Original Article

Beneficial Therapeutic Effects of Sildenafil on Bone Healing in Animals Treated with Bisphosphonate

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Abstract

Objectives: The purpose of the present study was to evaluate the effects of sildenafil on mandibular fracture healing in animals treated with zoledronic acid by using histologic, histomorphometric, immunohistochemical, and radiodensitometric methods. **Materials and Methods:** A total of 36 Sprague–Dawley rats (3 months old) were used. All animals were treated intraperitoneally with 0.1 mg/kg zoledronate three times per week, for a total of 8 weeks. Postoperatively, the animals were divided into two groups: zoledronate group (Z), which had no treatment applied (n = 18), and zoledronate + sildenafil (ZS), which were treated daily with 10 mg/kg sildenafil (n = 18). Each group was divided into two subgroups and the animals were sacrificed at the end of week 1 (Z1 and ZS1, n = 9) and week 4 (Z4 and ZS4, n = 9) after the operation. Histologic, histomorphometric, immunohistochemical analysis, and radiodensitometry were performed on the test subjects. **Results:** Sildenafil-treated groups showed a significant increase in fracture healing scores. This result was supported by the densitometric, histologic, histomorphometric, and immunohistochemical findings. **Conclusions:** Sildenafil may have positive effects on accelerating and improving fracture healing, and it may be used as a supporting factor in bone healing in patients treated with bisphosphonate (BP) to prevent negative effects of BP's

Keywords: *bisphosphonate, bone healing, fracture healing, osteonecrosis, sildenafil, zoledronic acid*

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Introduction

Fracture healing is an important subject in orthopedic and maxillofacial surgical procedures. Fracture healing is also a multistage repair process that involves both catabolic and anabolic responses. The initial callus that forms is remodeled into lamellar bone by the combined action of osteoclasts resorbing the initial matrix, coupled with additional deposition of bone by osteoblasts.[1-3]

Bisphosphonates (BPs) have potent effects on osteoclastic bone resorption and have been classed as anti-anabolic drugs. Current BP therapies are effective for bone loss, and BPs have been widely used in the treatment of various systemic metabolic bone diseases, such as Paget's disease, malignancy, postmenopausal osteoporosis, fibrous dysplasia, and osteogenesis imperfecta.[2-6] Zoledronic acid (ZA) is a third-generation drug and is the most potent inhibitor of osteoclast-mediated bone resorption.[7] It is a well-known drug that depresses bone blood flow and significantly decreases angiogenesis. Although ZA is the most effective BP in clinical use, undesirable effects such as BP-related osteonecrosis of the jaw (BRONJ) have been reported, especially associated with infused nitrogen containing BPs.[8]

Sildenafil is a selective inhibitor of phosphodiesterase-5 (PDE-5) and a potent stimulator of angiogenesis. PDE-5 catalyzes the breakdown of cyclic guanosine

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monophosphate (cGMP), one of the primary factors causing smooth muscle relaxation, vasodilatation, and increased blood flow to the tissues.[9] Sildenafil has been reported to cause the dilatation of peripheral arteries as well as veins and the inhibition of the thrombus-forming capabilities of the platelets, in vivo. Recently, several studies have shown that sildenafil exerts angiogenic actions through upregulation of distinct proangiogenic growth factors.[10,11] Previous studies have shown that the angiogenic and osteogenic factors, such as vascular endothelial growth factor (VEGF) and cysteine rich-61 (CYR-61), are involved in the process of bone formation and fracture healing. Some experimental and clinical studies have shown the potential benefits of sildenafil in conditions associated with diminished blood flow and poor vascularization.[12,13] Histing *et al*.[14] have been the first to demonstrate that sildenafil treatment accelerates fracture healing by enhancing bone formation.

Numerous clinical and experimental studies have been performed to study the acceleration of bone formation, improvement of mineral content, and mechanical strength of the healing area. BPs strongly bind to hydroxyapatite crystals and potently inhibit osteoclast-mediated bone resorption. However, it has been reported that they might have the potential to improve fracture healing. In previous studies, ZA was generally applied for fracture repair perioperatively or postoperatively with the aim of improving bone healing.[15,16] In cases wherein ZA was administered preoperatively, the healing mechanism of bone may be adversely affected, and the application of any angiogenic agent might have a preventive effect on the development of BRONJ. There are some published studies on the effects of PDE-5 inhibitors on fracture healing; however, to our knowledge, there are no studies on the effects of sildenafil on BP-treated fracture healing in the oral and maxillofacial region.

The aim of this study was to investigate the effects of sildenafil as an angiogenic drug on the ZA applied for bone fracture repair by using stabilized rat mandibular fracture models.

Materials and Methods

The study protocol was approved by the Ondokuz Mayis University Animal Care and Ethics Committee. Thirty-six female Sprague–Dawley rats (3 months old) were used as experimental animals in this study. All animals were treated intraperitoneally with 0.1 mg/kg zoledronate (Zometa; Novartis, Istanbul, Turkey) three times per week, for a total of 8 weeks. Postoperatively, the animals were divided into two groups: zoledronate group (Z), which has no treatment applied (n = 18), and zoledronate + sildenafil (ZS), which were treated daily with 10 mg/kg sildenafil (n = 18) (Viagra 1; Pfizer, Istanbul, Turkey: dissolved in saline oral using a gavage). Each group was divided into two subgroups and the animals were sacrificed at the end of week 1 (Z1 and ZS1, n = 9) and week 4 (Z4 and ZS4, n = 9) after the operation. The doses of the drug administration were based on the previous literature.[17]

Surgical Procedures

The rats were anesthetized with an intramuscular injection of 20 mg/kg ketamine (Ketalar; Pfizer) and 5 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey). 0.5 ml artikain Ultracain-DS containing 1:200,000 epinephrine (Hoechst Marion Roussel, Istanbul, Turkey) was injected in the surgical site for local anesthesia and hemostasis. A submandibular incision was made along the inferior border of the left hemimandible, and the masseter muscle was divided along its length and elevated to expose the body of the mandible. A fracture was created using surgical burr from the coronoid process to the gonial angle of the mandible. The fracture was stabilized by using two screws and two holes microplate [Figure 1]. The subcutaneous tissues and skin were closed with absorbable sutures. All animals were given analgesics (tramadol, 1 mg/kg; Contramal, Abdi Ibrahim, Istanbul, Turkey) and antibiotics (cefazolin sodium, 500 mg/kg; Sefazol, M Nevzat, Istanbul, Turkey) intramuscularly after the surgery, twice a day for 4 days. All animals were fed a soft diet for at least 7 days. The rats were observed frequently for food intake and animal movement.

The rats were sacrificed with an injection of high-dose sodium penthobarbitone (Pental; IE Ulagay, Istanbul, Turkey) at the end of week 1 (Z1 and ZS1) and week 4 (Z4 and ZS4) postsurgery (n = 9 per group). The mandible was dissected, and all soft tissues were removed after sacrifice. The fractured hemimandibles were then obtained for histopathologic examination.

Histologic and Histomorphometric Examinations

Histologic and histomorphometric analyses were performed by a single pathologist blinded to the samples. All tissue samples were immediately fixed in 10% formalin. After fixation, specimens were kept in 10% nitric acid and decalcification was completed in 4 days. Specimens were then embedded in paraffin. Specimens were cut into sagittal sections of 4-µm-thick sections, and hematoxylen and eosin, Masson's trichrome, and safranin-O staining were performed. The sections were then examined with a light microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan).

The amount of the ossification for each section was determined by modifying the scoring system described

by Perry *et al.*[18] [Table 1]. The total score on the grading scale ranged from 1 point [fibrous tissue (FA)] to 10 points (mature bone), and morphometric evaluations were made.

The total callus areas (TCA), determined separately for each sample, were measured in a morphometric evaluation. The ratio of the areas of FA, cartilage tissue (CrA), immature bone tissue (IBT), and mature bone tissue (MBT) forming the callus over the TCA was calculated for each sample. Morphometric evaluations were performed using the KLONK Image Measurement program (Version: 14. 2.1.6, Image Measurement Corporation WY, USA).

Immunohistochemistry

Sections (5 μm) were subjected to immunohistochemistry (IHC) using the streptavidin–biotin–peroxidase complex (SBPC) technique for detection of collagen 1 and gremlin 1 antibodies. Serial sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 15 min. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.2) and subsequently incubated in proteinase K for 15 min in 37°C. After washing with PBS, the sections were preincubated in 10% rat nonimmune serum (Zymed Laboratories, Inc., California, USA) at room temperature for 10 min. Tissue sections were then incubated with primary antibodies rabbit anti-collagen-1 polyclonal antibody (bs-0578R; Bioss, MA, USA) and bone morphogenetic protein 2 (BMP-2) antibody (NBP 1-19751; Novus, USA) overnight and then rinsed with PBS at room temperature. The sections were then incubated with broad-spectrum biotin-conjugated second-step antibodies (Zymed Laboratories, Inc, San Francisco, CA) for 10 min and then rinsed in PBS. SBPC was applied for 10 min at room temperature. Labeling was "visualized" with 3-amino-9-ethylcarbazole (Zymed Laboratories, Inc.) as the chromogen. Sections were counterstained briefly with Mayer's hematoxylin. Negative controls were prepared by omitting the primary antibodies and replacing them with PBS or normal rabbit serum.

Evaluation of Immunohistochemistry

IHC results were interpreted using a light microscope (Nikon Eclipse E600). A semiquantitative evaluation of the stained sections was performed only in areas of interest. Col-1 and BMP-2 immunostaining was assessed semiquantitatively by the ratio of the positively stained area over the total area (0: negative; 1: weak; <20%; 2: moderate; 20–40%; 3: severe; >40%). Quantification was done by the same pathologist.

Radiologic Examination

Standard axial computed tomography (CT) scans were taken of each animal for radiologic analysis. All animals were positioned with the occlusal plane perpendicular to the horizontal plane for CT scans. Images were taken by a CT (Aquilion 16 system; Toshiba Medical System Corporation, Japan) according to a standard protocol. Axial slices were obtained from the superior border of the ramus to the low border of the corpus mandible, including the fracture line at 1-mm intervals. Density measurements were made in Hounsfield units (HU) from the marked area between the miniscrews [Figure 2]. The mean HU values as a unit of bone density was obtained by making five measurements for each animal. Each animal's bone density values were measured twice by the same examiner.

Statistical Analysis

Results for histologic and radiologic analyses are presented as mean ± standard deviation. Histologic analysis results are presented as a mean histologic scoring, and bone density analysis results are presented as a mean HU value. A one-way analysis of variance and post hoc Tukey test were used to compare significant changes between the control and study groups. Statistical significance was accepted at $P < 0.05$.

Results

Histologic Analysis

The mean bone fracture healing scores for groups Z1 and ZS1 were 5.2 ± 0.4 and 5 ± 0.0, respectively; however, in groups Z4 and ZS4, the mean bone fracture healing scores were 6.8 ± 0.4 and 8.8 ± 0.4, respectively [Figure 3]. Histologic examination revealed mainly CrA and a small amount of immature (woven) bone in groups Z1 and ZS1. In group Z4, there were equal amounts of CrA and immature bone, and group ZS4 had completely immature bone and a small amount of mature bone [Figure 4] and [Figure 5]. The sildenafil-treated groups showed a significant increase in fracture healing scores postsurgery at the end of the 4 weeks ($P = 0.00$).

Table 1: Histologic scoring system

Score, No.	Points Histologic Findings of the Fracture Zone
1	Fibrous tissue
2	Mainly fibrous tissue and small amount of cartilage tissue
3	Equal amount of fibrous and cartilage tissue
4	Completely cartilage tissue
5	Mainly cartilage tissue and small amount of immature (woven) bone
6	Equal amount of cartilage tissue and immature bone
7	Significantly immature (woven) bone and small amount of cartilage
8	Completely immature (woven) bone
9	Immature bone and small amount of mature bone
10	Mature (lamellar bone)

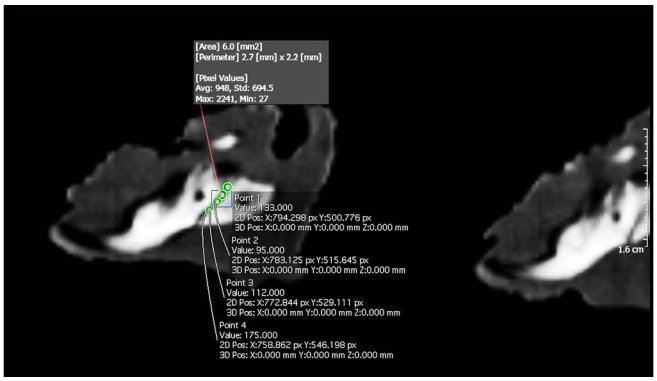

Figure 1: The view of fracture area after the miniplate fixation.

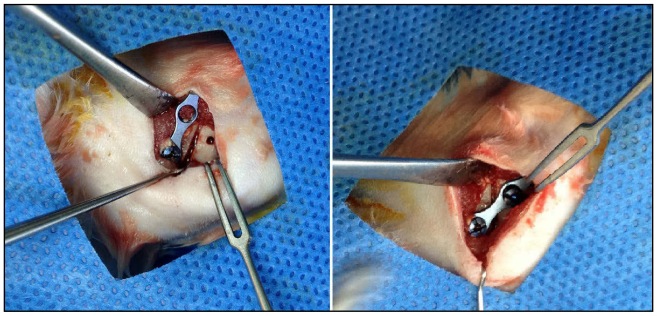

Figure 3: Mean histologic healing scores at 1 week and 4 weeks of fracture healing in zoledronate (Z) and sildenafil-treated groups (ZS).

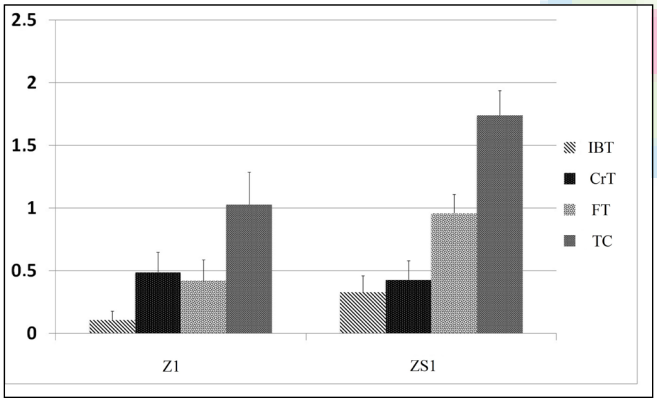

Figure 5: Histological and immunohistochemical representative sections of the zoledronate (Z) (A–C) and sildenafil-treated groups (ZS) (D–F) at 4 weeks after surgery (A–F). Completely immature bone formation (►) and a little mature bone was observed in the ZS groups and enhanced cartilage tissue (arrows) was observed in the Z groups (A, D: H&E, ×40; B, E: Safranin, ×40; C, F: Masson's trichrome, ×40).

Histomorphometric Analysis

Histomorphometric analysis of the tissue distribution within the fracture area after 1 week and 4 weeks of fracture healing are shown in Figures 6 and 7. TCA was higher in group Z4, and there were significant differences between groups Z4 and ZS4 for TCA (P = 0.05). The FA was higher in groups Z1 and ZS1, and there was a significant difference between groups Z1 and ZS1 compared with the other groups (P < 0.05).

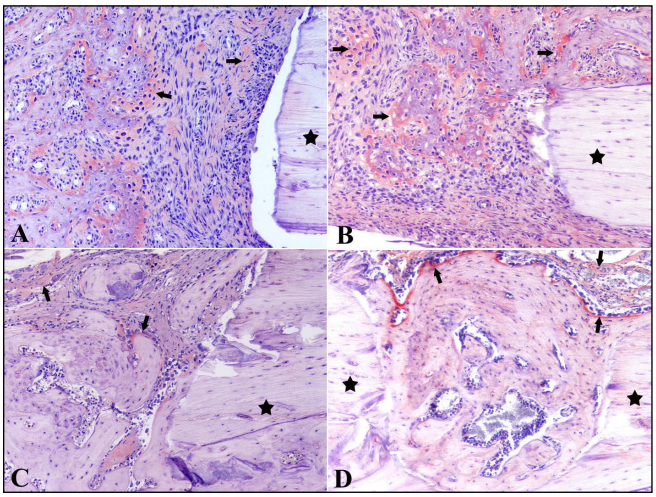

Figure 2: Density measurement was made from the marked area between the miniscrews.

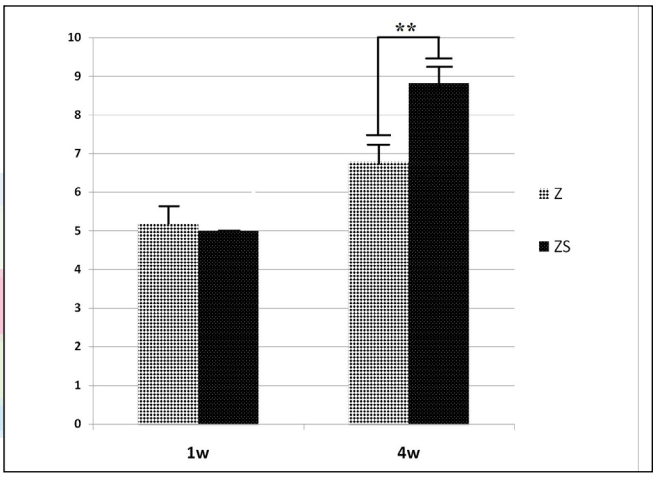

Figure 4: Histological and immunohistochemical representative sections of the Zoledronate (Z) (A–C) and sildenafil-treated groups (ZS) (D–F) at 1 weeks after surgery. Enhanced immature bone formation (►) was observed in the ZS groups and enhanced fibrous tissue (arrows) was observed in the Z groups. (A, D: H&E, ×40; B, E: Safranin, ×40; C, F: Masson's trichrome, ×40).

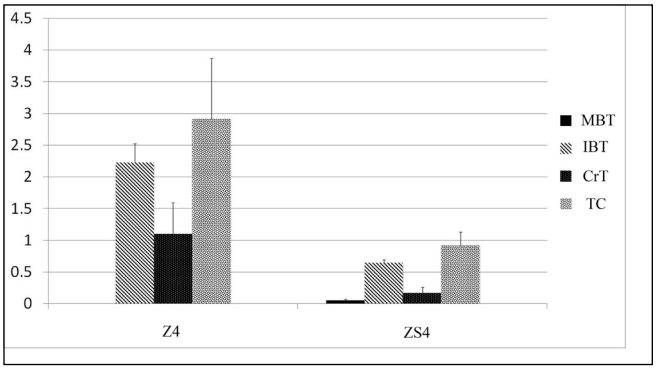

Figure 6: Histomorphometric analysis of the tissue distribution within the fracture area after 1 week of fracture healing in zoledronate (Z) and sildenafil-treated groups (ZS). IBT = immature bone tissue, CrT = cartilage tissue, FT = fibrous tissue, TC = total callus.

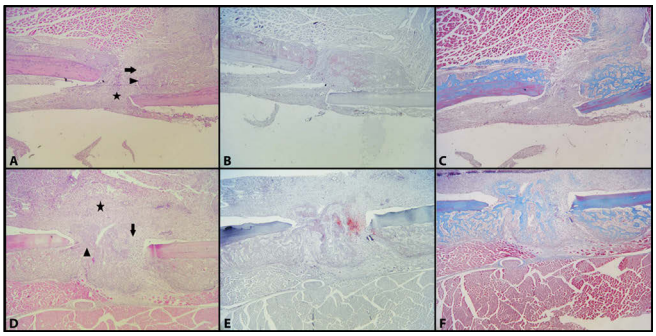

Figure 7: Histomorphometric analysis of the tissue distribution within the fracture area after 4 weeks of fracture healing in zoledronate (Z) and sildenafil-treated groups (ZS). MBT = mature bone tissue, IBT = immature bone tissue, CrT = cartilage tissue, FT = fibrous tissue, TC = total callus.

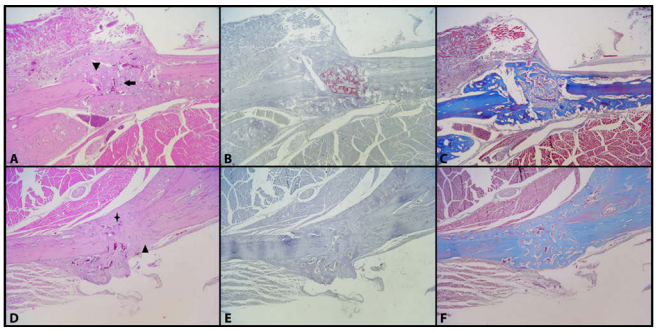

Figure 8: Histomorphometric analysis of the rates of tissues over the total callus area after 1 week of fracture healing in zoledronate (Z) and sildenafil-treated groups (ZS). IBT = immature bone tissue, CrT = cartilage tissue, FT = fibrous tissue, TC = total callus.

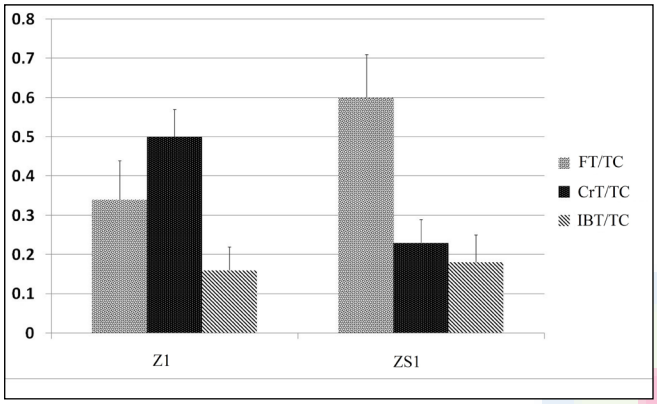

Figure 9: Histomorphometric analysis of the rates of tissues over the total callus area after 4 weeks of fracture healing in zoledronate (Z) and sildenafil-treated groups (ZS). MBT = mature bone tissue, IBT = immature bone tissue, CrT = cartilage tissue, FT = fibrous tissue, TC = total callus.

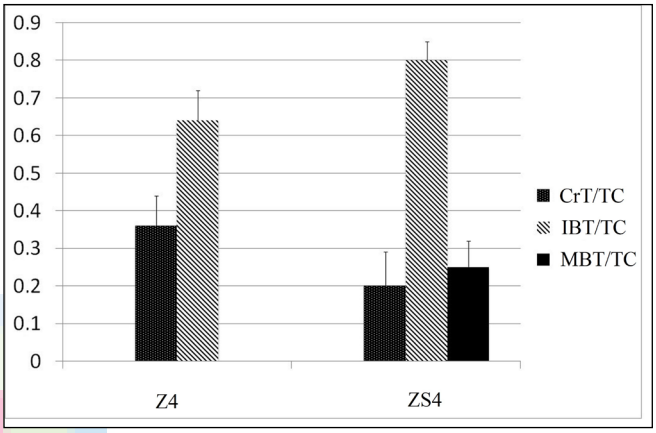

Figure 10: Immunohistochemical staining of the fracture areas (IHC ×10 magnification) in Zoledronate (Z) and sildenafil-treated groups (ZS), (A, B) at 1 week and (C, D) 4 weeks after surgery (B), intense immunopositive staining (arrows) for BMP-2 was observed in the group Z1. (*) mature bone tissue

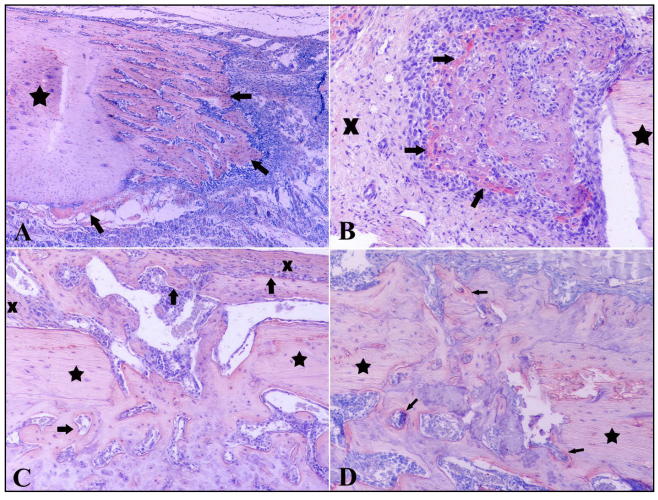

Figure 11: Histological sections of the fracture areas (IHC ×10 magnification) in Zoledronate (Z) and sildenafil-treated groups (ZS) (A, B) at 1 week and (C, D) 4 weeks after surgery, (B), intense immunopositive staining (arrows) for col-1 was observed in the group ZS4. (*) mature bone tissue.

The CrA formed in the callus was higher in group Z4 without significant differences. The highest value for IBT was in group Z4 (2.22), and there was a significant difference between group Z4 and the other groups (P = 0.00). There was no MBT in the groups, except for group ZS4.

Table 2: Mean bone density values of the zoledronate (Z) and sildenafil-treated (ZS) groups after 1 week and 4 weeks of fracture healing.

Animal No:	Bone mineral density (HU)	
	mean±SD	P value
Z1	1762±345	0,014
ZS1	2010±499	
Z4	2770±647	1,000
ZS4	2830±642	

The FA decreased in all groups, whereas the area of immature osseous tissue increased from weeks 1 to 4. Also from weeks 1 to 4, the CrA and IBT significantly increased in group Z4.

Histomorphometric analysis of the rates of tissue growth over the TCA of fracture healing after weeks 1 and 4 are shown in Figures 8 and 9. When comparing groups, the ratio of the FA/TCA and IBT/TCA were significantly higher in groups Z1 and ZS1 than the other groups ($P < 0.05$). The ratio of IBT/TCA was significantly higher in groups Z4 and ZS4 than the other groups ($P = 0.00$). The highest value of MBT/TCA was in group ZS4 ($P = 0.00$). These results indicate that callus mineralization in the sildenafil-treated groups was faster than in the zoledronate-treated groups from weeks 1 to 4.

Immunohistochemical Analysis

Mild BMP-2 staining was observed in the periosteal region and newly shaped bone tissue surrounding connective tissue cells in group Z1. Newly formed bone by intramembranous ossification, especially in the peripheral region of the osteoblasts, showed a severe BMP-2 immunoreactivity in group ZS1. Severe BMP-2 staining was seen in the new bone tissue and connective tissue in group Z4. Only mild BMP-2 staining was observed in the adjacent connective tissue and newly formed bone tissue in group ZS4 [Figure 10].

Only mild or moderate staining for col-1 was observed in the matrix components in groups Z1 and Z4. Strong staining for col-1 was observed in connective tissue and the peripheral region of the newly formed bone trabeculae in groups ZS1 and ZS4 [Figure 11].

Bone Density Analysis

The sildenafil-treated group did, however, show a significant increase in bone density measurements at week 1 ($P = 0.03$). We did not detect any significant differences between the treatment groups with regard to bone density at week 4 [Table 2].

Discussion

BPs are widely used to prevent the skeletal complications of bone metabolism disorders. BPs are well-characterized drugs that inhibit osteoclastogenesis and angiogenesis. As BPs affect angiogenesis, an inadequate level of migration of precursor cells to the inflammation area occurs. Several studies support that systemic administration of ZA resulted in a potent inhibition of angiogenesis and endothelial cell adhesion, as well as migration and decrease of circulating levels of VEGF.[19,20]

The healing process in the jaw is similar to that of any long bones in the skeleton. Bone healing after invasive surgical procedures is typically followed by inflammation, repair, and remodeling. Vascular ingrowth from vessels and neo-angiogenesis in the periosteum is an essential component of fracture healing, and defective angiogenesis at the fracture site has been a primary cause of poor outcomes.[21,22]

Sildenafil is shown to be effective in various ischemic conditions associated with diminished skin blood flow and poor vascularization.[10–12] Histing *et al.*[14] showed that sildenafil treatment accelerates fracture healing by a cysteine rich-61 (CYR61)-associated pathway. It is well known that CYR61 is upregulated in fracture callus, stimulates endothelial cell migration, and promotes osteoblast proliferation and differentiation.

Some studies have also reported oxidative damage due to BPs in various cancer tissues, neurons, and oral epithelium. Oxidative stress (OS) changes would be found in patients treated with BPs, especially in those with inflammatory and infectious conditions, such as in BRONJ.[23,24] Dussault *et al.*[25] reported that sildenafil significantly reduces OS and the antioxidative properties of sildenafil restore the angiogenic actions in hypercholesterolemic conditions.

The assessment of bone microarchitecture is important for the evaluation of the effects of drug therapies on bone fracture healing. Some methods are available to analyze the bone architecture, including histomorphometry, high-resolution computed tomography, magnetic resonance imaging, and quantitative computed tomography. In the present study, we used the histomorphometric method and bone densitometry for quantitative measurements. Tatli *et al.*[26] found significantly increased ossification and higher histologic grading scores in the ZA-treated animals compared with control groups in their study. Additionally, they reported that the radiodensitometric value of the bone at the fracture gap in the ZA-treated animals was 1.19 times greater than that of the control group.

In our study, we found higher histologic scores in groups Z4 and ZS4, and there was a significant difference between weeks 1 and 4 in regards to histologic scoring. It was observed that the positive effects of sildenafil, especially in group ZS4, namely increased the transition of fibrous callus to completely immature and a little mature bone callus, whereas completely cartilage and immature bone callus in the group Z4 in the fourth week. In accordance to parameters reflected on callus formation, the MBT and IBT measurements on sildenafil groups were shown to be significantly higher, but TCA was lower than their zoledronate-treated groups during the period from weeks 1 to 4, representing a better ossification. Interestingly, CrT and IBT areas increased in Z groups from week 1 to 4. These findings were consistent with previous studies indicating reduced callus and cartilaginous area in sildenafil-treated groups compared with control groups.[8,9,14] Additionally, bone density measurements revealed that an increase in bone density in group ZS1

was statistically significance. In line with the literature, our findings support sildenafil enhanced bone healing by improving the quality of callus tissue, as demonstrated by higher histologic bone healing scores.

BMP-2 is expressed primarily during the early stages of fracture healing by osteoblasts, osteoclasts, and other mesenchymal cells within the fracture callus. Im *et al.*[27] showed significantly increased osteoblastic cell number, enhanced gene expression of BMP-2 and type-1 collagen, and osteocalcin with BP administration in the cell culture. Our results showed that the strong expression of BMP-2 in groups Z4 and ZS1 and the weak stain in groups ZS4 are in line with the literature, which indicate that BMP-2 expression increases in the early stages of intramembranous bone formation and chondrogenesis, and its expression decreases as woven bone is remodeled to mature lamellar bone.[28,29] Strong staining for col-1 in sildenafil-treated groups and weak staining in the zoledronate-treated groups shows that sildenafil increases new bone formation. Sildenafil seems to reduce negative effects of zoledronate on fracture healing, such as cartilaginous or delayed healing, by improving the quality of callus tissue. Sildenafil improves bone healing by increasing the expression of the CYR61. Lienau *et al.*[9] reported that the reduced expression of CYR61 led to reduced cartilage resorption and delay in healing.

The histologic, histomorphometric, immunohistochemical, and radiological results obtained in this study are consistent with each other and our results indicate that sildenafil contributes to bone healing in zoledronate-treated animals. Although this is the first study demonstrating the beneficial effects of sildenafil on zoledronate-treated bone fracture healing, in vitro experiments are required to reveal the exact underlying mechanism.

The understanding of biologic events during bone repair is critical for selecting appropriate therapeutic options to accelerate bone formation and directing clinical applications. The bone healing process is important for clinical practice, especially in medically compromised patients suffering from impaired or delayed fracture healing. Sildenafil may be used as a supporting factor in bone healing in BP-treated patients to prevent negative effects of BPs. Further clinical studies with different sildenafil doses used at different time points and on different samples are needed to clarify the usefulness of sildenafil in BP-treated patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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