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Experimental Research

Effectiveness of granulocyte colony stimulating factor to enhance healing on delayed union fracture model Sprague-Dawley rat

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ABSTRACT

Introduction: Delayed union is a problem that can occur after fracture healing. Many studies were conducted based on the diamond concept approach to solve the problem of delayed union. Granulocyte-colony stimulating factor (G-CSF) is one of the various substances known to have a positive role in healing skeletal tissue or adjuvant regeneration. This study was conducted to see the effect of G-CSF in affecting delayed union fracture healing.

Materials and method: The experimental study was conducted by randomized posttest only control group design on 24 experimental animals Sprague-Dawley white rats that had experienced delayed union models. The study compared the treatment group injected with subcutaneous G-CSF with a control group and was divided into four groups (n = 6). Harvest and follow-up histomorphometry and immunohistochemistry were performed in the second week and in the fourth week the histomorphometry analysis consisted of the percentage of immature bone area, cartilage, and fibrous area. The semiquantitative evaluation of immunohistochemistry with the expression of BMP-2 through the immunoreactive score (IRS).

Result: In the evaluation of histomorphometry and immunohistochemical parameters, there were significantly more woven bone area (p = 0,015), less fibrosis area (p = 0,002) and higher BMP 2 expression (p = 0,004) in treatment group week four compared to control. .

Conclusion: G-CSF was shown to increase the speed of healing in Sprague-Dawley rats on delayed union models evaluated from histomorphometry and immunohistochemical aspects.

1. Introduction

Fracture is a disease that has become a major problem in the health sector in the world. In the world, traffic accidents cause the most injuries. injury due to traffic accidents is one of the priorities in the health sector. WHO in 2004 reported that traffic accidents will be the 3rd cause of injury in 2020, while in developing countries this problem will be the 2nd.¹ though most fractures heal normally, there are some complications that occur with fracture healing, including delayed union or non-union. From a study on 5571 cases of fracture, the prevalence of delayed union was 4.4% and non-union was 2.5%. Widenfalk et al. showed 31% prevalence of delayed union cases, Clancey et al. Showed 13% [2]. There are various problems that arise in cases of delayed union such as decreased range of motion, immobilization, joint arthritis and prolonged

hospitalization which reduces the quality of life. The funds needed are quite large in an effort to speed up fracture healing or prevent non-union in the United States. [3,4]. Therefore, non-union and delayed union problems are important to overcome here have been many studies conducted in an effort to overcome the problem of delayed union through the approach of the diamond concept proposed by Giannoudis, 2007. The components of this concept are osteogenic (cells), osteoconductive (matrix, scaffold), osteoinductive (growth factors) and stable fixation. [5,6]. If there is a deficit in one of the components, it causes interference with fracture healing, causing delayed union or non-union in the fracture [7,8].

Success in the fracture healing process is the result of a complex interaction between the osteogenesis and angiogenesis processes [10, 11]. The number of osteoblasts originating from the periosteum and

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local bone marrow is adequate, as well as adequate vascularization, especially in the bone marrow capillaries, affecting the process of osteogenesis and angiogenesis. fascia) through good circulation, and play a role in fracture healing [9,12].

Kuznetsov et al. Demonstrated that circulating cell osteoprogenitor cells contribute to bone formation and fracture healing processes [13]. These cells can contribute up to 10% of the presence of osteoblasts in the fracture consolidation callus and as much as 50% of the osteocytes to ectopic bone regeneration [14]. Intravenous injection of osteoprogenitor cells stimulates fracture healing [11]. In another study, circulating osteoprogenitor cells could be increased by the use of bioactive molecules that trigger the mobilization of these cell medullary precursors, thereby supporting the bone healing process [16,17]. Novicoff et al. found that there were 27 studies that met the Level-I criteria. The most research evidence (25%) was about growth factors and BMP and currently there are commercially available osteoinductive preparations [18].

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that is used therapeutically for its ability to mobilize medullary hematopoietic stem cells in the systemic circulation. G-CSF also induces the mobilization of vascular stem cells and mesenchymal stem cells, both of which are involved in the healing of skeletal tissue [19,20].

This study aims to determine the effect of G-CSF on the quality of fracture healing on delayed union model of experimental animals in terms of histomorphometry and immunohistochemistry.

2. Material and method

This experimental study used a randomized posttest only control group design. The study population was Sprague-Dawley white rats weighing about 250–350 g. Treatment of experimental animals was carried out at the Animal Research Facilities IMERI, Faculty of Medicine, Universitas Indonesia, Jakarta. Histomorphometry and immunohistochemical examinations have been carried out at the Laboratory of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo National Central Hospital. By calculating Federer's formula, the total number of samples was 24 Sprague-Dawley white rats. each group consisted of 6 experimental animals. This study is conducted by the approval of the Faculty of Medicine Universitas Indonesia ethical committee.

2.1. Intervention

In each animal, a fracture (delayed union model) was made on the femur and then fixed using an intramedullary K-wire. Then the experimental animals were randomly allocated into 2 groups. Group 1 (control): rats that only experienced delayed union model and were injected with 0.9% NaCl. Group 2: the rats who experienced delayed union model were given G-CSF injection at a dose of 50 µg/kg per day for 5 days after surgery.

Delayed union fracture was made with osteotomy in diaphysis using a saw, forming a simple transverse fracture. Delayed union model in this study refers to the research of Kasman D et al. In the form of mechanical treatment with circular stripping of the periosteum using scalpels each 5 mm from the fracture line to the proximal and distal directions [21]. Then performed intramedullary reaming with a 21G needle, followed by internal fixation using an intramedullary k-wire measuring 1.2–1.4 mm retrograde. The surgical wound was closed by suturing the soft tissue using catgut 3.0 and the skin with Silk 3.0. In the treatment group, experimental animals were given subcutaneous injection of G-CSF (Leucogen®) at a dose of 50 µg/kg per day for 5 days after surgery. The treatment group will be divided into 2 groups which will differentiate the fracture healing formation (2 weeks and 4 weeks).

At week 2 and 4 post treatment, the experimental animals were sacrificed by giving Phenobarbital at a dose of 75 mg/kilogram Body-weight intraperitoneally. After the femur is separated, a

histomorphometry and immunohistochemical analysis will be examined at the Department of Anatomical Pathology by expert from musculoskeletal pathology division. The euthanized experimental animals will be buried through the Animal Research Facilities, Faculty of Medicine, Universitas Indonesia with Replacement, Reduction and Refinement principle in experimental research. After the fixation procedure, making paraffin blocks and staining the specimen, then shooting the slides using a digital light microscope using the Leica ICC50 HD.

2.2. Histomorphometry analysis

Hematoxylin eosin streaks are mainly for assessing callus, fibrosis areas, reinforcement areas and assessing cartilage areas. Immunohistochemical staining using IRS scoring system. The immunoreactive score (IRS) is used to assess the number of cells expressing BMP-2. Histomorphometry assessment was carried out using Image J software version 1.48s with the help of a scale from the counting chamber (see Fig. 1), which was carried out semi-automatically [22,23]. Determination of each area to be assessed was carried out by a supervisor who is an anatomical pathologist.

The data obtained from the six sample groups were processed using the SPSS 21.0 for windows computer program. The normality test used the Shapiro Wilk test for each group prior to the analysis test. The test was carried out with one-way ANOVA for data with normal distribution and Kruskal Wallis test for data with abnormal distribution. If there is significance in the one-way ANOVA test, it is followed by Post Hoc analysis, another test carried out by T-test or Mann-whitney test (non-parametric data) for two independent sample group.

3. Result

3.1. Histomorphometry parametric evaluation

Total callus area evaluation.

The first evaluation of histomorphometry parameters is the total callus area. In this study, the total callus area was divided into four groups, namely the second week control group, the fourth week control group, the second week intervention group and the fourth week intervention group. The total area of callus in this study had abnormal data distribution, so the Kruskal Wallis test was performed. In the Kruskal Wallis test, the p value was obtained = 0.277, which means that there was no significant difference in the mean comparison between the four groups see Table 1.

3.2. Total woven bone evaluation

This study compared the percentage of woven bone as a second parameter for histomorphometry. Comparisons were made for the four groups namely the second week control group (2nd WC group), the fourth week control group (4th week control group), the second week intervention group (2nd WI group) and the fourth week treatment group (4th WI group) (Fig. 2). In the normality test, the distribution of data was normal, a one-way ANOVA test was performed. In this test, there was a significant mean difference ($p = 0.001$) (see Table 2) where this difference could affect the study outcome. For further analysis, a one-way Post hoc Bonferroni test was conducted (see Table 3) and the result is significantly different between 2nd weeks control group and 4th weeks interventional group (2nd CG vs 4th IG) and found significantly different between 4th weeks control group and 4th weeks interventional group (4th weeks CG vs 4th weeks IG).

On the results of the one-way Post hoc Bonferroni test, it was found that there was a significant difference in the percentage of woven bone in the fourth week control sample with the fourth week treatment ($p = 0.015$). In addition, there was also a statistically significant difference between the control at the second week and the treatment at the fourth week ($p < 0.001$). Meanwhile, there was no significant difference in the

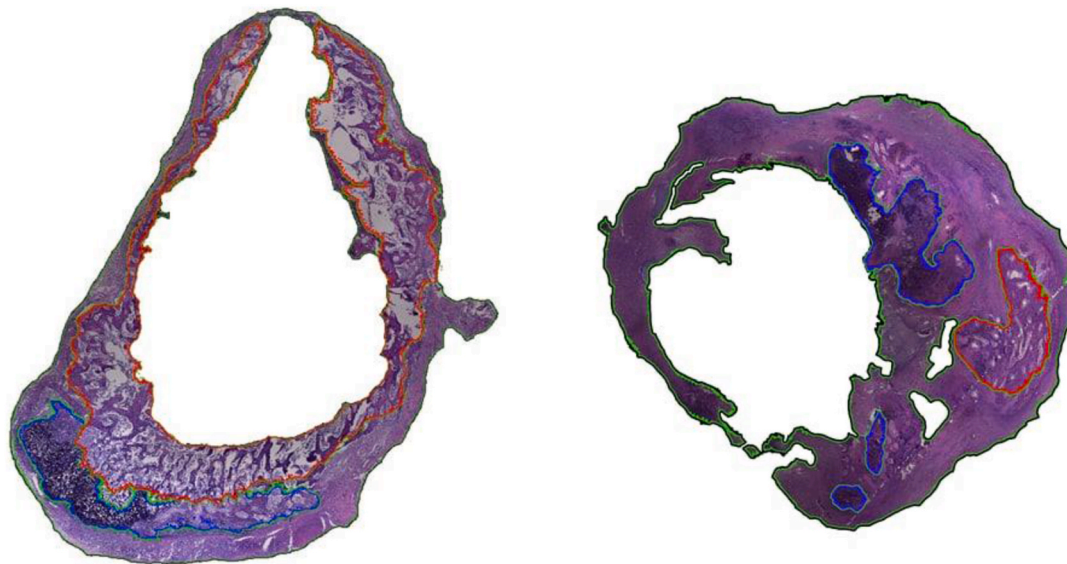


Fig. 1. Determination of the assessment area on a slide at 40× magnification, put together using PTGui. Black line: total callus area, green line: fibrosis area, blue line: cartilage area, red line: reinforced area (woven bone). (Left) control group; (right) treatment groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Analysis of Total Callus Area with the Kruskal Wallis test.

		N	Median (min-max)	P
Total Callus Area	second week control	6	13,10 (10,91-18,44)	0,277^a
	Fourth weeks control	6	15,70 (12,42-19,47)	
	second week intervention	6	10,29 (8,70-25,44)	
	Fourth week intervention	6	13,51 (10,54-16,32)	

^a The Kruskal Wallis test was used for independent abnormal distribution data that was not paired with groups of more than two.

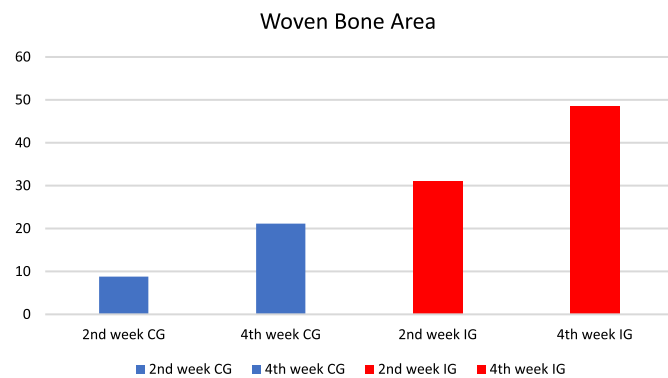


Fig. 2. Comparison between woven bone area % between all group.

Table 2
One-Way ANOVA of comparison % of woven bone area.

		N	Mean ± SD	P
Total Woven Bone area (%)	second week control	6	8,80 ± 4,11	0,001*
	Fourth weeks control	6	21,16 ± 4,96	
	second week intervention	6	31,04 ± 16,32	
	Fourth week intervention	6	48,63 ± 21,15	

Table 3
Woven bone area % analysis with post hoc.

	mean	CI 95%		p
		min	Max	
2nd week CG vs 4th week CG	-12,35	-35,58	10,8802	0,812
2nd week CG vs 2nd week IG	-22,23	-45,46	0,9960	0,066
2nd week CG vs 4th week IG	-39,82	-63,05	-16,5951	<0,001^a
4th week CG vs 2nd week IG	-9,88	-33,11	13,3475	1,000
4th week CG vs 4th week IG	-27,47	-50,70	-4,2437	0,015^a
2nd week IG vs 4th week IG	-17,59	-40,82	5,6405	0,231

^a One-way Anova Post hoc Bonferoni.test.

percentage of woven bone between groups 2nd week CG-2nd week IG, 2nd week CG-4th week CG and 2nd week IG-4th week CG.

3.3. Fibrotic area evaluation

In this study, a comparison between control and treatment samples was also carried out in the second week and at the fourth week. Each of them tested independent samples T-Test and Mann-Whitney.

The results of the independent samples T-Test showed a significant difference in the mean between control and treatment in the second week where the p value < 0.001 (see Table 4). And in the fourth week using the Mann-Whitney test where the p value = 0.004 (see Table 5). Fig. 3 showed comparison total fibrosis area between all group, it reveal

Table 4
Comparison fibrosis area.

		N	Median (min-max)	Total P value	P value inter group
Fibrosis area (%)	2nd week CG	6	79,07 (72,80–91,32)	<0,001^a	Reference
	4th week CG	6	68,13 (63,41–77,70)		
	2nd week IG	6	58,43 (45,77 - 66,73)		0,004^b
	4th week IG	6	47,89 (11,17–53,88)		0,004^b

^a Kruskal Wallis test.

^b Mann Whitney test.

Table 5
Comparison fibrosis area between two week and four weeks.

Perbandingan	N	Mean ± SD/Median (min-max)	P value
2nd week CG	6	80,14 ± 6,19	<0,001 ^a
2nd week IG	6	56,77 ± 8,44	
4th week CG	6	68,13 (63,41–77,70)	0,004 ^b
4th week IG	6	47,89 (11,17–53,88)	

^a Independent Samples T-Test.

^b Mann-Whitney test.

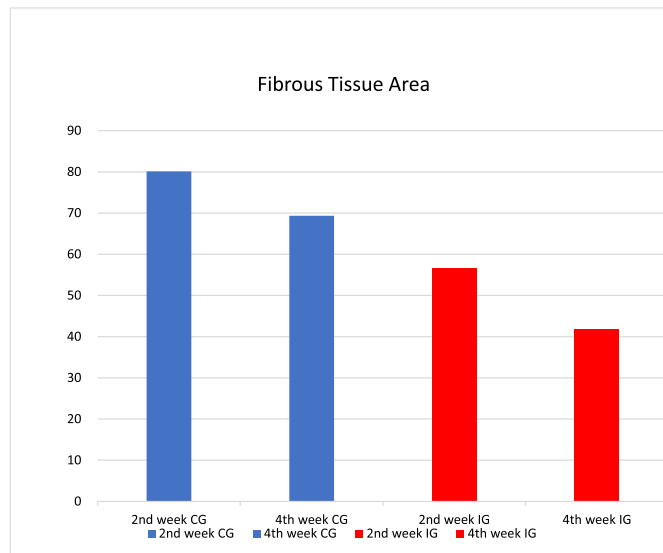


Fig. 3. Comparison of the mean fibrosis tissue formed (%) in the control and treatment groups.

4th week intervention groups has lowest fibrotic area.

3.4. BMP-2 expression evaluation

In the last parameter, the BMP-2 expression was compared using the IRS score. In the normality test, the distribution of data was not normal, so the Kruskal Wallis test was performed to determine the mean difference that was significant in the whole group. From this test, it was obtained p value < 0.001, which means that there were significant differences in the control and treatment groups, both the second and fourth week as a whole (see Table 6).

Post Hoc Mann Whitney test was performed with a 2nd CG as a reference to determine which group comparisons had significant mean differences. From the test results, there was a significant difference in 2ndCG-4thCG (p = 0,016), 2nd CG-2nd IG (p = 0,008) dan 2ndCG-4thIG (p = 0,003). Furthermore, the comparison between control and treatment was carried out in the fourth week and the p value was obtained =

Table 6
Comparison BMP-2 with Kruskal Wallis test.

		N	Median (min-max)	Total P value	P value inter group
IRS score	2nd week CG	6	4 (3–6)	<0,001 ^a	Reference
	4th week CG	6	6 (6–9)		
	2nd week IG	6	9 (6–9)		
	4th week IG	6	12 (9–12)		

^a Kruskal Wallis test.

^b Mann Whitney test.

0.004 (see Table 7), which means that there is a significant difference in the mean that can affect the study outcome. Fig. 4 showed comparison BMP-2 expression between all group, it reveal 4th week intervention groups has highest IRS score

4. Discussion

This study used animal models of white rats or Sprague Dawley rats. The rat animal model was chosen because it has been standardized with the ability of a short bone turnover rate so that economically representative results can be obtained with the desired target. Rats are known to have a habit of standing upright more often than rabbits which are also often used as experimental animal models [24]. So that the femoral bone morphology of mice has the advantage of being used in research when compared to species that stand on four legs [25].

To reduce the bias in the results of the study, the researchers used uniform male rats aged 12–16 weeks with a body weight of 250–350 g. This is to ensure that nutrition is fulfilled properly for the bone healing process and reduces the risk of hormonal influences on the research outcome. This is evidenced by the absence of a statistically significant mean difference in body weight of the control and treatment group rats in this study. In addition, the uniform size of the mice will also make it easier for operators to intervene in experimental animals.

In a study conducted by Dologo et al. it was found that G-CSF was able to increase the proliferation of mesenchymal stem cells taken from peripheral blood vessels [26]. This proves that G-CSF not only has activity on bone marrow, but also has an effect on peripheral blood vessels acting on hematopoietic cells which are osteoinductive. G-CSF works to increase mesenchymal stem cells by induction of osteogenic cells. Administration of G-CSF is known to suppress osteoblasts and interfere with CXCR4/CXCL12 signaling, thereby inducing hematopoietic stem cells and progenitors [26,27]. therefore the fracture healing effect due to inducing osteoblast cells is not the answer to accelerating the healing process. As previously explained, G-CSF can increase the number of stem cells and it is these stem cells that will produce BMP-2 where BMP-2 expression will recruit stem cells at the fracture site [28–30]. In addition, from a clinical application point of view, Patients who presented with delayed union did not require surgery [31]. This was the basis for indirect G-CSF administration at the fracture site in this study.

In a study conducted by Kaygusuz et al., G-CSF was shown to increase the level of TGF-β1 which functions for the fracture healing process [32]. In this study, subcutaneous administration of G-CSF helped in the fracture healing process. So that without local administration at the fracture site, the bone healing process can already occur in this study. This is evident from the percentage of woven bone and the IRS score which was significantly better in the group with G-CSF injection compared to the control group. The results of the increase in woven bone which were found to be higher in the G-CSF group were supported by research by Herrmann et al. which stated that on day 20, the size of the bone defect in the group of mice given G-CSF subcutaneously at a dose of 50 µg/kg body weight was smaller than the control group. Herrmann et al. also stated that bone formation in mice in the G-CSF group was faster than in the control group [28].

Another study by Moukoko et al. using a G-CSF dose of 5 µg/kg body weight in rats also resulted in optimal fracture healing compared to the control group. Histologically, the difference in the area of callus formation found by Moukoko et al. is the same as that found in this study [33]. However, one of the factors that produced different results was the difference in the dose of G-CSF administration of 50 µg/kg body weight

Table 7
Comparison of BMP-2 expression at week four with the Mann Whitney test.

		N	Median (min-max)	P value
IRS score	4th week CG	6	6 (6–9)	0,004*
	4th week IG	6	12 (9–12)	

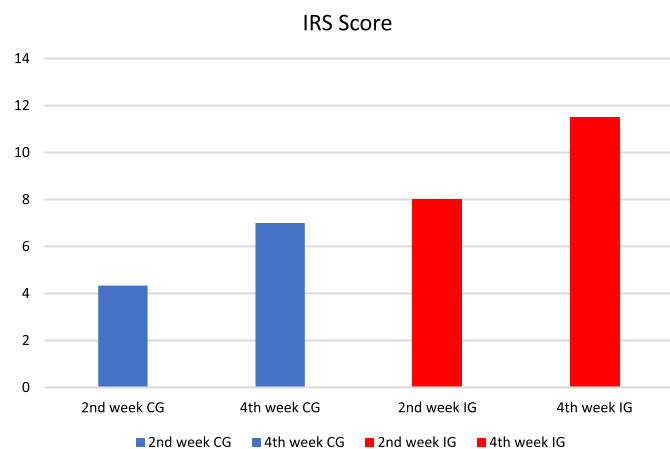


Fig. 4. Comparison of IRS BMP2 Expression scores in groups.

which was able to increase the difference in mean woven bone significantly between the control and treatment groups, especially at the fourth week of this study.

These results are in line with the research of Bozlar M et al. which states that G-CSF has an important role in fracture healing. There was a significant difference in the median radiographic score in the control group of rats was 4.1, while the median radiographic score in the group of rats injected with G-CSF was 6.1. However, Bozlar M reported that cortex remodeling, callus formation, bone fusion, and bone marrow changes did not differ significantly between the control group and the group with G-CSF injection [34]. The same thing was found in this study, where there was no significant difference in callus formation in the treatment and control groups seen from the histomorphometry analysis.

In this study, it was found an increase in the percentage of woven bone from week two to week four both in the G-CSF injection treatment group and in the control group. However, the woven bone in the treatment group was significantly higher than the control group. The significant difference in the percentage of woven bone occurred between the control group in the second week and the fourth week of the treatment group. This matter still cannot be explained in detail due to inadequate literature. However, other literature mentions the addition of woven bone over time according to the physiology of bone healing. In animal models, the peak of soft callus formation occurred at 7–9 days posttraumatic. At the same time, intramembranous ossification occurs subperiosteal adjacent to the distal tip and proximal to the fracture to form a hard callus [35].

In this study, the total cartilage area did not differ significantly between the treatment group and the control group. This shows that the process of bone formation through endochondral ossification cannot be compared through the aspect of cartilage formation in this study. The results obtained were different from the research conducted by Meeson R et al. on Wistar rats. In this study, an increase in the percentage of cartilage formation in the treatment group with a combination of G-CSF and the antagonist CXCR4 [36]. Research conducted by Herrmann et al. also showed similar results, where there was an increase in the formation of cartilage areas in the G-CSF treatment compared to controls [28].

In the area of fibrosis aspect, the percentage of fibrosis was found to be significantly lower in the treatment group than in the control group. This can occur because G-CSF has an antifibrosis effect. Zhao F et al. Stated that G-CSF increased the antifibrosis effect by upregulating CXCR4 expression [37]. However, different results were obtained by Herrmann et al. Where the bone gap in mice given G-CSF was filled with fibrosis tissue that was rich in collagen while in the control group it was only filled with fat tissue [28].

In another study, incomplete bony grafting between the two cortices by fibrotic tissue was observed in a control group on periosteal callus. The central portion consists of either calcified or uncalcified cartilage

adjacent to the new woven bone, indicating an endochondral ossification process. Peripherally, the periosteal callus consists of woven and lamellar bone. Whereas in the group given G-CSF, there was no fibrosis tissue and no evidence of endochondral ossification process. In the treatment group there was a complete bone grafting. The osteotomy gap is filled with anastomotic trabecular bone in the periosteal callus region and between the cortices. On the internal and periosteal callus, signs of bone formation and bone resorption indicate that bone regeneration is remodeling [33].

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G-CSF is a hematopoietic growth factor that plays an important role in the production, differentiation of neutrophils and osteogenesis. In this study, the group of mice treated with G-CSF injection had a significantly higher IRS score of BMP2 expression than the control group. This is consistent with a study by Moukoko et al. Who reported that G-CSF induces the mobilization of vascular stem cells and mesenchymal stem cells. In mice, injection of G-CSF resulted in a significant increase in CD34 + progenitor cells within five days. These progenitor cells play a role in differentiation into osteogenic or vasculogenic pathways. Stimulation of bone recovery is associated with an increase in vascular and mesenchymal progenitor cells at the site of neo-osteogenesis. Infiltration of cells at the bone site plays a role in endochondral osteogenesis. In the early stages, cells increase the cytokine BMP-2, which plays a role in bone repair [33]. According to Czekanska EM et al., G-CSF increases the expression of BMP-2 mRNA which plays a role in the process of bone formation from mesenchymal stem cells [39]. BMP-2 plays an important role in the bone healing process, especially in the process of callus formation. In mice, mutations in BMP-2 lead to failure of callus formation.⁴⁰ G-CSF (Leucogen®) are potential agent for enhancing bone healing and regeneration and have been proven in our study. This kind of drug recombinant already used clinically in human for another purpose (increasing granulocyte in leucopenia patient), and then proceeding for clinical trial in human should be safe and to prove efficacy on in vitro study would be same in in vivo study.

4.1. Study limitation

The first limitation of this study is that the different samples were used to compare the progression of week two to week four. This is difficult to do because the test animal mice must be sacrificed, so it is impossible to use the same sample. Giving a higher dose of G-CSF in this study, it is also thought that it will cause side effects, if it is tested on humans. Another limitation of this study is the absence of an assessment of the radiological and biomechanical aspects which can provide more comprehensive results. This was not done due to limited time and research costs.

5. Conclusion

G-CSF histologically (as seen from the percentage of woven bone) was significantly better in the fracture healing process in the delayed union model of Sprague-Dawley rats than controls. G-CSF significantly increased the expression of BMP-2 in the fracture healing process on the

delayed union model of Sprague-Dawley rats compared to controls. Further research in human (in vivo study) with clinical trial, translational study or randomized control study should be performed in next future research due to proven efficacy in the invitro. Beside the efficacy of G-CSF in our study, lessons learnt from this study are beside of cell progenitor (osteoblast, osteoblast and osteocyte) and mechanical stability, the granulocyte colony stimulating factor have main role in bone healing and regeneration.

Ethical approval

Ethical approval clearance from ethics committee board Faculty of medicine, Universitas Indonesia with protocol number 19-12-1426.

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Author contribution

Aryadi Kurniawan contributes in the study concept or design, data collection, analysis and interpretation, oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.

Evelina kodrat contributes in the study concept or design, data collection, analysis and interpretation from pathology anatomy perspective.

Yogi Ismail Gani contributes to the study concept or design, data collection and writing the paper.

Registration of research studies

1. Name of the registry: not applicable
2. Unique identifying number or registration ID: not applicable
3. Hyperlink to your specific registration (must be publicly accessible and will be checked): not applicable

Guarantor

Aryadi Kurniawan is the sole guarantor of this submitted article.

Consent

Written informed consent wasn't necessary due to in vitro experimental research.

Provenance and peer review

Not commissioned, externally peer reviewed.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2020.12.005>.

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