EFFECTS OF SILDENAFIL ON THE INFLAMMATORY AND REPAIR PHASE OF BONE HEALING SPEED IN A RAT MODEL

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ABSTRACT

Introduction*: Fracture healing is still one of the most important problems in orthopedic surgery clinics. Alcohol consumption, smoking, systemic disorders, and different drugs are among the factors that diminish fracture healing. Sildenafil citrate may enhance bone healing speed as it has known effects of increasing the blood supply to the tissues.*

Materials and methods*: 36 male, Wistar-Albino rats were randomly divided into two groups as the control group and sildenafil group. A retrograde intramedullary Kirschner (K)-wire was inserted, and then standard closed shaft fracture was established with the use of a three-point bending system. Sildenafil was given orally to the sildenafil group after production of fracture. Each group was subdivided into two groups; seventeen rats were killed at the first week (Control Group A; Sildenafil Group C) and the other seventeen rats at the fourth week (Control Group B; Sildenafil Group D).*

Results*: At the end of the first week, the results of control group (B) was not statistically significant but trended to have a better histological score, with a median grade of 5 (range: 2-5), than the sildenafil group (D), with a median grade of 3.5 (range: 2-5) (p=0.16). At the end of the fourth week, histological callus formation was found to be significantly superior in the sildenafil group, with a median grade of 8 (5-9), than in the control group, with a median grade of 5 (5-7) (p=0.02). The difference between the control and experimental groups as determined radiographically was not significant at both 1 and 4 weeks, respectively.*

Conclusions*: The microscopic results of this study indicate that sildenafil might have dual effects on fracture healing. It accelerates the bone healing in the repair phase, where it has a trend to have some suppressive effects on the inflammatory phase.*

Key words*: Fracture healing; sildenafil; bone histology.*

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Introduction

Fracture healing is a complicated process, which occurs by restoration of the normal tissue without scarring. This process is affected by many factors. Adequate blood supply of the bone and its surrounding tissue is the mainstay of bone healing[1, 2]. Bone healing begins with the hematoma phase, which is characterized by complex process of clot formation, platelet degranulation, and growth factor release at the fracture site. For this purpose, the required inflammatory mediators, fibroblasts and growth factors accumulate at the fracture site.

Phosphodiesterase (PDE)-5 inhibitors act by inhibiting the catabolism of cyclic guanosine monophosphate (cGMP), which is a potent vasodilator initially activated by nitric oxide (NO)[3]. These drugs are not only being used in erectile dysfunction[4, 5], but also in pulmonary hypertension, congestive heart failure and diabetic neuropathy[6-8]. Furthermore, sildenafil - a phosphodiesterase inhibitor has also shown promising results in the treatment of ischemia-reperfusion injury in the heart and experimental embolic stroke in the rat[9, 10]. Clearly, all of these diseases involve a significant ischemic component, indicating the

effectiveness of sildenafil in resolving these tissue pathologies[11]. Currently, no study exists in the literature that examines the effects of PDE-5 inhibitors on bone healing.

Sildenafil has also been proven to promote angiogenesis in several studies, particularly in ischemic conditions. In an experiment on mice, Dussault et al.[12] found that sildenafil is almost as potent as vascular endothelial growth factor in inducing cellular migration and promoting capillary-like tube formation in human umbilical vascular endothelial cells. Likewise, Senthilkumar et al.[11] found that sildenafil therapy stimulated angiogenesis in ischemic limbs of eNOS–/– and iNOS–/– mice. This effect was independent of NO-production. As a fracture impairs the blood supply of a long bone, angiogenesis is a critical point for revascularization of bone fragments.

Several studies in the literature have reported some deleterious effects of the sildenafil on collagen synthesis, inflammation and fibroblast differentiation. In one of them sildenafil reduced both collagen 1 synthesis and differentiation of myofibroblasts[13]; in another neonatal lung injury-recovery model in rats, sildenafil treatment has been shown to inhibit lung inflammation by demonstrating a reduction in the influx of inflammatory cells, including macrophages and neutrophilic granulocytes[14].

We have previously published our hypothesis that sildenafil citrate may enhance bone healing by various mechanisms such as by increasing the blood supply, micro-circulation and the mediatory concentration at the fracture site[3]. The results of a very recent study on mice supports our hypothesis that sildenafil may accelerate bone healing[15]. The present study is conducted to supply further data about the effects of sildenafil on early and late phases of fracture healing. Our experimental study aimed to investigate the histological, radiological and biomechanical effects of sildenafil citrate on a closed femoral fracture model in rats. The hypothesis of the study was that the effects of sildenafil might be different in early (inflammatory) and late (repair) phases of the fracture healing.

Material and methods

Experimental model

The study was performed with the approval of the Institutional Review Board. This study was conducted on 36 male Wistar rats that were skeletally mature, weighing 350 ± 20 g. The rats were supplied from the same animal laboratory in order to minimize the hereditary variations between subjects. The study has been approved by ethics committee. The procedures and handling of rats were in conformity with the Helsinki Declaration.

A closed femur fracture model as described by Bonnarren and Einhorn[16] was used for study. Before the anesthesia, a single dose of a first-generation cephalosporin (Cefamezin 50 mg/kg) was administered intramuscularly for infection prophylaxis. Rats were fed a standard chow and allowed water without restriction. They were allowed mobility in a similar environment without any restriction.

Surgical technique

The rats were weighed by electronic scale, and a combination of ketamine HCl 50 mg/kg (Ketalar) and xylazine (Rompun), injected intraperitoneally, was used for anesthesia.

The surgical procedures were performed by the same surgeons (SO and CYK). After the knee was prepared with povidone iodine, an anteromedial skin incision was made. Medial parapatellar incision was used, the patella was everted laterally, and the knee was flexed to expose the femoral condyles. An 8 mm Kirschner (K)-wire was applied from the entry point just anterior to the intercondylar notch. The K-wire was cut at about the entry point, leaving less than 5 mm length in the joint. The patella was reduced, and capsule and skin were closed.

The intramedullary position of the K-wire was checked radiographically. A closed fracture was created with the closed fracture device recommended by Bonnarens and Einhorn[16]. The fractures were also checked radiographically. All of the fractures in the experiment were simple mid-shaft fractures.

Study groups

Rats were arbitrarily assigned to four groups (Groups A, B, C, D) of nine animals each. Groups A and B were control groups, and Groups C and D were sildenafil groups (Table 1). Sildenafil citrate was pulverized within 10 cm 3 water and Groups C and D received sildenafil citrate in their drinking water as 1 mg/day.

On the seventh day after the fracture, the rats in Groups A and C were sacrificed. Their fractured left femurs were excised without damaging the callus, and radiological and histological examinations were applied.

Group A:	Control group, sacrified on 7th day
Group B:	Control group, sacrified on 28th day
Group C:	Sildenafil group, sacrified on 7th day
Group D:	Sildenafil group, sacrified on 28th day

Table 1: Groups in the study.

There were two rats with displaced K-wires and reduction loss (1 in Group A and 1 in Group C). These were excluded from the study.

Rats in Groups B and D were sacrificed on the 28th day, and the same process, which was described above for Groups A and C, was applied.

Simple biomechanical examination

Clinical examination of the healing was done as described by Akman et al[17]. The femur disarticulated from the hip and knee joints, and was stripped of the soft tissues after sacrification. Passive motion in both sagittal and coronal dimension scored 0, motion in only one direction scored 1 and no motion scored 2.

Radiological examination

Anteroposterior (A-P) and lateral radiographs were taken after excision of the femurs. Radiographs were scored according to Goldberg classification[18]. The scoring was done by three independent orthopedists (Table 2).

Table 2. Radiological assessment of union according to Goldberg et al.[13]

Score	Radiologic Assessment
1	Non-union
2	Possible union
3	Complete union

Histological examination

Two pathologists, who were blinded to the group assignments, individually assessed histological grade of the fracture healing. After two months, the senior pathologist assessed the specimens once again. The same team as a consensus decided the final scores. After the radiological assessment, the femurs were preserved in 10% formol solution. After fixation, they were decalcified by 10% acetic acid, 0.85 NaCl and 10% formalin solution, sequentially.

Then, samples were embedded in paraffin blocks and cut in slices of 4-5 microns. The samples were stained with hematoxylin- eosin. Each sample was examined under x40 and x100 magnifications. Healing was graded according to Huo et al.[19] (Table 3, Figure 1).

Table 3. Histological grading of the fracture healing due to classification of H

Score	Histological Findings in Fracture Site
1	Fibrous tissue
2	Mainly fibrous tissue
3	Equal amounts of fibrous tissue and cartilage
4	Mainly cartilage, little fibrous tissue
5	Cartilage
6	Mainly cartilage, little immature bone tissue
7	Equal amounts of cartilage and immature bone tissue
8	Mainly immature bone tissue, little cartilage
9	Fracture healing with immature bone tissue
10	Fracture healing with mature bone tissue

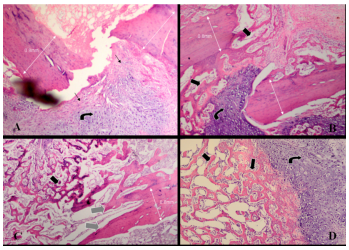

Figure 1 A-C: Hematoxylin-eosin (HE) stain and x 40 magnification. Cortical thickness of the femur (approximately 0.8 mm) was shown with two headed white arrows. Thin black arrows indicate fibrous tissue, bend arrows indicate chondroid tissue, thick black arrows indicate woven bone tissue, thick grey arrow indicate mature bone tissue. A. Huo grade 3, as there are equal amounts of fibrous and chondroid tissue between cortical bone fragments. B. Equal amounts of woven bone and cartilage between cortices of two fragments are seen (Grade 7 callus formation).C. Fracture healing with immature bone (Grade 9 callus formation). D. HE stain and x 100 magnification, Grade 7 callus formation.

Statistical analysis

All data obtained from this study were recorded and evaluated by using "Statistical Package for Social Sciences for Windows 13" program. Quantitative variables were given as mean± SD (standard deviation), while the categorical variables were given as numbers and percentages. A frequency analysis was also performed. The normalcy of distribution was evaluated when comparing the groups. Parametric tests (independent samples test) were used with normally distributed data, while non-parametric tests (Mann-Whitney U test) were used with non-normally distributed data. Wilcoxon signed ranks test was used for comparisons over time (1 and 4 weeks). Statistical significance was accepted as $p<0.05$. Method 2 (covariance matrix) was used for intra-observer and inter-observer reliability.

Results

Simple biomechanical findings

No significant differences exist between the

control (0(0-1)) and sildenafil (0 (0-1)) groups at the end of the first week (p=0.8). No significant differences exist between the control (2 (1-2)) and sildenafil (2(2-2)) groups at the end of the fourth week (p=0.77).

Radiological findings

No significant differences exist between the control (1.66 (1-2)) and sildenafil (1.33-2.66) groups at the end of the first week (p=0.28). No significant differences exist between the control (2.33 (2-3)) and sildenafil (2.66 (2-3)) groups at the end of the fourth week (p>0.22) (Table 4). Both A - B (control groups) and C - D (sildenafil groups) pair demonstrated a significant difference over time, with p values of 0.01 and 0.025, respectively.

Median (min-max)	One week later	Four week later	P value
Control group	1.66 (1-2)	2.33(2-3)	0.01 *
Sildenafil group	1.66 (1.33-2.66)	2.66 (2-3)	0.025 *
P value	0.28 **	0.22 **	

Table 4: Comparison of the radiological results due to Goldberg et al[13].
* *Wilcoxon signed ranks test*
** *Mann Whitney-U test*

Histological findings

At the end of the first week, the median score of the group (A) (control group) was 5 (2-5) and of the group (C) (sildenafil group) was 3.5(2-5). The difference was not significant (p=0.16) between Groups A and C (Figure 2).

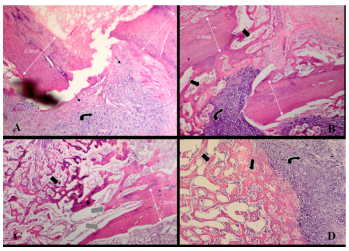

Figure 2: The scatter-plot graphic of the samples in control and sildenafil groups due to Huo histological classification at the end of the first week.

At the end of the fourth week, the healing scores of Group D had significant difference compared to healing scores of Group B (8 (5-9) and 5 (5-7), respectively; p=0.02) (Figure 3) (Table 5).

Both A - B (control groups) and C - D (sildenafil groups) pairs demonstrated a significant difference over time, with p values of 0.01 and 0.04, respectively.

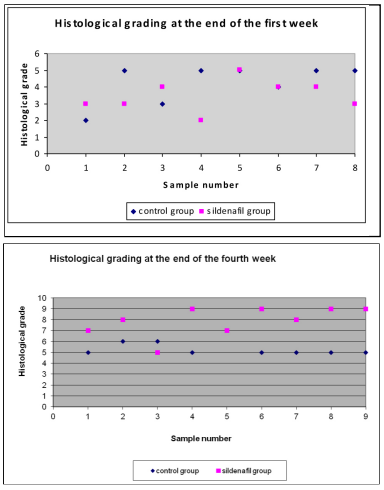

Figure 3: The scatter-plot graphic of the samples in control and sildenafil groups due to Huo histological classification at the end of the fourth week.

Median (Min-max)	One week later	Four week later	P value
Control group	5 (2-5)	5 (5-7)	0.01 *
Sildenafil group	3.5 (2-5)	8 (5-9)	0.04 *
P value	1.61 **	0.02 **	

Table 5: Comparison of histological results due to classification of Huo et al[14].
* *Wilcoxon signed ranks test*
** *Mann Whitney-U test*

The intra-observer reliability was 0.9894 (95% confidence interval, 0.9789 to 0.9947), and the intra-observer reliability was 0.9598 (95% confidence interval, 0.9211 to 0.9797).

No adverse effect of the sildenafil was encountered on the subjects.

Discussion

This is the first study in the literature that separately investigate the effects of sildenafil on bone early and late phases of healing. According to our histological examination results, sildenafil citrate does not have a significant effect in the inflammatory phase, whereas it significantly accelerated bone healing in the repair phase.

Bone healing acceleration in the late phase was evident in our study. This effect might be related to the beneficial effects of PDE-5 inhibitors on vasodilatation[20-24] and angiogenesis[25-27]. These effects are related to the NO-mechanism[24, 27]. Guanylate cyclase is activated by NO- and converts guanosine triphosphate (GTP) to cGMP[27]). cGMP increases vasodilatation and requires PDE to be catabolized.

Sildenafil acts by inhibiting PDE and consequently functions as a vasodilator[24]. Vasodilatation results in increased blood flow to the tissues.

A very recent study on mice[15], showed that sildenafil treatment accelerates fracture healing by enhancing bone formation. To their findings, Western blot analyses have shown a significantly higher expression of the pro-angiogenic and osteogenic cysteine-rich protein 61, confirming the increase of bone formation. This was in concurrence with our hypothesis, which has been published years before[3].

Our study has similar findings with this study in a different model, except that it gives some data that sildenafil is not useful (if not harmful) in the inflammatory phase of the bone healing.

Several studies exist proving that NO- improves bone healing[28, 29]. NO is a well- accepted angiogenic regulator that participates in several cellular responses, including but not limited to increased endothelial cell proliferation and survival, increased endothelial cell motility, and increased activation of signaling pathways necessary for angiogenic activity[25, 26, 30].

Although the effects of sildenafil citrate was not statistically significant, there was a slight trend of a potential retardation of bone healing observed in the early phase of callus formation in sildenafil group. This possible retardation in the inflammation phase can be related with sildenafil's known suppressive effect on inflammation[13, 14]. In an in vitro study on tissue culture of fibroblasts taken from Peyronie's plaques, Valente et al.[13] showed that sildenafil reduced both collagen 1 synthesis and differentiation of myofibroblasts. Also, in a neonatal lung injury-recovery model in rats, de Visser et al.[14] showed that sildenafil treatment inhibited lung inflammation by demonstrating a reduction in the influx of inflammatory cells, including macrophages and neutrophilic granulocytes. Fibroblasts act as both precursor of chondroblasts and osteoblasts, and a potential source of growth factors that are essential in bone healing[31]. Furthermore, they synthesize collagen and other extracellular matrix proteins and have a crucial role in fibrous callus formation[32].

The current study has limitations. Our experimental model did not provide absolute fracture stabilization, but this was done on purpose as to optimize external callus formation, and facilitate histological assessment. Pre-fracture fixation was preferred in order to minimize the risk of complications related to prolonged anesthesia and operative time (infection). The descriptive nature of the study was another limitation. Moreover, using standard radiographs might not be expected to provide enough details to classify bone healing. Micro-computed tomography (CT) o would have been more appropriate, since it would add data about bone architecture, which are not provided by histology[33]. In addition, radiological assessment was done after dissection of the bone, which makes an objective assessment difficult.

The results of this study indicate that sildenafil accelerates fracture healing, particularly in the repair phase. However, it has no positive effects on bone healing in the inflammatory phase.

References

1) Brinker MR. Bone. Review of Orthopaedics, ed. Miller M. 1996, Saunders, Philadelphia.
2) Webb, J.C.J., Tricker, J.A., *Review of fracture healing*. Curr Orthop, 2000. 14: p. *457-63*.
3) Akgul, T. and Alemdaroglu, B. *Phosphodiesterase 5 inhibitors may facilitate bone fracture recovery*. Med Hypotheses, 2008. 70: *461-2*.
4) Carson CC, Lue, TF. *Phosphodiesterase type 5 inhibitors for erectile dysfunction*. BJU Int, 2005. 96: *257-80*.
5) Kloner, R.A., *Cardiovascular effects of the 3 phosphodiesterase-5 inhibitors approved for the treatment of erectile dysfunction*. Circulation, 2004. 110: *3149-55*.
6) Galiè N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, et al., *Sildenafil citrate therapy for pulmonary arterial hypertension*. N Engl J Med, 2005. 353(20): p. *2148-2157*.
7) Takimoto E, Champion HC, Li M, Belardi D, Ren S, et al., *Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy*. Nat Med, 2005. 11: *214-22*.
8) Patil CS, Singh VP, Singh S, Kulkarni SK. *Modulatory effect of the PDE-5 inhibitor sildenafil in diabetic neuropathy*. Pharmacology, 2004. 72: *190-5*.
9) Elrod JW, Duranski MR, Langston W, Greer JJ, Tao L, et al., *eNOS gene therapy exacerbates hepatic ischemia-reperfusion injury in diabetes: a role for eNOS uncoupling*. Circ Res, 2006. 99: *78-85*.
10) Zhang L1, Zhang RL, Wang Y, Zhang C, Zhang ZG, et al., *Functional recovery in aged and young rats after embolic stroke: treatment with a phosphodiesterase type 5 inhibitor*. Stroke, 2005. 36: *847-52*.
11) Senthilkumar A, Smith RD, Khitha J, Arora N, Veerareddy S, et al., *Sildenafil promotes ischemia-induced angiogenesis through a PKG-dependent pathway*. Arterioscler Thromb Vasc Biol, 2007. 27: *1947-54*.
12) Dussault S, Maingrette F, Ménard C, Michaud S-E L, Haddad P, et al. S*ildenafil increases endothelial progenitor cell function and improves ischemia-induced neovascularization in hypercholesterolemic apolipoprotein E-deficient mice*. Hypertension, 2009. 54: *1043-49*.

13) Valente EG, Vernet D, Ferrini MG, Qian A, Rajfer J, et al. *L-arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures*. Nitric Oxide, 2003. 9: *229-44*.
14) de Visser YP, Walther FJ, Laghmani el H, Boersma H, van der Laarse A, et al. *Sildenafil attenuates pulmonary inflammation and fibrin deposition, mortality and right ventricular hypertrophy in neonatal hyperoxic lung injury*. Respir Res, 2009. 10: p. *30*.
15) Histing T, Marciniak K, Scheuer C, Garcia P, Holstein JH, et al. *Sildenafil accelerates fracture healing in mice*. J Orthop Res, 2011. 29: *867-73*.
16) Bonnarens F, Einhorn TA. *Production of a standard closed fracture in laboratory animal bone*. J Orthop Res, 1984. 2: *97-101*.
17) Akman S, Gögüs A, Sener N, Bilgiç B, Aksoy B, et al. *Effect of diclofenac sodium on union of tibial fractures in rats*. Adv Ther, 2002. 19: *119-25*.
18) Goldberg VM, Powell A, Shaffer JW, Zika J, Bos GD, et al. *Bone grafting: role of histocompatibility in transplantation*. J Orthop Res, 1985. 3: *389-404*.
19) Huo MH, Troiano NW, Pelker RR, Gundberg CM, Friedlaender GE. *The influence of ibuprofen on fracture repair: biomechanical, biochemical, histologic, and histomorphometric parameters in rats*. J Orthop Res, 1991. 9: *383-90*.
20) Kilickesmez K., Kucukoglu MS., *Phosphodiesterase type 5 inhibitors in the treatment of pulmonary arterial hypertension*. Anadolu Kardiyol Derg, 2010. 10: *16-8*.
21) Bandera BC, Pham T, Hill-Pryor C, Bah-Sow M, Franco N, et al. *Role of growth factors in improved skin flap viability caused by phosphodiesterase-5 inhibitor.* Am Surg, 2010. 76: *614-7*.
22) Carlino C, Tobias JD, Schneider RI, Heller RL, Alpert MA, et al. *Pulmonary hemodynamic response to acute combination and monotherapy with sildenafil and brain natriuretic peptide in rats with monocrotaline-induced pulmonary hypertension*. Am J Med Sci, 2010. 339: *55-9*.
23) Sejima H, Tominaga K, Egawa T, Ikeda M, Shibuya K, et al. *Gender differences in tail-skin flushing induced by nitrates and phosphodiesterase type 5 inhibitors in a climacteric mouse model*. Eur J Pharmacol, 2009. 624: *66-70*.
24) Montani D, Chaumais MC, Savale L, Natali D, Price LC, et al. *Phosphodiesterase type 5 inhibitors in pulmonary arterial hypertension*. Adv Ther, 2009. 26: *813-25*.
25) Luque Contreras D, Vargas Robles H, Romo E, Rios A, Escalante B. *The role of nitric oxide in the post-ischemic revascularization process*. Pharmacol Ther, 2006. 112: *553-63*.
26) Cooke JP, *Flow, NO, and atherogenesis*. Proc Natl Acad Sci U S A, 2003. 100: *768-70*.
27) Rotella DP. *Phosphodiesterase 5 inhibitors: current status and potential applications*. Nat Rev Drug Discov, 2002. 1: *674-82*.
28) Baldik Y, Talu U, Altinel L, Bilge H, Demiryont M, et al. *Bone healing regulated by nitric oxide: an experimental study in rats*. Clin Orthop Relat Res, 2002. 404: *343-52*.
29) Diwan AD, Wang MX, Jang D, Zhu W, Murrell GA. *Nitric oxide modulates fracture healing*. J Bone Miner Res, 2000. 15: *342-51*.
30) Yu J, deMuinck ED, Zhuang Z, Drinane M, Kauser K, et al. *Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve*. Proc Natl Acad Sci U S A, 2005. 102: *10999-11004*.
31) Scutt, A. and P. *Bertram, Basic fibroblast growth factor in the presence of dexamethasone stimulates colony formation, expansion, and osteoblastic differentiation by rat bone marrow stromal cells*. Calcif Tissue Int, 1999. 64: *69-77*.
32) Bland YS, Critchlow MA, Ashhurst DE. *The expression of the fibrillar collagen genes during fracture healing: heterogeneity of the matrices and differentiation of the osteoprogenitor cells*. Histochem J, 1999. 31: *797-809*.
33) Hillier ML, Bell LS. *Differentiating human bone from animal bone: a review of histological methods*. J Forensic Sci, 2007. 52: *249-63*.

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