

Role of Vasodilator Agents in Osseous Tissue, Cartilage and Fibrous Tissue Formation of Non Vascularized Fracture Healing on Sprague-Dawley Rats

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Abstract

In spite of various methods to achieve fracture healing, delayed union with its subsequent morbidity remains a major problem. Angiogenesis plays an important role in fracture healing. Sildenafil as a vasodilator agent has been shown to be a potent stimulator of angiogenesis through upregulation of pro-angiogenic factors known as vascular endothelial growth factor (VEGF) that is believed could hasten fracture healing. This study evaluated the effect of sildenafil and its influences on osseous tissue, cartilage and fibrous tissue formation in delayed union model of non vascularized fracture healing. This was an experimental study in delayed union femoral fracture model in Sprague dawley rats evaluated using histomorphometry. Twenty four rats were randomized into four groups; control (group 1), administration of vasodilator agents (sildenafil) 3.5 mg/kgbw (group 2), sildenafil 5 mg/kgbw (group 3), and sildenafil 7.5 mg/kgbw (group 4). The parameters evaluated were osseous tissue, cartilage and fibrous tissue formation. The measurement was carried out at 2 and 4 weeks after intervention. After two weeks, sildenafil significantly increased the parameter of fracture healing in delayed union model. The results showed a significant increase in total osseous tissue ($p=0.001$), cartilage area ($p=0.015$) and fibrous tissue ($p=0.005$). After four weeks, the results were also significant in total osseous tissue ($p=0.015$) and fibrous tissue ($p=0.001$). Conclusion, sildenafil as a vasodilator agent is proven to effectively accelerate osseous tissue, cartilage and fibrous tissue formation in delayed union model of non vascularized fracture healing.

Keywords: cartilage and fibrous tissue formation, non vascularized fracture healing, osseous tissue, vasodilator agents

Pengaruh Agen Vasodilator Terhadap Pembentukan Tulang, Tulang Rawan dan Jaringan fibrosis Pada Penyembuhan Fraktur Non Vaskularisasi Pada Tikus Sprague Dawley

Abstrak

Meskipun telah ditemukan berbagai metode untuk mencapai penyembuhan fraktur, *delayed union* tetap menjadi masalah besar. Angiogenesis memainkan peran penting dalam penyembuhan fraktur. Sildenafil sebagai agen vasodilator telah terbukti menjadi stimulator poten dari angiogenesis melalui peningkatan faktor pro-angiogenik yang dikenal sebagai *Vascular endothelial growth factor* (VEGF) yang diyakini bisa mempercepat penyembuhan fraktur. Penelitian ini mengevaluasi efek sildenafil dan pengaruhnya pada jaringan tulang, tulang rawan dan pembentukan jaringan fibrosis pada model *delayed union* fraktur yang tidak tervaskularisasi. Penelitian ini merupakan penelitian eksperimental pada model *delayed union* fraktur femur tikus Sprague Dawley yang dievaluasi menggunakan histomorfometri. Dua puluh empat tikus secara acak dibagi menjadi empat kelompok; kontrol (kelompok 1), diberi agen vasodilator (sildenafil) 3,5 mg / kgbb (kelompok 2), sildenafil 5 mg / kgbb (kelompok 3), dan sildenafil 7,5 mg / kgbb (kelompok 4). Parameter penyembuhan yang dievaluasi adalah jaringan tulang, tulang rawan dan jaringan fibrosis. Pengukuran dilakukan pada 2 dan 4 minggu setelah intervensi. Setelah dua minggu, kelompok dengan sildenafil secara signifikan terjadi peningkatan parameter penyembuhan fraktur. Hasil penelitian menunjukkan peningkatan yang signifikan dalam jumlah jaringan tulang ($p = 0,001$), tulang rawan ($p = 0,015$) dan jaringan fibrosis ($p = 0,005$). Setelah empat minggu, hasilnya signifikan pada total jaringan tulang ($p = 0,015$) dan jaringan fibrosis ($p = 0,001$). Kesimpulan, sildenafil sebagai agen vasodilator terbukti efektif mempercepat pembentukan jaringan tulang, tulang rawan dan jaringan fibrosis di pada model fraktur *delayed union* dengan non vaskularisasi.

Kata Kunci: penyembuhan fraktur non vaskularisasi, agen vasodilator, jaringan tulang, tulang rawan dan jaringan fibrosis

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Introduction

Fracture defined as discontinuity of bone and cartilage.^{1,2} On the healing process of a fracture needs two factors which are mechanical factors and biological factors.³⁻⁵ During healing process, sometimes it can often complicated by any condition, resulting in delayed union or non-union. Delayed union is a condition in which fracture healing takes longer time than the normal one, whilst non-union is failure for a fracture to reach union.^{3,6} General consensus agrees that delayed union happens if no signs of union in radiological examination are present in 20-26 weeks after fracture. If this condition occurs, physicians should consider additional therapy or even radically changing therapy to reach union.⁶ According to a study of 5571 cases of fracture, the prevalence of non union numbers 2,5% while delayed union reaches 4,4% of all cases.⁶ Almost all of non union case are tibial fractures caused by high energy trauma. Study conducted by Clancey et al (1982)⁷, showed prevalence of delayed union were 13% on open fracture of tibia. Otherwise Widenfalk et al (1979)⁸, showed 31%. Study by Edwards and Jarowski showed 41% prevalence on open fracture type III that requires bone graft to reach union.^{6,9}

Many factors contribute to this condition, the main factors is inadequate vascularization to the fracture site.^{6,10,11} Adequate vascularization contributes to increase activity of the cytokines that can help heal fracture, which are TGF- β (Transforming Growth Factor- β), BMP (Bone Morphogenetic Protein), PDGF (Platelet Derived Growth Factor), FGF (Fibroblast Growth Factor), IGF (Insulin like Growth Factor) and VEGF (Vascular Endothelial Growth Factor).¹²⁻¹⁴ Among those cytokines, VEGF is one of the major factors that can promote angiogenesis, it has 4 isoform, and VEGF-A is the isoform that we discuss in this study.¹⁵ VEGF is a protein, which work to stimulate growth, endurance, and duplication of blood vessel cells.^{12-14,16} In healing process of a fracture, VEGF plays a significant role in every step of it, started from hematome phase until bone turn over on the remodeling phase.¹⁷⁻²⁰ Sildenafil, previously known as a drug for erectile dysfunction, has an effect on vasodilation that may improve vascularization by increasing VEGF activity. It works as a specific inhibitor on phosphodiesterase-5

(PDE5) which catalyzed cyclic guanosine monophosphate (cGMP).¹⁵⁻¹⁸ Histing et al (2011)¹⁷, shows sildenafil stimulated angiogenesis by increasing VEGF on healing process of fracture.

The aim of this study is to know how sildenafil as a vasodilator agent accelerated healing process of delayed union fracture. This drug can give an alternative approach on treating this condition, which is more cost efficient than adding another therapy. This study has never been tested, so it will become the first study in the world, that's why it will has an intellectual rights. The population of this study is male Sprague dawley rats aged 12-16 weeks, with body weight around 250-350 gram.

Methods

Our research was an experimental study with post test only control group design. The study was initiated with pilot study of Sprague-Dawley rats to get model of delayed union. The procedure was approved by Ethical Committee of University of Indonesia No. 731/H2.F1/ETIK/2012 on 10 December 2012. We did the experiments at animal house, laboratory of pathology anatomy department, and laboratory of orthopaedic and traumatology department of CiptoMangunkusumo hospital in Jakarta-Indonesia from July until December 2013.

We calculated the samples by unpaired categorical-numeric analysis and categorized them into random four groups. We included six rats for each group so the total 36 rats were included, consisting of 12 rats for preliminary study to determine the model for delayed union of non vascularized fracture healing, 24 rats for main study with sildenafil. Group 1 was rats with delayed union only, group 2 was rats with delayed union and treated with sildenafil 3,5 mg/kg bb 3 times weekly (minimal doses), group 3 was rats with delayed union and treated with sildenafil 5 mg/kg bb 3 times weekly (optimal doses), group 4 was rats with delayed union and treated with sildenafil 7,5 mg/kg bb 3 times weekly (maximal doses).

All groups were euthanised with phenobarbital 75mg/kgBB intraperitoneally on the second and fourth week for examination of histomorphometry and immunohistochemistry.²¹ The dose was higher in rats than human because the metabolism of

sildenafil in rats is about 4-5 times faster than human.^{22,23} The variables were doses of sildenafil given, quantitative measurement of fracture healing by histomorphometry.

The examination of histomorphometry was done. After stained by HE, the images of tissue were taken digitally by microscope and the width and diameter of callus were assessed. We used software Image J (R) for the assessment, and the results were the minimal, average, and maximal wide of callus diameter. The assessment was supervised by anatomical pathology consultant.^{24,25}

Data were presented as mean \pm standard deviation (SD). All statistical analyses were performed with SPSS version 16 for Windows. Normality test was assessed by Shapiro Wilk test for each group. After that, the analytic test was assessed by one way ANOVA for data with normal distribution and Kruskal Wallis for data with abnormal distribution. Any significant difference by one way ANOVA was then continued to be analyzed by Post Hoc to assess the comparison of each group. After that the data from second and fourth week will be assessed by Independent t-test.²⁷

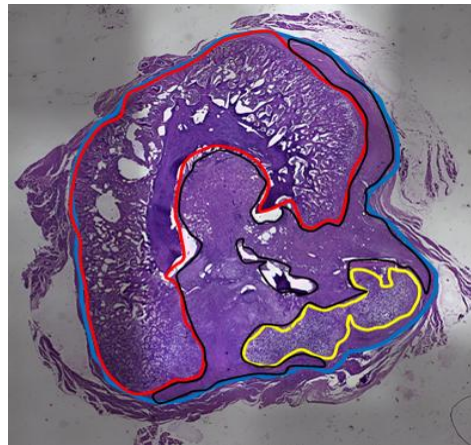


Figure 1. Determining callus area by HE stain. Blue line: total callus area; red line: calcification area; yellow line: cartilage area; black line: fibrosis area.

Results

In this study, we used 4 rats with different body weight for 2 groups. No significant difference was found between weight groups in this study, so the effect of body weight on the observed effects can be ignored. Along the study period, 1 rat was died. One rat from the third group was excluded due to fixation failure. One rat from the fourth group was excluded from the examination of histomorphometry because of infection.

In healing process, we collected data twice, at the end of the 2nd week and the end of the 4th week. So we could compare about histomorphometry on both week. There was an acceleration of the healing process in delayed union of non vascularized fracture in study subjects receiving sildenafil. The calculation is performed at twice in each group (table 1, 2 and 3).

Table 1. Evaluation of Dynamic Process of Osseous Tissue Area on 2nd and 4th week

Parameter	Group	Mean \pm SD Week 2	Mean \pm SD Week 4	Independent t-test (p-value)
Osseous Tissue	Control	0,712 \pm 0,751	0,851 \pm 0,170	0,482
	Sildenafil 3,5 mg	1,498 \pm 0,469	0,968 \pm 0,229	0,052
	Sildenafil 5 mg	1,041 \pm 0,065	1,416 \pm 0,690	0,196
	Sildenafil 7,5 mg	5,451 \pm 0,018	2,651 \pm 0,372	0,001

Table 2. Evaluation of Dynamic Process of Cartilage Area on 2nd and 4th week

Parameter	Group	Mean ± SD Week-2	Mean ± SD Week-4	Independent t-test (p value)
Cartilage Area	Control	1,391 ± 0,322	0,597 ± 0,048	0,209
	Sildenafil 3,5 mg	1,898 ± 0,267	0,643 ± 0,229	0,061
	Sildenafil 5 mg	1,164 ± 0,162	0,712 ± 0,067	0,158
	Sildenafil 7,5 mg	3,296 ± 0,382	0,868 ± 0,055	0,002

Table 3. Evaluation of Dynamic Process of Fibrous Tissue on 2nd and 4th week

Parameter	Group	Mean ± SD Week 2	Mean ± SD Week 4	Independent t-test (p-value)
Fibrous Tissue	Control	1,330 ± 0,223	1,425 ± 0,012	0,776
	Sildenafil 3,5 mg	1,208 ± 0,117	1,190 ± 0,064	0,967
	Sildenafil 5 mg	3,751 ± 0,816	2,190 ± 0,510	0,203
	Sildenafil 7,5 mg	1,708 ± 0,190	1,221 ± 0,714	0,218

Discussion

This study used Sprague Dawley rats because rats had bone architecture and remodeling process similar to humans with faster rate of bone turnover.^{28,29} They were breed and cared in the same place where the research conducted, so that all samples have a relatively homogeneous conditions. Experimental animal was 12-16 weeks old because in those age the bone was mature. Weight were ranged from 250-350 mg, in statistical analysis, there was no significant differences so that influence of weight could be ignored. In addition, the animal also from the same gender, so hormonal influences in experimental animals was the same.

Histomorphometry measurement at week-2 found increasing tissue cells that play a role in the healing process. There result consistent with the study by Histing et al, who proved that sildenafil with its vasodilating ability stimulated angiogenesis process by increasing the activity of proangiogenic growth factor (VEGF).¹⁷⁻²⁰ On day-14 more bone tissue is formed; partially calcified cartilage and start resorption process, as well as an increase in woven bone formation and significant angiogenesis processes.^{14,22} In this study, sildenafil improved the expression of VEGF and vascularization.^{15-17,30,31} Vascular invasion allow for the delivery of MSCs, especially pericyte, which share the same basic membrane with endothelial cells of capillaries that penetrate soft callus in which vessels proliferate during the repair process. Study by Brighton et al (1992)³². prove that pericyte able to express

alkaline phosphatase, collagen, glycosaminoglycans, and osteocalcin, and thus able to make calcification in vitro. These findings suggest that vascular invasion in a fracture not only provide oxygen and nutrients needed to repair the injured tissue cells, but also provide an additional source of MSCs which will develop into osteoblasts, and cells of macrophage / monocytes develop into osteoclast.

Different doses of sildenafil gave different response to healing parameter. The minimum dose improved of the ossification area, but had no effect on the total area of callus, cartilage and fibrous tissue. Optimal dose of sildenafil increased areas of cartilage and fibrous tissue, but had no effect on the total area of the callus and ossification areas. The maximum dose of sildenafil increased total area of callus, ossification areas, and areas of fibrous tissue, and does not affect the cartilage area. This variation is due to differences in healing response to sildenafil in different doses.^{33,34}

Maximum dose of sildenafil gave affect in acceleration of fracture healing. Sildenafil increased the expression of the angiogenic activity of VEGF. VEGF increased preosteoblasts differentiation and metabolism and in turn increased bone formation. Bone formation and resorption coupled with bone remodeling to convert woven bone into lamellar bone.^{16,18} So in this study there was an increase in the percentage area of ossification. At week 4, most of the cartilaginous callus had calcified and replaced by bone. In this process

not only resorption of mineralized matrix occurs but also the removal of chondrocyte. There were combinations of the calcified cartilage and newly formed woven bone.^{14,17,35}

Histomorphometry measurement results on the bone healing parameters decreased significantly at week 4. This was likely caused by angiogenesis stimulating activity of VEGF in increasing recruitment, survival and activity osteoclasts, which are responsible for bone resorption and remodeling.^{14,17,35} The process of bone healing in fractures with delayed union models in this study was accelerated. This was consistent with several studies, research by Street et al. showed that groups of rats administered VEGF would had increased mineral density and vascularization in the callus.^{16,18} Eckardt et al (2005)³⁶. conducted a study on rabbit tibial non-union. The result is a non-union group treated with rhVEGF had increased callus.

This study was the first in vivo study that evaluated the effects of sildenafil with different doses of the fracture healing process in delayed union and could be use as a pilot study in the future study. This study shows dynamic process of fracture healing in nonunion because we assess 2 times in week-2

and week 4. This study using histomorfometry assessment and done quantitatively to provide a better accuracy. But this study also had weakness that radiological and biomechanical assessment were not conducted to comprehensively assess the healing process. This was because of limited resources in this study.

Conclusion

Sildenafil as a vasodilator agent was proven to accelerate osseous tissue, cartilage and fibrous tissue formation on fractures with non vascularization. The difference in sildenafil dosage affects the acceleration of non vascularized fractures healing . Further research could be done by studying other variables to assess the healing of fractures with no vascularization, such as by counting the number of osteoblasts and osteoclasts. Researcher suggests the future research to assess the effect of sildenafil on radiological healing and biomechanics of fractures with delayed union. A study to look at the side effects of sildenafil in experimental animals should be established as a further research as well.

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