British Journal of Medicine & Medical Research
8(5): 419-428, 2015, Article no.BJMMR.2015.464
ISSN: 2231-0614

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Role of Sildenafil in Acceleration of Delayed Union Fracture Healing on Sprague-Dawley Rats Model

Ahmad Fauzi[1], Achmad Fauzi Kamal[1*], Aryadi Kurniawan[1] and Evelina Kodrat[2]

[1]Department of Orthopaedic and Traumatology, Cipto Mangunkusumo Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
[2]Department of Anatomical Pathology, Cipto Mangunkusumo Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Authors' contributions

This work was carried out in collaboration between all authors. Author AF managed the experimental process, analyses of the study, performed the histomorphometry analysis. Author AFK designed the study, wrote the protocol and wrote the manuscript. Author AK managed the literature searches, provide scientific support and author EK performed the immunohistochemistry analyses. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2015/15427
Editor(s):
(1) Ibrahim El-Sayed M. El-Hakim, Ain Shams University, Egypt and Riyadh College of Dentistry and Pharmacy, Riyadh, Saudi Arabia.
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Reviewers:
(1) Anonymous, Turkey.
(2) Anonymous, Brazil.
(3) Anonymous, USA.
(4) Anonymous, Sweden.
(5) Anonymous, USA.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1117&id=12&aid=9083

Original Research Article

Received 25th November 2014
Accepted 15th April 2015
Published 2nd May 2015

ABSTRACT

Introduction: Delayed union with its subsequent morbidity remains a major problem in fracture healing. Angiogenesis plays an important role in fracture healing. Sildenafil has been shown to be a potent stimulator of angiogenesis through upregulation of pro-angiogenic factors known as vascular endothelial growth factor (VEGF). This study evaluated the role of sildenafil in accelerating the healing process in delayed union model.
Methods: This was an experimental study in delayed union femoral fracture model in male Sprague Dawley rats evaluated by histomorphometry and immunohistochemistry. Twenty four rats

**Corresponding author: Email: fauzikamal@yahoo.com;*

were randomized into four groups; control (group 1), administration of 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 total area of callus, osseous, cartilage, fibrous tissue, and VEGF expression. The evaluation was carried out at week-2 and -4 after intervention.

Results: On week-2 evaluation, ANOVA test showed a significant difference in the total callus area with the p-value of 0.004. ANOVA test also found significant difference among groups in the osseous, cartilage, and fibrous tissue area with p-value of 0.001, 0.015 and 0.005 respectively. On week-4, ANOVA test found no significant difference among all groups in the total callus area with p-value of 0.192. However, in ANOVA test, we found significant difference between groups in the osseous and fibrous tissue area with p-value of 0.015 and 0.001 respectively.

Conclusion: Sildenafil is proven to accelerate fracture healing of delayed union and to increase VEGF expression.

Keywords: Sildenafil; delayed union; accelerated fracture healing; VEGF.

1. INTRODUCTION

Fracture is defined as a discontinuity of bone, cartilage and epiphyseal growth [1]. In the healing process, fracture needs mechanical and biological factors [2-4]. During healing process, many conditions may cause delayed union or non-union. Delayed union is a condition in which fracture healing takes a longer time than normal [3,5]. General consensus agrees that delayed union happens if no signs of union in radiological examination are present in 20-26 weeks after fracture [6]. Many factors contribute to delayed union. The main factor is inadequate vascularization of the fracture site [5-7].

Adequate vascularization contributes to increase in activity of the cytokines that promotes fracture healing process such as 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) [8-10]. Among those cytokines, VEGF is one of the major factor that can promote angiogenesis. VEGF has four isoforms, but only VEGF-A that we evaluate in this study [11]. VEGF is a protein that acts to stimulate growth, endurance, and duplication of blood vessel [8-10,12]. In fracture healing process, VEGF plays a significant role from hematoma phase until the remodelling phase [13,14].

Sildenafil is previously known as a drug for erectile dysfunction. It has an effect on vasodilatation that may improve vascularization by increasing VEGF activity. It works as a specific inhibitor of phosphodiesterase-5 (PDE5) which catalyzes cyclic guanosine monophosphate (cGMP) [11-13].

There was no previous study reporting sildenafil's effect in accelerating healing process in delayed union fractures. The aim of this study is to determine the role of sildenafil in accelerating the fracture healing process in delayed union models by histomorphometry and immunohistochemistry evaluation. All of these approaches have not yet been applied in humans, thus this study was conducted in Sprague Dawley (SD) rat.

2. METHODS

All procedures undertaken in this study have been approved by The Ethical Committee of Faculty of Medicine Universitas Indonesia No. 731/H2.F1/ETIK/2012. The study had been carried out in two stages. Thirty six male SD rats were randomly divided into four groups consisting of 12 rats for the first stage (preliminary study) to design a delayed union model and 24 rats for the second stage (main study).

2.1 The First Stage

The first stage was aimed to build a delayed union model in rats. Rats were anaesthetized by intraperitoneal injection of ketamine (Ketamil®, Troy Laboratories PTY Limited, Australia) 80 mg/kg body weight (bw) and xylazine (Seton 2%®, Laboratorios Calier S.A. Spain) 10 mg/kgbw. Antisepsis was done with 10% povidone iodine and 70% alcohol from mid-body to the entire region of the right lower extremity which had been shaved previously. Incision was performed 20 mm long with anterolateral approach to the right femur. Vastus lateralis and biceps femoris muscles were retracted from femoral bone. Group 1 (control group) underwent femoral osteotomy only (without

periosteal stripping) and fixated by 1.4 mm Kirschner (K) wire. Group 2, 3 and 4 (treatment group) underwent similar procedures as group 1 followed by 4 mm, 6 mm and 8 mm of periosteal stripping proximally and distally to the osteotomy site respectively. Subsequently, we fixated with 1.4 mm K wire intramedullary. Prophylactic antibiotic (ampicillin 50 mg/kgbw/day) and analgetic (paracetamol 50 mg/kgbw/day) was given for 3 days. We euthanized all groups with phenobarbital injection 75 mg/kgbw intraperitoneally on week-4 and evaluated the femoral speciments by histomorphometry.

2.2 The Second Stage (Main Study)

The second stage (main study) was aimed to evaluate the role of sildenafil in accelerating the healing process of delayed union fracture of rat's femur. Evaluation was performed with histomorphometry and VEGF expression by immunohistochemistry. Twenty four rats were randomly allocated to 4 study groups. We decided 4 mm periosteal stripping as delayed union model regarding preliminary study result. The surgical procedure in the second stage was similar to the preliminary study. Group 1 acting as the control group (4 mm periosteal stripping) will receive no treatment of sildenafil. Group 2, 3, and 4 as the treatment groups were rats with 4 mm periosteal stripping that received sildenafil 3 times a week. Group 2, 3, and 4 had 3.5 mg/kg bw (minimum dose), 5 mg/kg bw (medium dose), and 7.5 mg/kg bw (maximum dose) respectively. All groups were euthanized with phenobarbital injection 75 mg/kgbw intraperitoneally on week-2 and 4 for histomorphometry and immunohistochemistry evaluation [14].

2.3 Histopathologic Examination by Hishomorphometry

After euthanasia, the right femur was resected immediately. By maintaining a K-wire inside, harvested femur was fixed in 10% neutral buffered formalin for 48 hours. They were decalcified with Plank Rychlo's solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) [15,16]. Samples were embedded in paraffin and cut transversely with a microtome 5 µm section, and stained with hematoxylin eosin (HE). Then, they were examined with a Leica Microsystems microscope.

The histological images were taken by digital microscope camera. The width and diameter of the callus, total callus area, osseous, cartilage, and fibrous tissue area (Fig. 1) were evaluated using Image J® software (Fig. 2) [17].

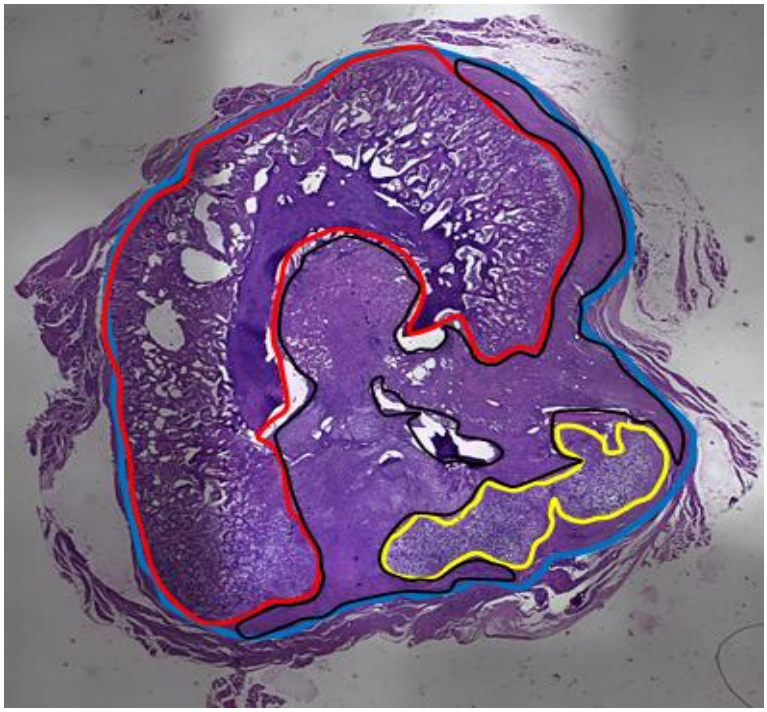

Fig. 1. Determining callus area by Haematoxyllin-Eosin (HE) staining. Blue line: total callus area; red line: osseus area; yellow line: cartilage area; black line: fibrous tissue area

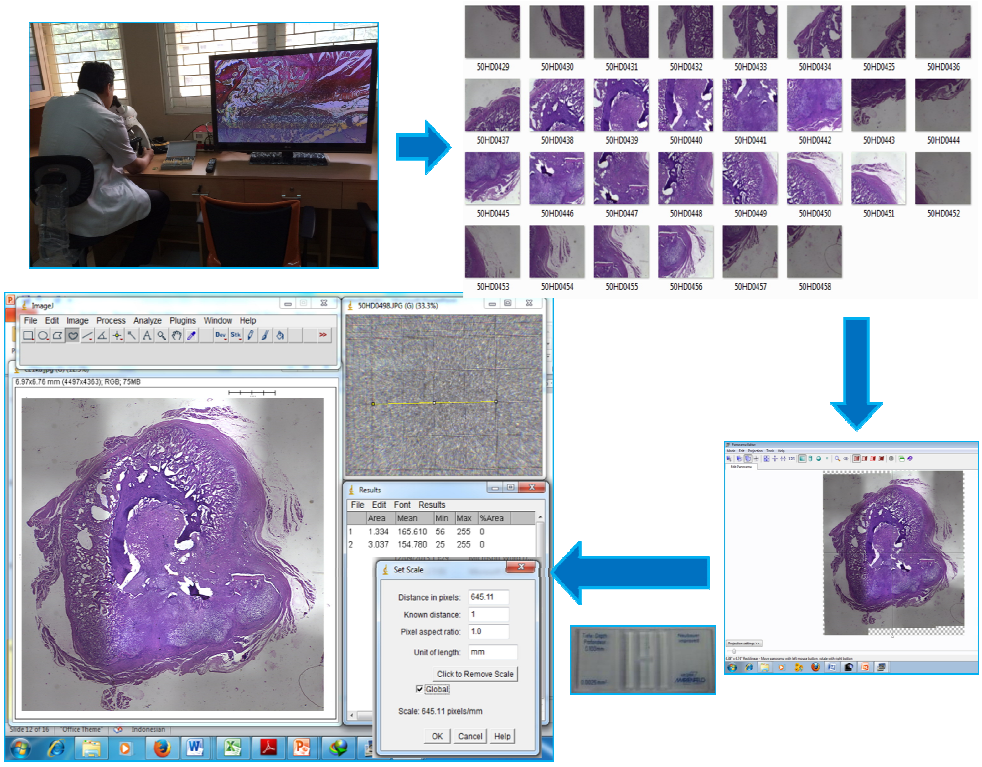

Fig. 2. Procedure of histomorphometry examination. (1) Picture was taken by Leica Microsystems microscope, 400x magnification; (2) Photomerging of picture by software PTGUI Pro 9.1

2.4 Immunohistochemistry Staining of VEGF

The slide was deparaffinized in xylene I-III respectively, for 5 minutes each and dehydrated in graded alcohol for each 4 minutes. Blocking was done with 0.5% H_2O_2 in methanol for 30 minutes and then washed in water for 5 minutes. Pretreatment of the slide was performed with citrate buffer in microwave Cook I and Cook II for 5 minutes each, followed by blocking background target to non-specific antigens and then incubated for 15 minutes. Then it received primary antibody to VEGF (Rabbit Monoclonal Anti-VEGF Antibody, Biocare Medical, CA, US catalog number CME356AK) with 1:200 concentration (the diluent was Van Gogh Yellow, Biocare Medical, CA, US), followed by 1 hour incubation. The slide was given a universal secondary antibody to bind to the primary antibody for 15 minutes. The secondary antibody was a biotinylated universal link secondary antibody (Starr Trek Universal HRP Detection System kit, Biocare Medical, USA, catalog number STUHRP700H) conjugated to Horseradish peroxidase labeled-streptavidin (TrekAvidin-HRP, included in the kit) and then a chromogen (Betazoid DAB chromogen, included in the kit) was added, the final reaction product was diamino benzidine (DAB) which was identified as an intense brown color. After that, counterstaining was performed with haematoxylin for 1 minutes. The positive control was human renal tissue and negative control was the same rat's fracture callus without VEGF antibody.

VEGF expression was evaluated by screening 3 to 5 visual fields at 100x magnification to look for hotspot areas defined as an area with intense vascular structure. We counted the number of blood vessel in the hotspots at 400x magnification using a method described by Weidner [18]. Group of endothelial cells, which were positively stained as well as morphologically identified vessels were calculated as individual blood vessels. Then we calculated the average number of blood vessels per high power field (400x magnification) and took these numbers as the VEGF expression (Fig. 4).

2.5 Statistical Analysis

Statistical analysis was performed with One Way ANOVA test using SPSS software version 16 for Windows. Any significant difference found by one way ANOVA was then analyzed by Bonferroni post hoc test to assess the significance of each group compared to control. Data from week-2 and -4 were analyzed by Independent t-test [19].

3. RESULTS

3.1 The Result from the Preliminary Study

The result of the preliminary study was summarized in Table 1. The ANOVA test showed a significant difference in the total callus area among groups with the p-value of 0.007. This test also showed a significant difference in the osseous, cartilage, and fibrous tissue area with p-values of 0.015, 0.004, and 0.001 respectively. The Bonferroni post hoc test obtained significant difference in all variables among groups with p-value of < 0.005. Based on these results, we conclude that all treatment groups (with periosteal stripping) cause delayed union of the femoral osteotomy. For the main study, we considered 4 mm periosteal stripping to make delayed union of the femoral osteotomy and administered sildenafil (Fig. 3).

3.2 The Result from the Main Study

3.2.1 Histomorphometry evaluation week-2

ANOVA test showed a significant difference in the total callus area with the p-value of 0.004. The Bonferroni post hoc test revealed a significant difference only between group 4 and 1 (control group) with p-value of 0.004.

ANOVA test also found significant difference among groups in the osseous, cartilage, and fibrous tissue area with p-value of 0.001, 0.015 and 0.005 respectively. The Bonferroni post hoc test obtained significant difference in the osseous area between group 2 and 4 compared to group 1 with p-values of 0.021 and 0.001 respectively. Meanwhile, in the cartilage area only group 3 showed a significant difference compared to group 1 in Bonferroni post hoc test with p-value of 0.015. The Bonferroni post hoc test showed a significant difference in the fibrous tissue area between group 3 and 4 to the control group with p-value of 0.005 and 0.004 respectively.

3.2.2 Histomorphometry evaluation week-4

ANOVA test found no significant difference among all groups in the total callus area with p-value of 0.192. However, in ANOVA test, we found significant difference between groups in the osseous and fibrous tissue area with p-value of 0.015 and 0.001 respectively. The Bonferroni post hoc test also showed a significant difference in the osseous and fibrous tissue area between group 4 and 1 with p-value of 0.003 and 0.001 respectively. ANOVA test found no significant difference among all groups in the cartilage area with p-value of 0.807.

Independent t-test found significant difference in the total callus, osseous, and cartilage area between group 4 in week-2 and week-4 with p-value of 0.001; 0.001; and 0.002 respectively. However, Independent t-test found no significant difference in the fibrous tissue area between all groups in week-2 and week-4.

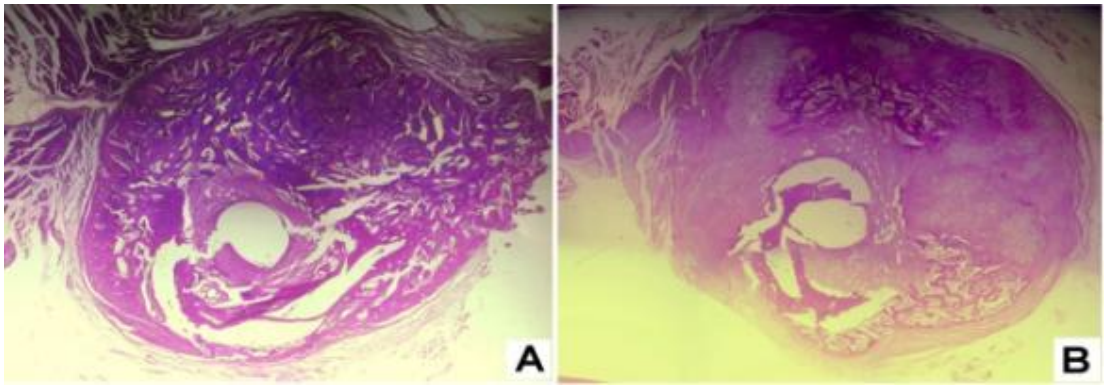

Fig. 3. Histomorphometry model of delayed union. (a) Control group with normal healing, callus was mainly composed of bony tissue, (b) Group with 4 mm periosteal stripping distally and proximally, callus was mainly composed of cartilaginous and fibrous tissue. HE 40x magnification

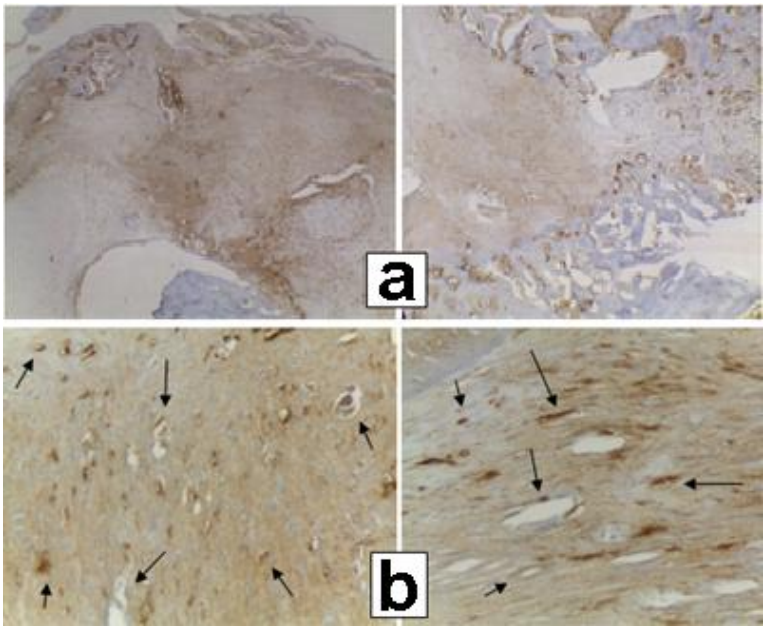

Fig. 4. The blood vessels were identified by immunohistochemistry staining. (a) screening of three to five visual field at 100x magnification to look for a hotspot area; (b) the amount of blood vessels at 400x magnification. Black arrows showed bloods vessels that were stained positively with VEGF. Black arrows are blood vessels stained positively by the immunohistochemistry

3.2.3 Immunohistochemistry evaluation

We could get an accurate measurement of the acceleration of the healing process in delayed union fractures and increase in the ability of angiogenesis in study subjects receiving sildenafil with immunohistochemistry (Tables 2 and 3).

3.2.4 The evaluation in week-2

showed that ANOVA test had a significant difference between groups in the total callus area with the p-value of 0.037. The Bonferroni post hoc test showed a significant difference between group 4 and control group with p-value of 0.046 (Table 4). The ANOVA test showed no significant difference between groups in the total callus area in week-4 (Table 5).

4. DISCUSSION

One of the complications of fracture healing was delayed union or non-union. Delayed union was considered when fracture healing took longer time, whereas non-union was failure of reaching union [2,5]. Factors that lead to delayed union were vascular disruption, periosteal stripping instability, infection, malnutrition, soft tissue interposition, and distraction or gap between fracture fragments [5,7,20].

In this animal study, the delayed union model was obtained by periosteum stripping of the osteotomized femur of the rats to disrupt bone vascularization and nutrition [21]. With this intervention, we found that the healing process was delayed. Periosteal stripping should decrease osteogenesis signals, osteoprogenitor or mesenchymal stem cells (MSCs) mobilization into the fracture site due to vascular disruption, hence the normal bone healing was expected to be disturbed during the healing period [10]. In this study, we chose 4 mm periosteal stripping proximal and distal to the fracture site as a delayed union model for the second stage study.

Based on histomorphometry evaluation on the week-2, there was a significant difference between treatment and control group. This result indicated that sildenafil administration increased in tissues and cells needed for fracture healing at that period. In other words, sildenafil may improve healing process in delayed union of osteotomy or fracture. It is similar to sudy conducted by Histing et al. who reported that sildenafil contributed in all phases of fracture

healing, from hematoma formation to bone turnover in remodelling [13,22,23].

In our study, sildenafil increased VEGF significantly on week-2 evaluation. It is consistent with previous study that reported sildenafil administration increased VEGF expression [11,13,24-26]. VEGF will improve differentiation and metabolism of pre-osteoblasts as well as bone formation [6,12]. VEGF also played important role in increasing angiogenesis and stimulating proliferation osteoblast, osteoclast and chondroblast [5]. We think that the more increased VEGF expression the better fracture healing (bone formation). Since sildenafil administration increased VEGF expression, it could play important role in fracture healing. Our hypothesis was supported by other experts. Street reported that VEGF administration increased in mineral density and vascularization of the callus [12]. In addition, Eckardt et al. found that rhVEGF administration accelerated and improved fracture healing of non union of the rabbit tibia [6,27]. Senthikumar et al. showed that sildenafil administration in peripheral arterial disease improved angiogenesis activity (by increasing ischemia-induced angiogenesis factor) [24]. Koneru et al. reported that sildenafil had the ability to regulate VEGF and angiotensin-1 which in turn stimulated neovascularisation in mice's myocardium [11].

Vascular invasion permitted the mobilization of mesenchymal stem cells and pericytes. [11,13,24-26]. Brighton reported that pericytes were able to express alkaline phosphatase, collagen, glycosaminoglycan, and osteocalcin. Pericytes proliferated during the vascular repair phase and could induce calcification (*In vitro*). His findings implied that vascular invasion not only provided oxygen and nutrition needed for tissue repair, but also could supply mesenchymal stem cells which would differentiate into osteoblasts [28].

In our study, we found that sildenafil administration did not affect VEGF expression significantly on week-4 evaluation, despite VEGF expression in treatment group was higher compared to control group. We think that VEGF expression has started increase since week-2 and become slower on week-4. Another reason probably is the fracture start to remodelling, so VEGF expression did not increase significantly. This result was different from previous studies which mentioned that VEGF (angiogenic factors) contribute in stimulating angiogenesis especially in the end of endochondral ossification and in remodelling [6,12].

Different doses of sildenafil administration resulted in different response to osteotomy healing as well. Minimum dose of sildenafil played important role of bony component (area) improvement. Medium dose of sildenafil improved both cartilage and fibrous tissue component (area).

Table 1. The result of the preliminary study

Parameter	Groups	Mean±SD	ANOVA test (p-value)
Total callus area	Control	16.323±1.908	0.007
	4 mm stripping	11.833±3.656	
	6 mm stripping	10.475±2.915	
	8 mm stripping	7.963±1.563	
Osseous area	Control	7.730±1.602	0.015
	4 mm stripping	1.780±0.116	
	6 mm stripping	2.457±0.422	
	8 mm stripping	1.695±0.744	
Cartilaginous area	Control	5.110±1.299	0.004
	4 mm stripping	4.695±1.466	
	6 mm stripping	1.402±0.795	
	8 mm stripping	1.902±0.085	
Fibrous area	Control	3.111±0.841	0.001
	4 mm stripping	3.470±0.893	
	6 mm stripping	2.587±0.478	
	8 mm stripping	1.330±0.271	

Bonferroni post hoc test: Control vs 4 mm stripping p<0.05; Control vs 6 mm stripping p<0.05; Control vs 8 mm stripping p<0.05

Table 2. Evaluation of dynamic process of ossification on week-2 and 4

Parameter	Group	Mean±SD week -2	Mean±SD Week- 4	Independent t-test (p-value)
Osseous area	Control	0.712±0.751	0.851±0.170	0.482
	Sildenafil 3.5 mg	1.498±0.469	0.968±0.229	0.052
	Sildenafil 5 mg	1.041±0.065	1.16±0.690	0.196
	Sildenafil 7.5 mg	5.451±0.018	2.651±0.372	0.001

Table 3. Evaluation of dynamic process of VEGF expression on week-2 and 4

Parameter	Group	Mean ± SD Week-2	Mean ± SD Week-4	Independent t-test (p-value)
VEGF expression	Control	9.376±2.784	12.433±4.393	0.154
	Sildenafil 3.5 mg	13.733±4.244	16.466±7.516	0.117
	Sildenafil 5 mg	15.186±2.492	14.673±5.363	0.593
	Sildenafil 7.5 mg	22.933±1.847	15.800±1.847	0.533

Table 4. Evaluation of VEGF expression on week-2

Parameter	Group	Mean±SD	ANOVA test (p-value)
VEGF expression	Control	9.376±2.784	0.037
	Sildenafil 3.5 mg	13.733±4.244	
	Sildenafil 5 mg	15.186±2.492	
	Sildenafil 7.5 mg	22.933±1.847	

ANOVA test. Post hoc test: Control vs sildenafil 3.5 mg p=0.275; Control vs sildenafil 5 mg p=0.050; Control vs sildenafil 7.5 mg p=0.046

Table 5. Evaluation of VEGF expression on week-4

Parameter	Group	Mean ± SD	ANOVA test (p-value)
VEGF expression	Control	12.433±4.393	0.807
	Sildenafil 3.5 mg	16.466±7.516	
	Sildenafil 5 mg	14.673±5.363	
	Sildenafil 7.5 mg	15.800±1.847	

Meanwhile, maximum dose of sildenafil improved total callus, bony, and fibrous tissue area. We found that only maximum dose of sildenafil administration resulted in significantly different (on week-4) between treatment and control group especially in bony and fibrous component (area). In other words, sildenafil administration with maximum dose will accelerate fracture healing of delayed union more clearly at least 4 weeks after treatment. Variations in the response of fracture healing in this study were probably influenced by various factors such as dose-response relationship, type of animal, and different individual potential to sildenafil metabolism. The latter factor (accumulation and elimination) in the rats have not been studied well [29-31].

5. CONCLUSION

Sildenafil was proven to accelerate healing on delayed union fractures in SD rats and to increase VEGF expression which was evaluated by histomorphometry and immunohistochemistry.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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