

This final year project is dedicated to my beloved family for supporting and encouraging to belief in oneself throughout the journey of tertiary study and final year project study.

To the lecturers of Faculty of Veterinary Medicine, for the endless knowledge sharing and moral support

To my fellow friends for their kindly advise and encouragement throughout the project, and countless accompany moral and emotional support.

May all of us be well and happy as always.

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Abstrak daripada kertas projek yang dikemukakan kepada Veterinar Fakulti Perubatan untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Tahun Akhir

KESAN PEMBERIAN SECARA SISTEMIK FAKTOR PERANGSANG KOLONI GRANULOSIT KEPADA KIRAAN SEL DARAH PUTIH ARNAB

Oleh

ZUL KHIDIR MOHD ROBAI

2020

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Faktor perangsang koloni granulosit (G-CSF) adalah glikoprotein yang digunakan untuk mendorong pengeluaran sumsum tulang, proliferasi granulosit dan sel stem dalam perubatan manusia. Potensi penggunaan rawatan G-CSF dalam amalan haiwan eksotik dinilai sebagai rawatan alternatif terutamanya dalam kecederaan muskuloskeletal dengan memprovokasi produksi sel-sel inflamasi dalam peredaran darah untuk memperbaiki dan regenerasi tisu. Lima (N = 5) arnab digunakan dalam eksperimen percubaan klinikal ini untuk menentukan kesan G-CSF pada kiraan sel darah

putih (WBC) dan tindak balas sumsum tulang terhadap rawatan yang diberikan. Arnab diberi suntikan plasebo secara subkutan pada fasa pertama dan setelah tempoh tenggang selama 14 hari, arnab yang sama diberikan suntikan G-CSF subkutan. Rejim rawatan dirancang selama 3 hari, di mana darah dikumpulkan setiap hari dalam jangka masa 5 hari, termasuk hari pra-rawatan dan pasca-rawatan. Sampel sumsum tulang diambil untuk kajian sitologi selepas arnab dieutanasia. Hasil pengiraan leukosit menunjukkan peningkatan yang signifikan dalam komponen WBC, terutamanya heterofil tetapi menurun sekitar 48 jam setelah rawatan terakhir. Bagi sitologi sumsum tulang, peningkatan jumlah siri sel myeloid dan peningkatan nisbah myeloid ke eritroid (M:E) diperhatikan. Penemuan ini membuktikan bahawa G-CSF mampu meningkatkan sel inflamasi dan siri sel myeloid dalam sumsum tulang arnab. Oleh itu, potensi penggunaan G-CSF pada masa hadapan untuk kecederaan muskuloskeletal pada arnab boleh diterokai.

***KATA KUNCI**: G-CSF, arnab, sel darah putih, sumsum tulang, kiraan pembezaan*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

EFFECT OF SYSTEMIC ADMINISTRATION OF GRANULOCYTE-COLONY STIMULATING FACTOR ON WHITE BLOOD CELL COUNT IN RABBITS

By

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Granulocyte-Colony Stimulating Factor (G-CSF) is a glycoprotein used to induce bone marrow production, proliferation of granulocytes and stem cells in human medicine. The potential use of G-CSF treatment in exotic animal practice is being evaluated to serve as an alternative treatment predominantly for any musculoskeletal injury to provoke production of inflammatory cells in the circulation for tissue repair and regeneration. Five (N=5) rabbits were used in this experimental clinical trial to determine the effect of G-CSF on the white blood cell count (WBC) and bone marrow

response to the treatment given. The rabbits were subjected to subcutaneous injection of a placebo in the first phase and after a grace period of 14 days the same rabbits were subjected to subcutaneous injection of the G-CSF. The treatment regime was designed for 3 days, in which blood was collected daily for a span of 5 days, including day of pre-treatment and post treatments. Bone marrow samples were harvested for cytological study after euthanasia. Result on leukocyte count shows significant increase in the WBC components, primarily heterophils but declined about 48 hours after the last treatment. As for bone marrow cytology, notable elevated numbers of myeloid series cells and increment of the myeloid to erythroid (M:E) ratio observed. These findings prove that G-CSF is able to increase inflammatory cells and myeloid series cells in the bone marrow of rabbits. Thus, the potential future use of G-CSF for skeletal muscle injury in rabbits could be explored.

KEYWORDS: *G-CSF, rabbits, white blood cell, bone marrow, differential count*

1.0 INTRODUCTION

Granulocyte-Colony Stimulating Factor (G-CSF) is a glycoprotein that has been used to stimulate the bone marrow production and proliferation of granulocytes and stem cells (Zhang & Chen, 2011). It also induces haematopoietic stem cell including white blood cells (WBC), red blood cells (RBC) and platelets mobilization into the bloodstream.

Stem cells can be harvested for various regenerative purposes (Hermann et al, 2018). G-CSF is generally used to regulate the production and release of functional neutrophils from bone marrow upon chemotherapy. It has been reported as a relatively safe and effective alternative to bone marrow harvesting (Hosing, 2012).

An opportunity to explore regenerative medicine and stem cell therapy in pet rabbits may offer alternative options for veterinary practitioners and pet owners when dealing with various musculoskeletal conditions. G-CSF is hypothetically considered to also accelerate stem cell stimulation in rabbits. Therefore, in this study, the effect of G-CSF is being evaluated for the provocation of inflammatory cells in circulation and stimulation of granulocyte progenitor cells in bone marrow for tissue repair and regeneration. The objective of this study is to stimulate the bone marrow to produce stem cells while determining the duration and peak of WBC counts in rabbits treated with G-CSF. Five rabbits were subjected to a placebo treatment (0.9% NaCl injection) for 3 days to serve as the control group (Control Group) and after 2 weeks, the same rabbits were then subjected to G-CSF treatment after up to 3 days to stimulate the bone

marrow proliferations (Treatment group). The alternative hypothesis of this study is there are differences in WBC counts between Treatment Group and Control Group.

2.0 LITERATURE REVIEWS

2.1 Granulocyte-Colony Stimulating Factor (G-CSF)

Granulocyte-Colony Stimulating Factor (G-CSF) is a glycoprotein that has been used to stimulate the bone marrow production and proliferation of granulocytes and stem cells (Zhang & Cheng, 2011). It is a growth factor which gives effect to neutrophils in terms of production, maturation and even the activation phase in addition triggering displacement of neutrophils from the bone marrow (Semerad, 2002). G-CSF is generally used to regulate the production and release of functional neutrophils from bone marrow upon chemotherapy.G-CSF also induces haematopoietic stem cell (HSC) such as s (WBC), red blood cells (RBC) and platelets mobilization into the bloodstream. Mobilization mechanism of the glycoprotein at cellular level can invoke a series of cascades including neutrophils and precursor expansions, bone marrow macrophage, peripheral sympathetic nervous, osteocytes and osteomacs stimulations (Bendall, 2014).

2.2 G-CSF in Veterinary Medicine

Various studies have been conducted in many animal species where rodents are most common model while few in rabbit species for experimental reasons. In human medicine, G-CSF functions ranges from treating leukopenia as well as provoking inflammatory cells and mobilizing stem cell for potential use in regenerative medicine.

Hermann et al found that G-CSF able to provoke increment of leukocytes in rat species and helps in bone formation due to induced vascularization (Herman, 2018). Alternatively, with lentivirus as vector for G-CSF, given through intramuscular injection

extended the therapeutic levels of neutrophils longer which were recommended for severe and cyclic neutropenia cases patients (Barry, 2005).

Similarly, a case report shown G-CSF is effective in the case of neutropenia in a foal (Davis, 2003). A unique application of G-CSF conducted in horse species which served as preventive measure against shipping fever in horse (Endo, 2014). The hematopoetic growth factor is found to be important for augmenting host defences for example leukopenia due to post-operative gastrointestinal surgery (Sullivan, 1993)

In a study involving dogs with moderate to severe radiation-induced myelosuppression, G-CSF have proven to tone down the neutropenia severity and duration at the same time shorten thrombocytopenia (Macvittie, 1990).

In non-human primate, G-CSF offers mobilization of peripheral blood stem cells (PBSC) that can be used for stem cell harvesting for allogenic transplantation (Larsen, 2008). Combined with thrombopoeitin (TPO), it served best treatment combination for rhesus monkeys induced to pancytopenia for 3 weeks (Wagemaker, 1998).

There is one previous experiment using goat as the animal model, the bone marrow derived mesenchymal stem cell (BM-MSC) was stimulated by injecting G-CSF prior to peripheral blood mesenchymal cell harvest (PBMC) (Roohi et. al, 2017). The glycoprotein was also found to promote bone defect fracture healing in goats due to its proliferation properties (Roohi et. al, 2017).

Paradoxically, not much reported studies in regards of use of G-CSF in skeletal tissue repair or regeneration in rabbit species model. Sasaki et al., (2015) reported G-

CSF stimulated regeneration of cartilage defect in rabbit model. G-CSF can be used for treatment of steroid-induced osteonecrosis by suppressing resorption while at the same time stimulate bone formation (Wu et al., 2012).

To note, studies that focused on systemic administration of G-CSF shows increment of WBC in rat species (Herman, 2018), goats (Roohi et al., 2017) and rabbit (Marimoto, 1990) with follow up experimental design to gauge on bone repair for the rat and goat model. Close assessment of WBC in response to systemic administration of G-CSF is being evaluated in rabbit model by determining the peak level and duration of effects that may serve as optimal time for HSC harvest or tissue repair and regeneration.

2.3 White Blood Cells of Rabbits

In general, the WBC count will not be the same depending on the circadian rhythm, nutritional status and diets, also the age, gender and breed (Mitruka, 1977). WBC count appears lowest late afternoon and evening (Washington, 2012).

2.3.1 Neutrophils/ Heterophils

Neutrophil contains small acidophilic granules and large red granules, thus sometimes being called pseudoeosinophils. It has polymorphic nucleus with light purple stains confined in a pink cytoplasm (Zimmerman, 2010). G-CSF administration either syetematically or centrally have displayed escalated cell count of heterophils (Marimoto, 1990)

2.3.2 Lymphocytes

Lymphocytes are available in both large and small size with as large as

heterophils and small like RBC (Kozma, 1974). It has a rounded, condensed nucleus surrounded in a narrow blue-stain cytoplasm but larger ones come with azurophilic granules (Reagan, 2008).

2.3.3 Monocytes

Monocytes is the largest WBC to be observed with lobulated, horseshoe or bean shaped nucleus stained lightly purple (Zimmerman, 2010). Cytoplasm of monocytes is blue in colour and some contains vacuoles (Reagan, 2008).

2.3.4 Eosinophils

Like heterophils, the contents are acidophilic granules but are 3 to 4 times larger occupying so much space in the cytoplasm. The nucleus is bilobed or horseshoe shaped (Kozma, 1974).

2.3.5 Basophils

The size of the basophils is similar to the heterophils, having light purple stained nucleus and cytoplasm that hold purple-to-black metachromic granules, sometimes conceal the nucleus (Reagan, 2008).

Table 1: Reference range for WBC components of adult female NZW rabbits

WBC components	Adult Female NZW ref range (%)
WBC ($x10^3/\mu L$)	5.2 – 10.6
Heterophils (%)	36.4 – 50.4
Lymphocytes (%)	31.5 – 52.1
Monocytes (%)	6.6 – 13.4
Eosinophils (%)	0.8 – 3.2
Basophils (%)	2.4 – 6.2

2.4 Bone Marrow Cytology

The majority cells residing in bone marrow is the myeloid and erytheroid lineages with different levels of maturation. They are classified for the different stages by a previous characteristic description study (Harvey, 2012).

2.4.1 Erythroid lineage

2.4.1.1 Rubrilast

Rubrilast is described with presence of high nuclear-to-cytoplasm ratio which nucleus is round, having visible nucleoli with fine chromatin while the cytoplasm is deeply basophilic and vacuolated (Tadjalli, 2013).

2.4.1.2 Prorubricyte

Prorubricyte resembles rubriblasts but in the absent of nucleoli with denser chromatin (Tadjalli, 2012).

2.4.1.3 Basophilic rubricyte

Basophilic rubricyte is smaller than prorubricyte,with dark blue cytoplasm and clumped chromatin-filled rounded nucleus (Tadjalli, 2012).

2.4.1.4 Polychromathophilic rubricytes

The nuclear chromatin of these cells is more condensed than basophilic rubricytes while cytoplasm is red-blue (Tadjalli, 2012).

2.4.1.5 Metarubricytes

They can be observed as small cells with dark pyknotic nucleus with presence of cytoplasm stained red to red-blue (Tadjalli, 2012).

2.4.2 Myeloid lineage

2.4.2.1 Myeloblast

Identification of myeloblast is through the high nucleus-to-cytoplasm ratio, which the nucleus is round to oval with fine chromatin while moderately basophilic nucleus (Riedel, 2017).

2.4.2.2 Promyelocytes

They do not contain nucleoli, larger cytoplasm than myeloblasts and most importantly presence of magenta cytoplasmic primary granules (Riedel, 2017).

2.4.2.3 Heterophil myelocyte

The nucleus is round to oval shape with a lot of small-sized dark pink cytoplasmic secondary granules (Riedel, 2017).

2.4.2.4 Heterophil metamyelocyte

The nucleus is kidney bean shape also with secondary granules (Riedel, 2017).

2.4.2.5 Band Heterophils

Similarly, with the present of secondary granules but the shape of nucleus is elongated U or S-shaped (Riedel, 2017).

2.4.2.6 Segmented Heterophils

These cells have prominent long nucleus, carrying irregular indentations and lobulations (Riedel, 2017).

2.4.2.6 Others

Eosinophils and basophils precursors are not that much obviously appreciated unless the myelocyte stage where the secondary granules are lighter pink and the other is purple. Monocyte precursors relatively unreliable to differ from myeloid lineage hence they were counted along as the myeloid series (Riedel, 2017).

Table 2: Reference range for bone marrow cell composition in female NZW rabbits

Lineage	Cell type	Ref range (%)
Erythroid	Rubriblast	0.0 – 1.6
	Prorubricyte	0.8 – 4.5
	Basophilic rubricyte	8.6 – 20.4
	Polychromatophilic rubricyte	31.1 – 44.4
	Metarubricyte	39.6 – 53.6
Myeloid	Early myeloid precursor	1.4 – 8.8
	Promyelocytes	0.6 – 3.0
	Heterophil myelocytes	3.6 – 9.2
	Heterophil metamyelocyte	4.0 – 10.2
	Heterophil bands	20.8 – 36.8
	Segmented heterophils	33.8 – 57.4
	Eosinophils	1.2 – 5.6
	Basophils	0.8 -6.0

3.0 MATERIALS & METHODS

The study was approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia for Animal Utilisation Protocol (AUP). Five (N=5) New Zealand White (NZW) breed female rabbits (*Oryctolagus cuniculus*) between ages of 10 months – 1 year weighing about 1.8 – 2.2 kilograms involved in the experimental study. All rabbits were housed, maintained and monitored following standard operation procedures for research rabbits.

3.1 Procedure

These rabbits were subjected for normal saline (NaCl) (0.1ml) subcutaneous injection daily for 3 days as a placebo to determine the baseline. Daily blood samples were taken (approx 1ml) at 9am from the saphenous vein using EDTA blood tubes and submitted to Clinical Pathology Laboratory, Veterinary Laboratory Services Unit (VLSU), Fakulti Perubatan Veterinar, Universiti Putra Malaysia. WBC counts were recorded from Day 0 (before injection) to Day 4 (post injection). The rabbits were rested over a grace period of 2 weeks before the next part of the study.

The same group of female rabbits was to be given 5µg/kg of body weight of subcutaneous G-CSF daily for 3 days. Similarly, daily blood samples were taken at 9am and WBC counts were recorded from Day 0 to Day 4. Rabbits were euthanized on last day of blood collection. Right after euthanasia, bone marrow samples were collected from the right femur.

3.2 WBC Count

In the same day of each blood venipuncture, thin blood smear was done and stained with Wright's stain. Each characteristics of WBC must be identified among each other, heterophils, lymphocytes, monocytes eosinophils and basophils under microscopy for differential count. All WBC was manually counted and recorded accordingly until 100. With the ratio counted for every type WBC to 100, calculated the actual number relative to total leukocyte count of the sample. All data was tabulated in Microsoft Excel Sheet.

3.3 WBC Analysis

Two tabulated descriptive statistics data of daily blood picture recorded for Control Group and Treatment Group, respectively. Data included mean and standard deviation (SD) total leukocytes count and the breakdown of each components consisting of heterophils, lymphocytes, monocytes, eosinophils and basophils. Heterophil to lymphocyte (H:L) ratio was included. The formula for H:L ratio is heterophils to lymphocyte count each rabbit then we calculated the average to represent mean daily H:L ratio within the group. Independent t-test was run for all WBC components to test the differences between the two groups.

3.4 Bone Marrow Cytology

Bone marrow was harvested from the right femur of all rabbits. Following blood sample collection, rabbits were euthanized and immediately the right hindlimb was separated from the body. The femur bone was exposed from medial aspect of proximal

hindlimb part with sharp dissection. Rongeurs were used to cut open the periosteum of femur, revealing the bone marrow.

Bone marrow specimens were divided into two preparation groups, impression smear and squash preparation. Impression smear was conducted by directly impress on the bone marrow sample while squash smear was by squashing and spreading the bone marrow on a glass slide with another glass slide into a thin layer.

Both were stained with Wright's stain and observed under the microscopy. Every important cells within the bone marrow was identified, counted and recorded which include 2 major groups the erythroid linage and myeloid lineage both having different maturation level of cells using 500-cell differential count. Erythroid lineage consists of rubriblast, prorubricyte, basophilc rubricyte, polychromatophilic rubricyte and metarubricyte while Myeloid lineage are made up of myeloblast, promyelocyte, heterophil myelocyte, heterophil metamyelocyte, heterophil bands, segmented heterophils, eosinophils and basophils. Other cells include megakaryocyte was also recorded. Myeloid to Erythroid (M:E) ratio was calculated with formula total myeloid to total erythroid cells of each rabbits.

4.0 RESULTS

Table 3: Descriptive statistics for WBC count for Treatment Group

	Mean (SD)				
Cell type	**D0**	**D1**	**D2**	**D3**	**D4**
WBC	8.75±3.00	3.49±1.54	3.49±0.85	4.40±2.51	7.02±1.14
Heterophil	3.83±2.67	1.01±0.22	1.15±0.38	1.49±1.05	2.01±0.33
Lymphocyte	4.54±0.99	2.28±1.16	2.13±0.51	2.59±1.31	4.58±1.28
Monocyte	0.27±0.09	0.14±0.08	0.09±0.03	0.21±0.12	0.28±0.06
Eosinophil	0.06±0.09	0.02±0.78	0.04±0.03	0.07±0.09	0.06±0.03
Basophil	0.05±0.07	0.04±0.06	0.08±0.06	0.03±0.03	0.09±0.11
H:L ratio	1.00±0.53	0.51±0.16	0.54±0.16	0.53±0.15	0.48±0.15

In the placebo group, the mean and SD WBC count is shown in **Table 3**. The trend remained steady and within the normal range throughout the duration of experiment although slight rise on D0 and D4. Lymphocytes accounts for the highest component followed by heterophils, monocytes, eosinophils and basophils. H:L ratio accounts roughly 2 lymphocytes for every 1 heterophil thorough the span of 5 days which is normal except for D0.

Table 4: Descriptive statistics for WBC count for Treatment Group

Cell Type	Mean (SD)				
	D0	**D1**	**D2**	**D3**	**D4**
WBC	8.20±1.72	11.90±3.92	14.12±6.19	14.64±4.76	5.78±0.87
Heterophil	4.08±1.77	6.58±3.95	8.22±4.11	9.40±3.29	2.38±0.85
Lymphocyte	3.22±0.52	4.04±1.63	4.93±2.04	4.14±1.82	2.97±0.75
Monocyte	0.44±0.21	0.30±0.03	0.48±0.24	0.65±0.29	0.29±0.07
Eosinophil	0.32±0.30	0.26±0.12	0.40±0.38	0.23±0.09	0.08±0.07
Basophil	0.14±0.21	0.25±0.03	0.21±0.24	0.22±0.29	0.05±0.07
H:L ratio	1.35±0.62	1.89±1.17	1.73±0.69	2.45±0.67	0.87±0.37

The effects of GCSF on rabbits is pictured in **Table 4** where it is observed a significant spike of mean WBC count from D1 to D3 post-injection but then decline to the normal level by D4 which approximately 48 hours from last injection. The peak level was on D3, and in comparison, to D3 of Control Group, G-CSF has stimulated total WBC count as high as about three folds. However, the increasing trend was progressively plateau from D0 to D3 where it was recorded 45%, to 10% and lastly only 4% to peak level on D3. Independent t-test analysis revealed $p<0.5$ hence proved that there is significant different between Control Group and Treatment Group.

Peak WBC count on average was on D3, where two of five rabbits continued elevating up to D3 but two of the rabbits showed peak until D2 while one of them D1

only. By D4 majority of rabbits have achieved their normal WBC count except that one of them had fall to normal as early as D3.

Major component that contributed to the rise is heterophils, followed by small increments of monocytes, eosinophils and basophils while lymphocytes showed no remarkable change from the Control Group. Based on H:L ratio, heterophils is now the dominant component thanks to G-CSF especially on D3 where ratio was 2.45, around 5 times more than Control Group.

In bone marrow cytology, all the myeloid series were found under 1000x magnification including myeloblast, progranulocyte, myelocyte, metamyelocyte, band heterophils and segmented heterophils **(Figure 1, 2, 3, 4 and 5)**. While every erythroid series can be observed from erythroblast, basophilic rubricyte, polychromatic rubricyte and metarubricyte as in **(Figure 2, 3, 4 & 5)**. Another extra hematopoietic cell is the precursor of platelets, the megakaryocyte which is relatively large can be seen already in 400x magnification **(Figure 6)**. There was an increase in M:E ratio for the bone marrow of rabbits in Treatment Group **(Table 5)**.

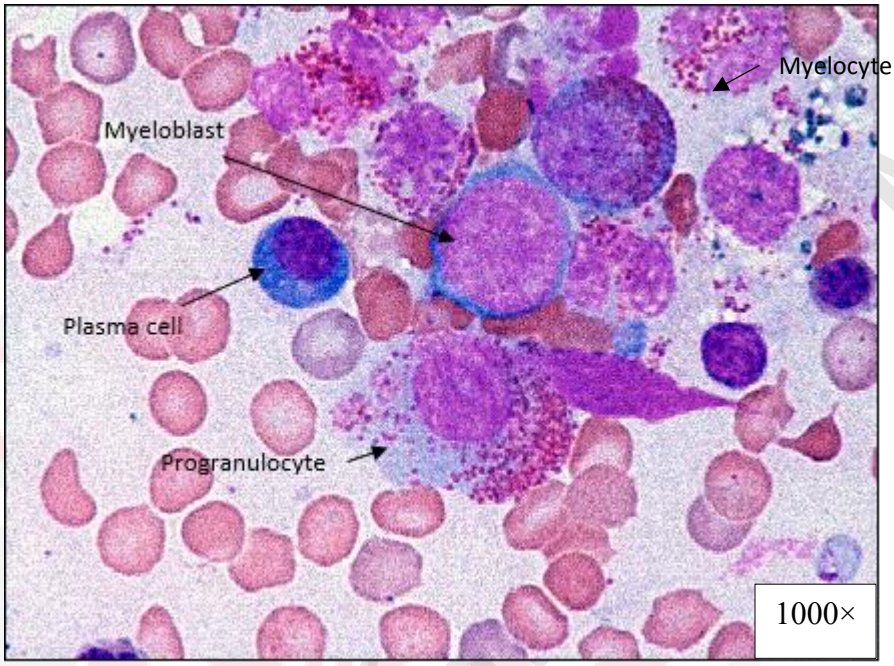

Figure 1: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits.

Myeloblast; myelocyte; progranulocyte; plasma cell. Wright stain; 1000x

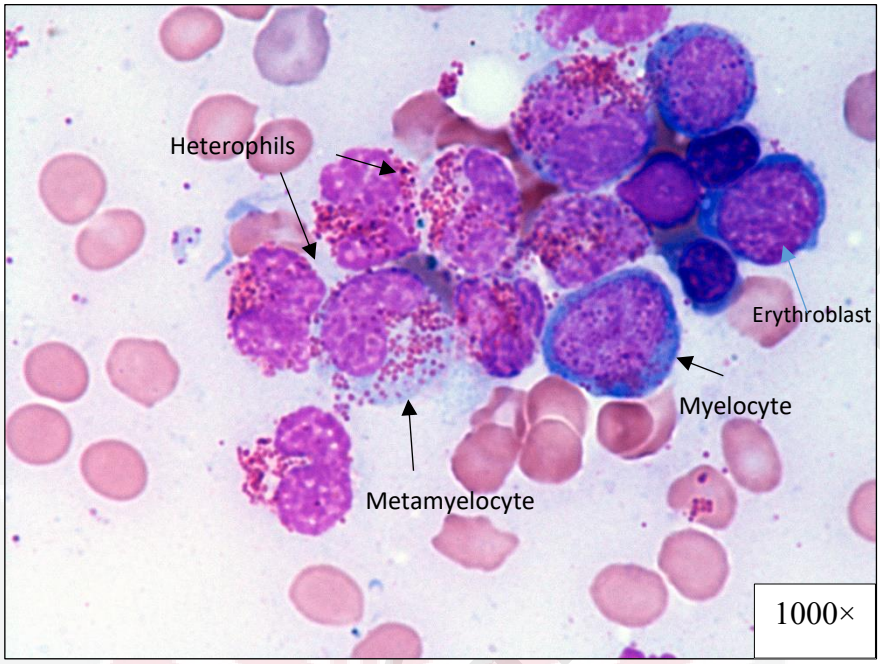

Figure 2: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits.

Myelocyte; metamyelocyte; heterophils; erythroblast. Wright stain; 1000

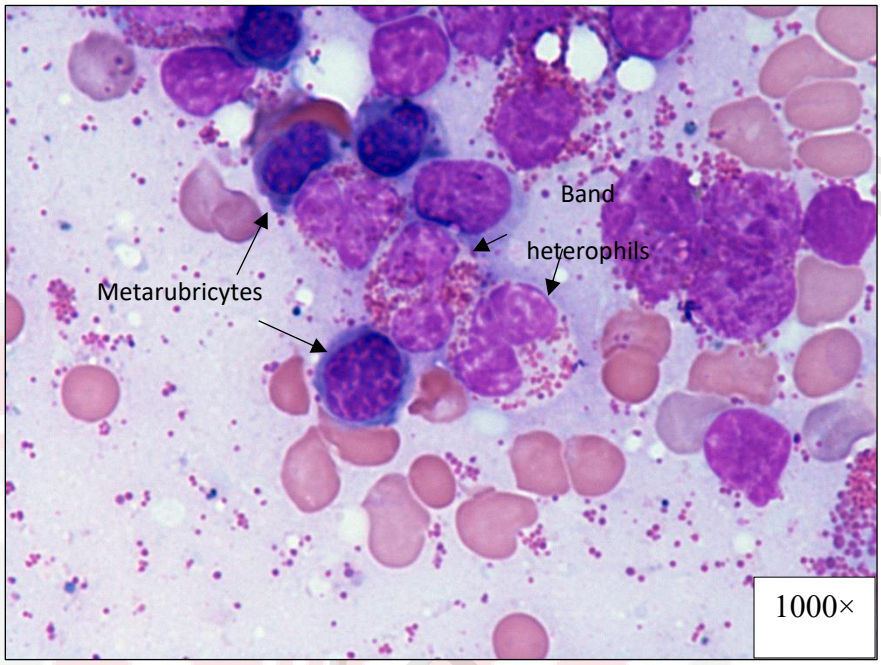

Figure 3: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits.

Band heterophils; metarubricyte. Wright stain; 1000x

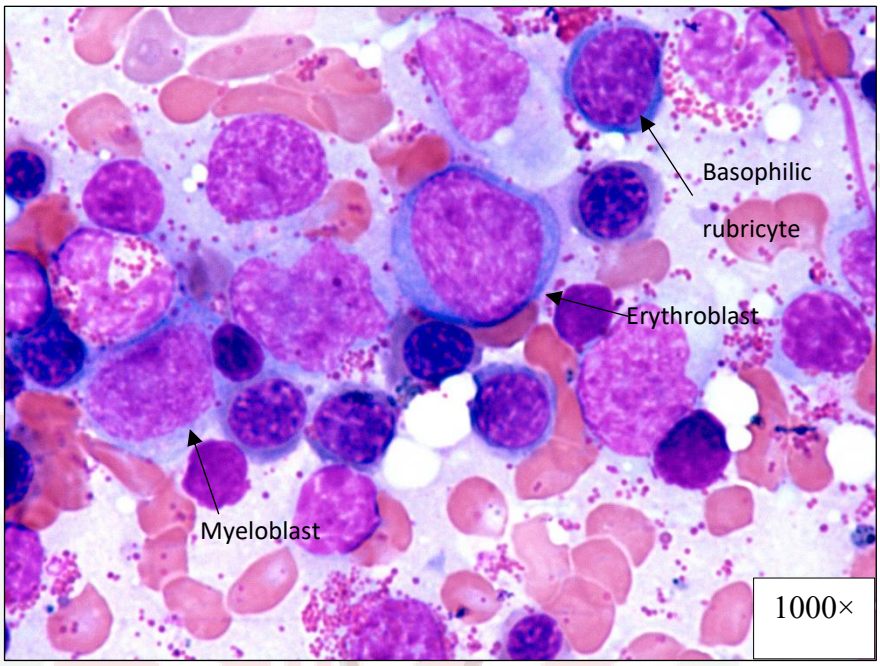

Figure 4: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits. Erythroblast; basophilic rubricyte; myeloblast. Wright stain; 1000x

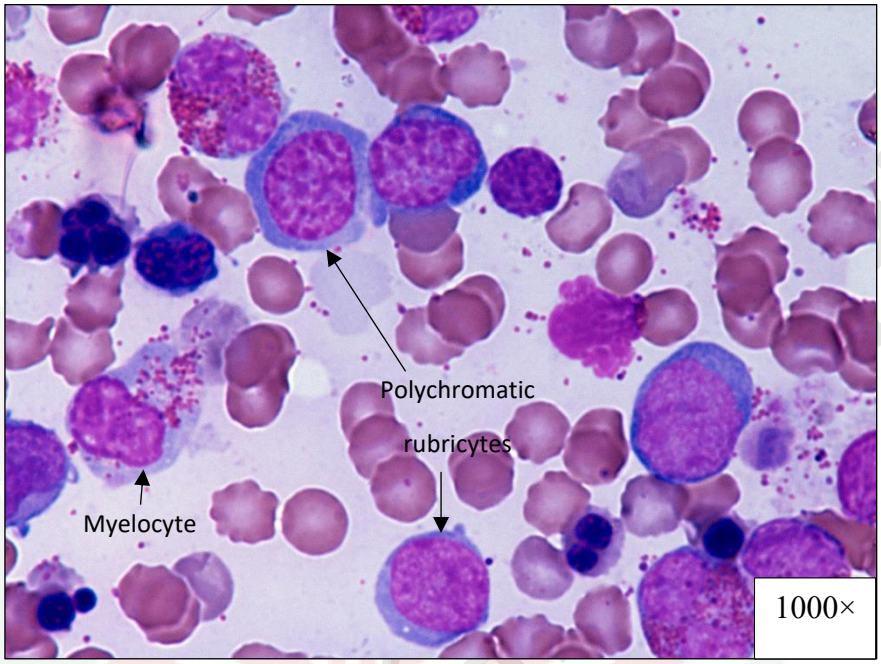

Figure 5: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits. Polychromatic rubricyte; myelocyte. Wright stain; 1000x

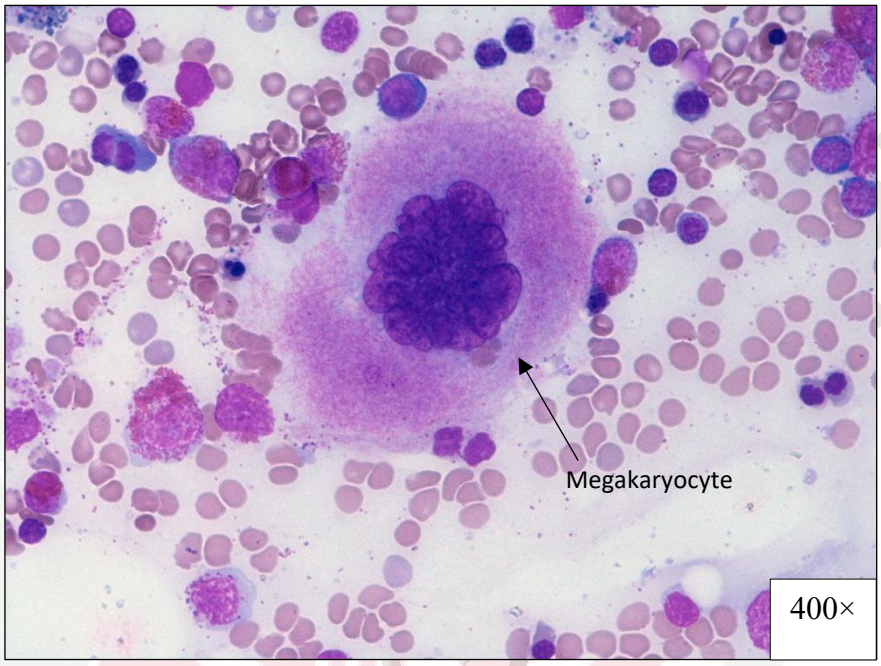

Figure 6: Photomicrograph of hematopoietic cells in bone marrow of female NZW rabbits. Megakaryocyte. Wright stain; 400x

Table 5: Descriptive statistics for hematopoietic cells in the bone marrow for Treatment Group

Lineage	Mean (SD)
Erythroid series	47.6 ± 12.6
Myeloid series	50.9 ± 13.3
Megakaryocytes	1.5 ± 1.1
M:E ratio	1.1 ± 0.7

5.0 DISCUSSION

In the present study, the effects of GCSF on rabbits was tabulated in **Table 3 & 4** where it is observed a significant folding number of WBC in Treatment Group compared to Control Group with independent t-test analysis revealed $p<0.5$ hence there is markable different between the two. Previous study also reported increment of WBC due to GCSF in multiple species such as goat (Roohi et al., 2017), rats (Herman, 2018) and in non-human primate (Wagemaker, 1998). Based on each component and H:L ratio of Treatment Group, heterophils was the dominant component that contributed to the spike, followed by small increments of monocytes, eosinophils and basophils while lymphocytes showed no notable change from the Control Group. This is the same with a study in goat where highest count contributed by neutrophils however other WBC component was not described (Roohi et al.)

The peak level for was achieved on D3, and in contrast to Control Group, G-CSF has stimulated total WBC count as high as about three times more than placebo injection. The peak level accounts 24 hours after the last injection of the 3-day-treatment. This finding is comparable to the rat species study that maximum peak level was obtained after 24 hours from the last injection, but they injected G-CSF for 5 days (Herman, 2018). Yet the same study, WBC level maintained high though with slight declining trend over span of 10 days (Herman et al., 2018) unlike the current study where it immediately drops back to normal the day after on D4. In addition to another study in goat species, also reported after 3 days injection of G-CSF, the WBC remain high until 2 days post last injection (Roohi et al., 2017). The closest paper regarding

rabbit species systemic administration of G-CSF found leukocytosis but the study only covers for 4 hours, 8 hours and last 24 hours post injection (Marimoto, 1990). Base don this study, the duration of effect in rabbit pertain only within window of 24 – 48 hours before it will return to normal level.

The bone marrow of rabbits was also responsive toward G-CSF as there was an increase in M:E ratio relative to published normal bone marrow cell composition and morphology in NZW rabbits reference range (Riedel, 2016). The entire progenitor cell can be found in abundance despite the circulatory hematological values returned to normal by the day of euthanasia. We could speculate that probably the amount of myeloid progenitor cells is possibly higher. This glycoprotein helps in bone marrow progenitor cells proliferation and maturation particularly neutrophils in addition to lengthening neutrophil survival time and retarding apoptosis (Ulich, 1998).

Ultimately, the mechanism of G-CSF toward WBC has been discussed by a few researchers with few theories explained in detail to the molecular level. One of the comprehensive theories stated that mechanism of G-CSF is by provocation of a few potential collateral pathways both directly or indirectly toward WBC or granulocytes (Bendall, 2014). The growth factor, G-CSF itself binds to a receptor called G-CSFR (Fukunaga, 1990). G-CSF can be produced biologically with appropriate stimulation with inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and many others (Fossiez, 1996) and presence of G-CSF in bloodstream triggers neutrophil production in, and mobilization from thebone marrow (Colotta, 1992).

Another mechanism involved a more indirect cascade which through stimulation of peripheral nervous system to release catecholamines which causes encouragement of CD169+ bone marrow macrophage, and suppression of osteocytes and osteomacs altogether (Bendall, 2014). Due to the cascades stated earlier, the supportive factor CXCL12 is also suppressed where its main function is as chemo-attractant for maintaining HSC within the bone marrow; thus, due to absence of CXCL12, HSC are readily mobilized into the circulation. A different research found neural output was to be partly involved in modulating migration of the mature neutrophils because central administration of GSCF causes neutrophilia (Morimoto, 1989).

The WBC count in rabbits may vary dramatically as a result of stress, circadian rhythms (diurnal fluctuations and variation within a month), nutritional status and dietary differences, and differences in age, gender, and breed. All these parameters were being controlled to make sure that the increase of WBC was mainly contributed by G-CSF and external factors are not the major factors of unwanted changes.

Being prey animals, rabbits are vulnerable to environmental changes that contributed to stress, hence directly affecting the WBC count. Rabbits are to be handled with care and knowledge of behavior while at the same time minimal time of procedure required. Acute stress and its relationship to adrenaline increase give rise to lymphocytosis, while prolonged stress with association to cortisol cause lymphopenia (Melillo, 2007). In contrast another study suggested the adrenaline release in acute stress generate heterophilia along with relative lymphopenia (Marshall, 2008). As an example, rabbits

transported at 28˚C at a duration of 1-3 hours noticed to have inclined PCV, lymphopenia and leukocytosis (Nakyinsige. 2013). As rabbits have sensitive hearing capability, another common stress is loud noise where loud noises notably lead to rise in H:L ratio (Melillo, 2007). The main purpose was to reduce all stress factors as low as possible during the sampling period.

A paper cites that in the late afternoon and evening, total leukocyte counts are at lowest (Washington, 2012). Circadian rhythm was covered by taking blood collection only on the same time of the day within the whole week of sampling. All 5 are taken simultaneously, not at different days or month. According to some journals, it was stated lymphocyte numbers are at peak in morning and bottom in late evening while the opposite occurred in heterophil numbers (Fox and Laird, 1970; Malillo, 2007). This explains how in Control Group, lymphocytes are the dominant WBC component.

Another study showed that, hematological values were correlated with the nutritional status of the animal (Adejumo, 2004). Choice of feed can even affect the blood picture in rabbits. Abdelhalim cited rabbits fed with high-cholesterol diet proven aside from hypercholesteremia, there was significant increase in WBC and lymphocyte (Abdelhalim, 2008). Hence every rabbit was monitored and given same type of water and feed, that was commercial diet and hay.

A study compiled there was a bimodal increase in rabbit species, first at 3 months old while next peak after 1 year old where represented dominantly by lymphocytes and neutrophils respectively (Jain, 1986). Even among male and female, it was reported

there were slight differences where male typically has higher counts than females (Moore, 2015). A study evaluated there were differences for hematological values in 3 different breeds, but the same author stated it was not significant (Etim, 2014). Though age, gender and breed play a minor role in this case, we still rule out any possibility of inconsistency by selecting only the same group of samples which were only NZW female rabbits with age more than 6 months old upon purchasing.

6.0 CONCLUSION

The effect of G-CSF in rabbits is that the glycoprotein increased the WBC count as high as 3 folds where the major component responsive towards G-CSF is heterophils followed by monocytes, eosinophils and basophils but no significant rise in lymphocytes. The peak level reached was at D3 however the duration of effect does not last long as by D4 (48 hours from last injection) the blood values returned to normal level. As for bone marrow cytology findings, high numbers of myeloid progenitor cells supported by the increase in M:E ratio proved that G-CSF were giving influence on the bone marrow of rabbits.

7.0 RECOMMENDATION

The peak level and duration of effect served as an important information for future application of G-CSF in veterinary practice to provoke influx of inflammatory cells in the circulations and help to promote musculoskeletal healing or repair in rabbit pets. The information of this study also serves future experimental extension to consider the ideal time for stem cell harvest in this species. This design study only considers control and one dosage effect, possibly in the next experimental design can include multiple dosage application where it can give a better picture on whole dose-dependent effect in addition discovering the harmful side-effects. Next is to increase the sample size as this may achieve a more conclusive finding where there would be smaller disparity even within same group of same day findings. The limitation of the study is that a comparison of the bone marrow samples between treated and non-treated group to evince a more valid effect were not conducted as the same group of rabbits were used for this study.

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