



Sildenafil Transiently Delays Early Alveolar Bone Healing of Tooth Extraction Sockets

Elysse Orchard¹, Wanda Green², Renjith Parameswaran Nair³, Fleurette Abreo⁴ and Gulshan Sunavala-Dossabhoy^{3*}

¹Department of Animal Resources, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, Louisiana 71130, USA

²Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, Louisiana 71130, USA

³Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, Louisiana 71130, USA

⁴Department of Pathology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, Louisiana 71130, USA

Abstract

Bone is a unique tissue that has the ability to repair itself and return to full function. Bone regeneration is a well-synchronized biological process that recapitulates embryonic bone development. The establishment of a functional vascular supply has been shown to be essential for proper ossification of newly deposited bone, and impaired angiogenesis as in advanced age, diabetes, and anti-cancer treatments affects bone repair. Endothelial guanosine 3', 5'-cyclic monophosphate (cGMP) is known to support angiogenesis, and sildenafil, a phosphodiesterase 5 (PDE5) antagonist, prevents cGMP hydrolysis and promotes the formation of new blood vessels. Since the development of functional vascular networks is critical to bone repair, we investigated the effects of sildenafil on early alveolar bone regeneration following exodontia. Our results demonstrate that per-oral administration of sildenafil (10 mg/kg/day) in rats delays the dissolution and replacement of the sanguine clot with granulation tissue. As a result, the number of replicating cells, a hallmark of regenerating tissue, observed on day 4 is remarkably lower in sildenafil-treated animals than their control counterparts (control: mean±SD, 47.35 ± 9.21; sildenafil: mean±SD, 11.47 ± 5.14). Similarly, cells expressing transcription factor Cbfa-1/Runx2 and osteopontin, markers of differentiating osteoblasts, were fewer in treated animals (control: mean±SD, 83.18 ± 4.60; sildenafil: mean±SD, 13.77 ± 4.63). Treatment with hydrolysis-resistant cyclic GMP (cGMP) showed findings similar to sildenafil-treated animals suggesting the negative impact of cGMP on early inflammatory phase of bone healing. Nevertheless, histological differences were not significant between the 2 groups on day 8. Based on these findings, we conclude that sildenafil transiently retards early events in alveolar bone healing.

Keywords: PDE5, cGMP, Bone regeneration

Introduction

Bone regeneration is a biological process that involves a cascade of well-synchronized responses essentially comprised of three overlapping stages namely, inflammatory, reparative, and remodeling. Activation of endothelial cells and the sprouting of new capillaries from pre-existing vasculature is a process referred to as angiogenesis. Angiogenesis is critical for bone healing [1,2], and angiogenesis inhibitors have been shown to block the outgrowth of new blood vessels and delay the calcification of newly deposited bone at fracture sites [3-5]. Embryonic development of facial bones and flat bones of the skull occurs by intramembranous ossification, and bone regeneration within the alveolus after tooth extraction recapitulates embryonic development. The fibrin clot formed soon after extraction undergoes organization and is replaced with a stem cell-rich connective tissue. In response to inflammatory signals, stem cells undergo differentiation to cells of angiogenic and osteogenic lineages, and cellular events ultimately culminate in bone deposition and maturation. The formation of new blood vessels is intimately connected to bone repair, and impaired angiogenesis as in old age, diabetes, or radiotherapy delays bone healing.

The role of cGMP in modulating vascular tone and permeability during angiogenesis has been

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*Correspondence:

Gulshan Sunavala-Dossabhoy,
Department of Biochemistry and
Molecular Biology, Louisiana State
University Health Sciences Center,
1501 Kings Highway, Shreveport,
Louisiana 71130, USA, Tel: 318 675-
8216; Fax: 318 675-5180;
E-mail: gsunav@lsuhsc.edu

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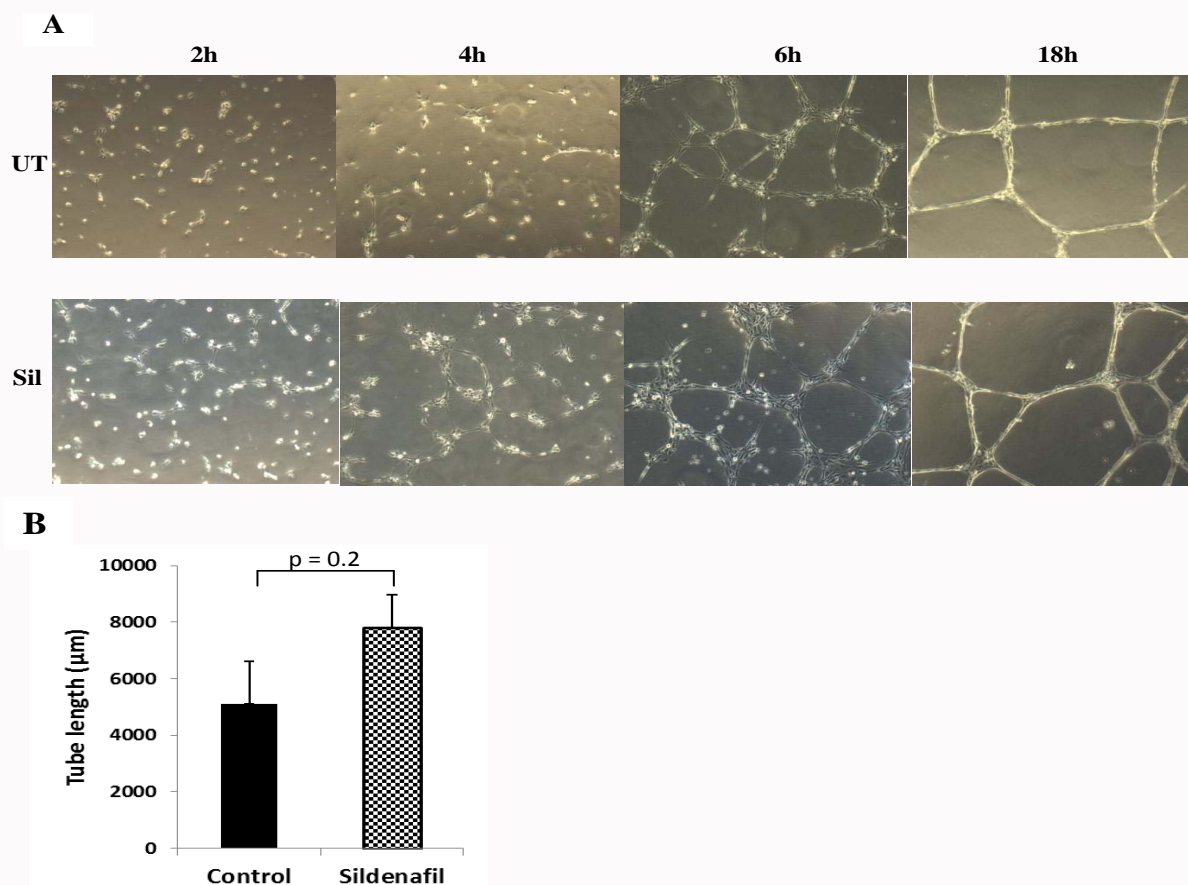


Figure 1: Sildenafil facilitates capillary tube formation *in vitro*. A) HUVEC cells plated on growth factor-reduced matrigel were treated, or not, with sildenafil (100 nM) and cells were imaged at 2, 4, 6, and 18 h. B) Quantification of total tube length at 4 h. Three random microscopic fields per well were imaged and total tube length quantified by ImageJ Angiogenesis Analyzer. UT: untreated; Sil: sildenafil-treated.

demonstrated before [6,7]. Phosphodiesterase 5 (PDE5) is a potent enzyme that regulates intracellular cGMP levels by promoting the conversion of cGMP to GMP. PDE5 antagonists such as sildenafil suppress the hydrolysis of cGMP and increase the intracellular cGMP pool. Sildenafil treatment has been shown to increase angiogenesis and improve soft tissue survival after embolic stroke, ischemic limb pathology, and random-pattern flap grafts in animals [8-11]. Nitric oxide (NO) interacts with soluble guanine cyclase to stimulate the production of cGMP, and NO is known to improve tissue recovery after ischemic pathology [12,13]. NO is a potent stimulator of cell proliferation and angiogenesis during wound healing [14-16], and wound repair is impaired and bone formation reduced in endothelial nitric oxide synthase (eNOS)-deficient animals and in animals administered nitric oxide synthase (NOS) inhibitors [17,18]. Moreover, osteoblasts of eNOS knockout animals demonstrated a delay in bone maturation, whereas exogenous supplementation of NO upregulated genes involved in osteoblast differentiation and matrix mineralization [15]. Based on the involvement of NO/cGMP pathway in bone regeneration, the aim of the study was to investigate the effects of PDE5 inhibition on alveolar bone healing after tooth extraction.

Materials and Methods

Reagents

Sildenafil was a kind gift from Pfizer (New York, NY), while

8-bromo-cGMP was purchased from Calbiochem. Primary antibodies, anti-Ki67, anti-von Willebrand factor, anti-Osteopontin and anti-Runx2 were purchased from Abcam. The HRP-conjugated secondary antibodies and HRP-detection kit were obtained from Biogenex.

Endothelial tube formation

Human umbilical vein endothelial cells, HUVEC, were grown in Dulbecco's Modified Eagles Medium (DMEM) that was supplemented with 5% FBS, antibiotic-antimycotic (Invitrogen) and minimal essential amino acids (Invitrogen). Capillary tube formation was conducted in 6-well culture plates coated with low growth factor matrigel (BD Biosciences). HUVEC cells were seeded at 1×10^4 cells/cm² in DMEM with 5% FBS with, or without, sildenafil (100 nM) and cells were imaged at various times. Capillary tube formation was quantified in 3 random areas of the well and the length of cords between cells was measured using ImageJ Angiogenesis Analyzer.

Animals and Experimental procedure

Forty outbred male Wistar rats (Harlan/Envigo) weighing approximately 250 gm were housed under 12 h dark and light cycle with free access to food and water. Experiments were performed under the aegis of the LSUHSC Animal Care and Use Committee, and they conformed to the guidelines for the care and use of laboratory animals. Animals were anesthetized with intramuscular administration of ketamine chloride (42 mg/kg), xylazine (8 mg/

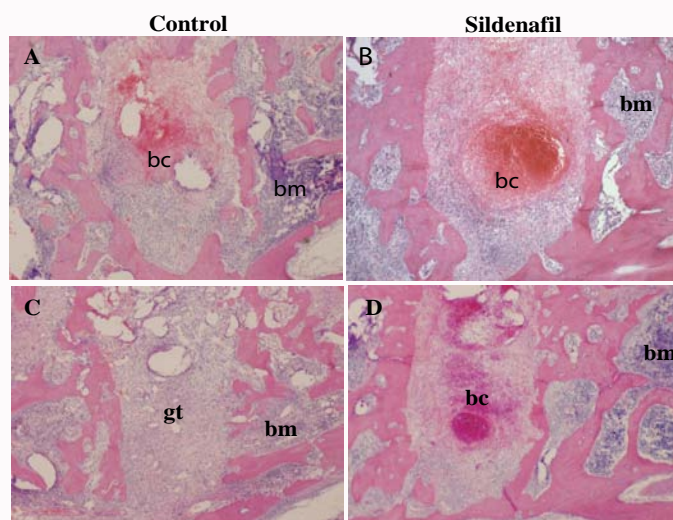


Figure 2: Blood clot resolution is delayed in response to sildenafil. Histological assessment of healing of extraction sockets at **A,B** day 2 and **C,D** day 4 in vehicle treated controls (**A,C**) and sildenafil-treated animals (**B,D**). Images were captured at 40x magnification. *bc*: blood clot; *bm*: bone marrow; *gt*: granulation tissue.

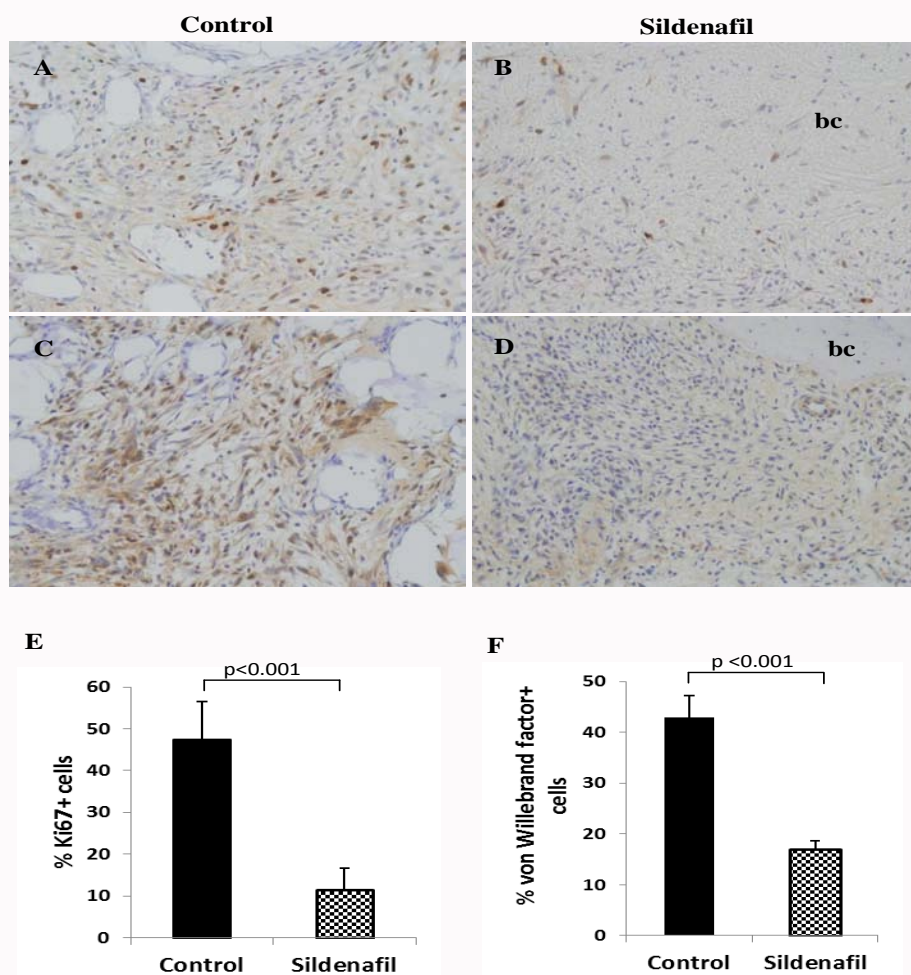


Figure 3: Sildenafil-associated decrease in cell proliferation and vascular density. Immunohistochemical detection of **A,B** Ki-67 and **C,D** von Willebrand factor

kg), and acepromazine (1.4 mg/kg), and the left mandibular first molars were luxated. Bleeding was stanchied by applying pressure at the extraction site. Animals were kept warm during recovery and

were housed based on treatment. Sildenafil was dissolved in sterile water, and 200 μ l of sildenafil (10 mg/kg/day; divided into 2 doses administered 10 h apart) or water was administered by gavage each

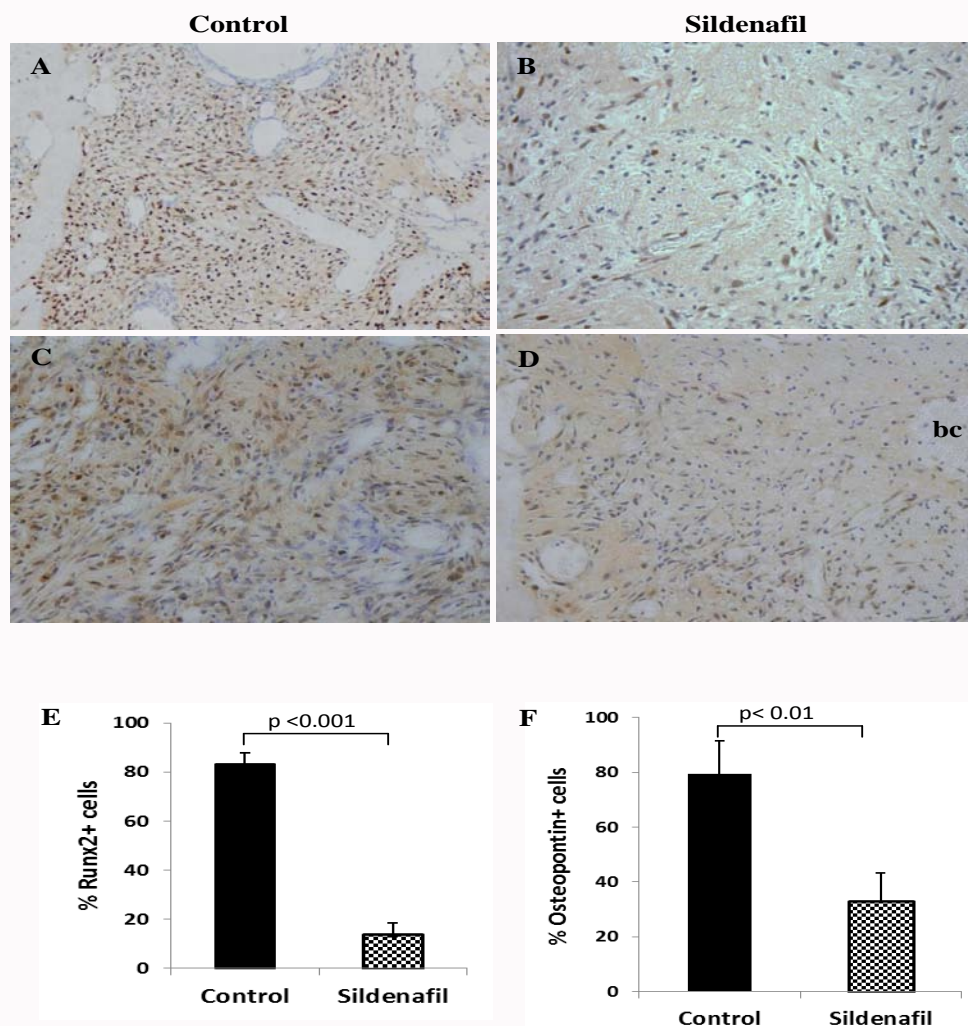


Figure 4: Decreased number of angio- osteo- progenitor cells after sildenafil treatment. Immuno-detection of **A,B** Runx2 and **C,D** Osteopontin in tissue samples harvested on day 4. Control (**A,C**) and sildenafil-treated animals (**B,D**). Percent Runx2+ and Osteopontin+ immuno-stained cells in 5 random fields (200x magnification).

day starting soon after tooth extraction. Due to the 8-fold increase in plasma clearance rate and a short half-life of sildenafil in rodents (>1 hour in mice versus 4 hours in humans; [19, 20]), the dosage of sildenafil was adjusted to achieve therapeutic dosing comparable to humans. Animals were euthanized under anesthesia by carbon dioxide inhalation on day 2, 4, or 8, and the left segment of the mandible dissected and harvested. In a separate set of experiments, 8-bromo-cGMP (100 gm/kg), a cell-permeable non-hydrolysable analogue of cGMP, was administered daily intravenously (n = 4).

Histology and Immunohistochemistry

The left segment of the mandible was dissected, and the bone cleared of soft tissue before fixing in 4% paraformaldehyde/ phosphate buffered saline (PBS), pH 7.4, for 48 h. Tissues were then decalcified in 20% EDTA/ PBS, pH 7.4, for 7 days before paraffin processing and embedding. Tissue sections were stained with hematoxylin-eosin for histological evaluation, or used for immunohistochemistry. Antigen retrieval of deparaffinized tissue sections was done by immersing slides in DeCal Retrieval Solution (Biogenex), 20 m, followed by incubation with anti-von Willebrand Factor (1:250), anti-Ki67 (1:100), anti-Runx2 (1:1000), or anti-Osteopontin (1:2500), 16 h at 4 oC. Tissue sections were washed and incubated in Biogenex HRP-

conjugated universal secondary antibody for 30 m before reacting with diaminobenzidine (DAB) and counterstaining with hematoxylin.

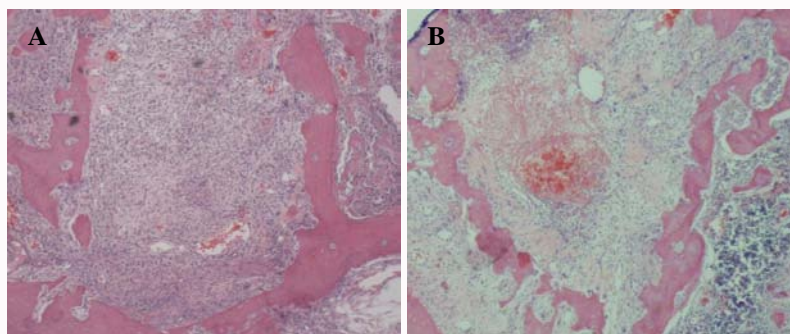
Morphometric analysis of regenerating tissue

Slides stained with antibodies to Ki67 and von Willebrand factor were used to measure cell proliferation and vascular indices. Immunostaining in a total of 5 random fields was analyzed, and proliferation index and vascular density were determined by percent Ki67 + cells or von Willebrand factor + cells to the total number of hematoxylin-stained nuclei. Immuno-reacted Runx2+ and osteopontin+ cells were similarly measured to evaluate percent osteo-angio-progenitor cells and percent osteogenic cells. All data were analyzed by *Student's t-test* to determine p-values.

Results

Sildenafil accelerates capillary tube formation *in vitro*

The effect of sildenafil on capillary tube formation was analyzed after seeding HUVEC on growth factor-reduced matrigel and assessing their organization at regular time intervals. Cells formed branching networks within 6 h, which organized into tube-like structures by 18 h (Figure 1A). The addition of sildenafil hastened the development of cell extensions and the formation of cell-cell connections. At 4 h, the



Supplementary Figure 1: Increase in cGMP signaling delays blood clot remission. Hematoxylin–eosin staining of bone isolated from **A)** control and **B)** 8-bromo-cGMP treated animals 4 days after tooth extraction.

total length of continuous tube connections was greater in sildenafil-treated cells than the non-treated counterparts (Figure 1B).

Sildenafil retards early bone healing

The establishment of blood supply is critical for proper bone healing [5,21], and sildenafil was shown to accelerate fracture repair in mice [22]. To evaluate whether the effect can be recapitulated in alveolar bone healing, we assessed the early stages of healing of molar extraction sockets over a period of 8 days. Hematoxylin and eosin stained tissue sections of samples collected on days 2 and 4 following tooth extraction demonstrated a lengthier retention time of the sanguine clot in sildenafil-treated animals compared to vehicle-treated (Figure 2A and B). A delay in clot resolution was also observed after administration of non-hydrolysable analog of cGMP, 8-bromo-cGMP (Supplementary Figure 1). A lag in infilling of granulation tissue was the major difference observed between the groups (Figure 2C and D). However, in contrast to days 2 and 4, healing on day 8 was comparable in drug-treated animals and controls (data not shown).

Delay in infiltration of stem/progenitor-rich connective tissue in response to sildenafil

Granulation tissue composed of actively dividing and differentiating stem/progenitor cells is a hallmark of tissue repair and regeneration. Bone tissues harvested on day 4 demonstrated far fewer cells that immuno-reacted with Ki67, a marker of proliferating cells, in response to sildenafil treatment (control: mean \pm SD, 47.35 ± 9.21 ; sildenafil: mean \pm SD, 11.47 ± 5.14 ; Figure 3A,B and E). Moreover, an assessment of neovasculature by von Willebrand factor immuno-staining demonstrated a decrease in the number of immuno-reactive cells following drug treatment (control: mean \pm SD, 42.9 ± 4.3 ; sildenafil: mean \pm SD, 17.0 ± 1.8 ; Figure 3C,D and F).

Essential to early osteoblast differentiation is the expression of osteoblast-specific transcription factor, Runt-related Transcription factor 2 (Runx2). Runx2 is highly expressed in immature osteoblasts, but is downregulated during maturation [23]. However, Runx2 is not an exclusive marker of osteogenic cells. It is expressed in endothelial progenitor cells too and has been found to be important for endothelial cell programming [24,25]. To assess the contribution of osteo- angio-progenitor cell population to bone healing, tissue sections were incubated with anti-Runx2. Quantitative morphometric measurement from 5 random fields demonstrated a near 6-fold decrease in cell-staining after drug treatment. (control: mean \pm SD, 83.18 ± 4.60 ; sildenafil: mean \pm SD, 13.77 ± 4.63 ; (Figure 4A,B and E).

Sildenafil retards bone matrix deposition

Runx2 induces the expression of important bone matrix

proteins namely, osteopontin, bone sialoprotein, osteocalcin and Colla1 in immature osteoblasts and mesenchymal progenitors during bone development [23]. To analyze whether the decrease in Runx2-immunoreactive cells translates to a decrease in osteopontin expression after sildenafil treatment, osteopontin immuno-localization was conducted on tissues collected on day 4. As expected, the results demonstrated that exposure to the drug results in fewer osteopontin-expressing cells (control: mean \pm SD, 79.58 ± 11.81 ; sildenafil: mean \pm SD, 33.12 ± 10.17 ; Figure 4C,D and F).

Discussion

Angiogenesis and the establishment of a functioning vascular bed have been shown to be vital to successful bone repair. Angiogenesis agonists such as vascular endothelial growth factor (VEGF) stimulate bone repair [26], whereas angiogenesis inhibitors suppress bone healing at fracture and implant sites [3-5]. PDE5 is the most active of all cGMP-hydrolyzing PDEs, and it is widely expressed in tissues including bone [27]. In light of previous studies that demonstrated PDE5 antagonism in promoting neovascularization through augmentation of endothelial cGMP and increasing in bone marrow-derived endothelial progenitor cells [27], we anticipated a positive impact of PDE5 inhibitor, sildenafil, on early bone healing. Similar to previous studies, we demonstrated that sildenafil increases HUVEC migration and formation of capillary-like structures on matrigel *in vitro* [13,19]. Angiogenesis and early alveolar bone healing when assessed *in vivo* showed that the sequential phases of healing of extraction sockets described before were reproducible in our study [28]. However, the addition of sildenafil retarded blood clot remission and the migration of stem cells into the defect. Since sildenafil principally acts through the cGMP signaling pathway, a delay in the resolution of the fibrin clot in cell-permeable 8-bromo-cGMP treated animals implicated cGMP in attenuating clot dissolution. Differentiation of monocytes to macrophages or dendritic cells is induced by inflammatory stimuli, and evidence indicates that cyclic nucleotides such as cGMP can suppress the process [29]. Our results suggest that sildenafil-initiated increase in cGMP negatively influences early bone healing. Alveolar bone formation after tooth extraction occurs by the direct differentiation of mesenchymal stem cells to cells of osteogenic lineage. The important role of Runx2 in osteogenesis was confirmed when Runx2 knockout mice displayed poor skeletal development [30,31]. Runx2 is also expressed in endothelial precursors and it regulates their migration, proliferation, and differentiation [24,32]. Runx2 is, therefore, expressed in cells committed to osteogenic and angiogenic fates, and our s suggest that recruitment of stem/progenitor cells to the site of repair is impaired by sildenafil. Earlier

studies investigating sildenafil focused on angiogenic outcomes, and only recently have studies noted its anti-inflammatory effects [33-36]. Inflammation is a critical initial event in a tissue's response to injury. It signals damage and activates tissue repair mechanisms. The inflammatory response initiated cascade of events plays a significant role in bone repair, and its suppression delays healing [37-40]. A recent study examining the effects of sildenafil on long bone fracture repair found that it negatively affects the inflammatory phase of bone healing [41]. Our findings corroborate the finding that the drug attenuates early events in bone healing. It is suggested that the anti-inflammatory effects of sildenafil suppress blood clot resolution and delays, the migration of mesenchymal stem cells to the site of injury. Previous studies that analyzed long bone fracture repair at >2 weeks have demonstrated a beneficial effect of sildenafil [22,41], and with no appreciable differences in healing observed at day 8, we suggest that the drug accelerates reparative phase of alveolar bone healing presumably through improved angiogenesis.

The preservation and maintenance of adequate bone volume after tooth extraction improves the success of prosthetic rehabilitation. Our findings of an early, but transitory, delay in bone healing by sildenafil suggest that a prudent measure would be to avoid the drug in the days immediately following tooth extraction or placement of dental implants.

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