ERYTHROPOIETIN – A POTENT FACTOR FOR ALVEOLAR RIDGE AUGMENTATION AFTER FIRST MOLAR EXTRACTION

A Thesis

by

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ABSTRACT

Purpose of the Research: Loss of teeth is commonly associated with a loss of the alveolar bone surrounding the functional tooth, resulting in an undesirably narrow bone ridge for subsequent implant placement. In our quest for alternatives to freeze-dried bovine bone, allografts, alloplasts, or RhBMP-2 we identified the kidney derived growth factor erythropoietin (EPO) as a candidate molecule for alveolar ridge augmentation in combination with suitable scaffolds. Currently, EPO overall safety in patients is established by current approval for Erythropoietin and biosimilars by the US Food and Drug Administration for the treatment of anemia caused by chronic kidney disease, chemotherapy, or use of zidovudine in patients with HIV infection. The aim of the present study is to evaluate the efficacy of erythropoietin's angiogenic and osteogenic potential compared to two popular ridge preservation techniques; anorganic bovine bone mineral (Bio-Oss®) with non-cross-linked collagen membrane (Bio-Gide®), and collagen membrane alone.

Methods and Materials: Rats underwent bilateral maxillary first molar extraction, and uniform extraction defects were made. Rats were randomly assigned to groups that were to receive an erythropoietin-soaked collagen pellet (Test Group), an anorganic bovine bone group (Bio-Oss® Geistlich Biomaterials) treatment group or no material (Control). Defects were then covered with Bio-Gide collagen membrane and secured with purified cyanoacrylate (Periacryl, Salvin®). The rats were then randomly assigned for sacrifice via CO_2 overdose after four or eight weeks. The following analyses were performed; reverse transcriptase polymerase chain reaction (RT-PCR), radiographs, paraffin sections with Masson's trichrome staining. Ground sections were subjected to Von Kossa staining.

Results: X-ray, and Von-Kossa stain comparison demonstrated comparable bone fill and radiopacity in the extraction sockets of EPO and of anorganic bovine bone treated rats. The extracellular matrix of erythropoietin treated groups appeared to show an organized, sheath-like matrix compared to the control and anorganic bovine bone groups. RT-PCR data showed statistically significant increases in crucial genes for osteoblast regulation, and new blood vessel formation.

Discussion: Our data indicate that EPO acts as a potent factor in combination with a collagen sponge, promoting both new bone and angiogenesis.

Conclusions: Our study suggests that EPO or biosimilars might serve as an alternative to established procedures for alveolar ridge augmentation.

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The project's data was collected, and analyzed with the help of Dr. Mirali Pandya, PhD, and laboratory procedures were assisted in part by Connie Tillman. Dr. John Bozanich played a pivotal role in study design.

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1. INTRODUCTION

1.1. BONE

Bone is an exceedingly complex tissue. Its organization allows it to provide not only mechanical functions in the form of structural support and protection, but also metabolic functions that include mineral homeostasis. Additionally, given its vascularity and its constant state of remodeling, bone has notable healing and regenerative properties. However, true regeneration is often not without shortcomings which has led researchers in the search for new therapies to aid in bone regeneration (Bueno, 2011).

Bone's extracellular matrix (ECM) is described as biphasic because it is made up of around thirty percent organic, and around seventy percent inorganic material by weight (Bueno, 2011). The organic portion is made up almost entirely by type I collagen fibers that are responsible for structure and elasticity and the rest is comprised of entities that aid in signal processing necessary for matrix organization, mineralization, and cell turnover which include; adsorbed serum proteins, glycoproteins, proteoglycans, peptides, lipid materials, and biologically active proteins (Hepenstall, 1984). Bone's inorganic component is made up of calcium phosphate mineral crystals that help provide material integrity.

The cell types found in bone include osteoblasts, a portion of which mature into osteocytes, and the third type is osteoclasts. Osteoblasts arise from multipotential mesenchymal cells that have the ability to differentiate into fibroblasts, adipocytes, and

chondrocytes. They are morphologically cuboidal and have robust endoplasmic reticulum that enables them to secrete organic matrix, including collagen. They also make paracrine and autocrine molecules capable of recruitment of osteoprogenitor cells, induction of the growth of preosteoblasts, and initiation of the resorption of the mineralized bony matrix via osteoclasts (Lian, 1999). A vast portion of osteoblasts undergo apoptosis after they are done secreting bone and ECM, and a fraction of osteoblasts end up as osteocytes after they become encased in the mineralized matrix that they have secreted (Jilka, 1998). Osteocytes play roles in mineral homeostasis, signal transduction, and mechanical sensing by maintaining an intricate intercellular network via cytoplasmic processes called canaliculi in the haversian system of lamellar bones. The haversian system provides osteocytes with their nutrients and oxygen, and the cells are generally located .1mm away from a capiallary vessel (Bueno, 2011).

Large, multinucleated osteoclasts are derived from hematopoietic stem cells. Osteoclasts are responsible for bone resorption via attachment to the bony surface and form pits of resorption. Following attachment, osteoclasts secrete hydrogen ions that results in a local environment with a decreased pH. Demineralization leaves bone's organic matrix prone to digestion via proteolysis, and the matrix byproducts are then eliminated (Bueno, 2011).

For the duration of one's lifetime, bone forming osteoblasts, and bone resorbing osteoclasts work in close concert in order to maintain bony growth and remodeling. In cancellous bone, osteoclasts can be seen in Howship lacunae on trabeculae. However, in cortical bone, osteoclasts reside close to small blood vessels and at the forefront of

entities called, “cutting cones”. Cutting cones are channels of resorbed bone mineral and matrix and osteoblasts are responsible for filling the void they leave in their wake. Osteoblasts work to lay down lamellar bone in concentric rings surrounding the central blood vessel to form a unit called an osteon. (Bueno, 2011). Due to its intricate, life-long remodeling process, bone is afforded the ability to control mineral homeostasis, allow for fracture healing, and incorporate bone grafts and implants (Murray, 2011).

1.2. ALVEOLAR RIDGE PRESERVATION

The alveolar process is the portion of the maxilla and mandible that houses the dentition. It provides a bony attachment to the periodontal ligament which surrounds root cementum. Alveolar bone is a dynamic tissue that undergoes a continuous cycle of resorption and new bone formation (An, 2017). When a tooth can no longer be maintained with comfort, health, function, or aesthetics, it is the deemed hopeless and extraction is recommended.

In a classic study by Cardaropoli et al in 2003, the phases of healing after extraction in mongrel dogs was documented in 1, 3, 7, 14, 30, 60, 90, 120- and 180-day intervals. Day one was characterized by a clot comprised mainly of erythrocytes and platelets trapped in a network of fibrin. By day 3, small segments of the coagulum had been replaced by a highly vascularized granulation tissue, with zones of lysed erythrocytes at the core of the extraction socket. At day seven, a provisional matrix of

newly formed blood vessels, and mesenchymal stem cells could be seen. After fourteen days, remnants of the periodontal ligament cannot be seen, and there are portions of new hard tissue present in sites that previously communicated with the adjacent bone marrow spaces. This new hard tissue is covered by connective tissue that is partly lined with epithelial cells. At the thirty-day healing mark, a keratinized epithelium with well-organized fibrous connective tissue covers the top of newly formed bone that was home to many primary osteons. Osteons make up the functional unit of mature human bone. They contain osteocytes that are housed in lacunae that are surrounded by rings of lamellar bone (Hepenstall, 1984). The one-month healing period also showed signs of osteoclastic activity, and therefore modeling and remodeling of new bone had commensed. To conclude the study, by days sixty and ninety, woven bone was sandwiched between the marginal mucosa, and underlying bony matrix with large blood vessels, adipocytes, and inflammatory cells (Cardaropoli, 2003).

Unfortunately, alveolar bone undergoes significant remodeling after extraction that leads to anatomical changes (Van der Weijden et al., 2009). Araujo showed the loss in the buccal dimension to be more severe compared to the lingual in a dog model and Tan confirmed this observation in a systematic review, when he found that horizontal bone loss ranged from 29-63% and vertical bone loss was 11-22%, based on human re-entry studies (Tan, 2012). While modern techniques have allowed us to quantify the amount of bone lost post extraction, the observation that tooth loss leads to significant changes has been documented for over fifty years, and dentists have sought a solution for this loss ever since (Pietrokovski, 1967).

Alveolar ridge preservation, the process by which a practitioner aims to inhibit or slow down the anatomic changes after tooth extraction dates back to 1974, in the form of, “root banking”, when Osburn proposed the preservation of roots in the alveolus to prevent horizontal and vertical changes in bone volume (Osburn, 1974). Ridge preservation via tooth roots was a solution to a problem that was often faced in the era before endosseous implant placement – an inadequate ridge width and subsequent poor removable denture retention and stability. However, even in the best of circumstances, root banking comes with risk of root fracture, caries, poor long-term prognosis, and interference of dental implant placement (Avila-Ortiz, 2014). Due to the often unfeasibility, impracticality and increased risk of preserving roots, practitioners began placing various substances and materials in extraction sockets to maintain an adequate volume of bone to facilitate restorative dentistry in the future.

These procedures are necessary because the placement of an implant requires sufficient alveolar bone support, and lack of bone volume is correlated with poor osseointegration (Iasella et al. 2003). The materials often used include; autografts from the patient’s own tissue, allografts from donated, cadaver bone, xenografts from another specie’s bone (typically porcine, or bovine), alloplasts or synthetic materials, and other less common biomaterials including; the glycoprotein erythropoietin, bone morphogenic protein, Leukocyte-platelet rich fibrin, and platelet derived growth factor.

The aforementioned treatment modalities all have their own unique set of upsides and downsides and no particular substance is perfect for alveolar ridge preservation. In the end, the success of bone grafting procedures is ultimately dependent upon

revascularization and remodeling of the grafted bone into vital, load bearing bone. Therefore, the ideal graft material should be safe, non-toxic, biocompatible, abundant, non-variable, and provide an environment for new bone to grow and thrive. Autografts maintain the possibility of cell viability, graft revascularization, and there is no chance of disease transmission (Goldberg, 1987). They also maintain the ability to be osteoinductive, whereby they elicit mesenchymal cell migration, attachment and osteogenesis when implanted in well-vascularized bone, and induce endochondral bone formation when implanted in tissues that would otherwise not form bone (Bueno, 2011) The superiority of autologous bone marrow for ridge preservation was shown when it preserved alveolar thickness better than no graft at all (Pelegrin, 2009), and Becker and Becker showed that sockets grafted with autologous bone healed with vital woven and lamellar bone, making it the gold standard in ridge preservation (Becker & Becker, 1994). On the other hand autografts often require a second surgical site, and harvesting from the patient can add pain and morbidity to the procedure, in addition to increased surgical risk, and limited graft supply (Rose LF, 2004). Allograft's are user friendly, osteoconductive, and potentially osteoinductive (Urist, 1965). Iasella observed that the use of freeze dried bone allograft and a collagen membrane improved height and width dimensions when compared to no graft at all (Iasella, 2003) However, allograft properties may be variable from batch to batch, and dependent upon the age of the donor and the post harvest processing techniques (Schwartz, 1996). Additionally a commercially available allograft paste was shown to not prevent significant ridge resorption (Brownfield 2012). Xenografts, are also easy to use, and abundantly

available. Cardaropoli showed that, “Socket preservation using bovine bone mineral and porcine collagen membrane considerably limits the amount of horizontal and vertical bone resorption when compared with tooth extraction alone” (Cardaropoli, 2012). Xenografts may however delay new bone formation in the healing extraction socket, and cause more inflammation compared to sites grafted with other materials (Vance, 2004). Lastly, both allografts, and xenografts may be unacceptable to various patient populations for cultural or religious reasons.

Resorbable, or non-resorbable barrier membranes have become a crucial step in the alveolar ridge preservation procedure. Avila-Ortiz documented that the use of any membrane at all had a strong beneficial effect on preservation of midbuccal (p = .008) and midlingual (p = .067) alveolar bone height (Avila-Ortiz, 2014). In the beginning, Nyman and colleagues used cellulose acetate, known by the brand name Millipore®, in monkeys in order to exclude gingival connective tissue ingrowth, thereby allowing for the study of the healing potential of periodontal ligament cells (Nyman, 1982). The first Teflon membranes were an expanded-polytetrafluorethylene (e-PTFE), which showed promise first in guided tissue regeneration and were later employed by Buser to aid in guided bone regeneration (Buser, 1990). Unfortunately, non-resorbable membranes are not immune to post-operative complications and membrane exposure created by variable amounts of flap sloughing is a recurrent complication associated with their use (Murphy, 1995). Due to e-PTFE’s porosity, exposure to the oral environment allows for bacterial adhesion, and in the very best of circumstances- they require a second surgery for removal. Dense-PTFE is non-porous and supplanted the use of e-PTFE after it was

shown to have comparable results in guided bone and guided tissue regeneration procedures and also did not necessitate primary closure in alveolar ridge preservation (Hoffman 2008; Walters 2003; Ronda, 2008).

In our study, a resorbable, non-cross-linked collagen membrane (Bio-Gide®) was used. Collagen membranes do not usually require a second surgery for retrieval, they are hemostatic, and can enhance fibrin linkage via stimulation of platelet attachment, which could aid in regeneration (Bunyaratavej P, 2001). Marinucci also showed they can promote bone regeneration through their effect on osteoblasts (Marinucci, 2001) It's ease of handling, and weak immunogenicity, in addition to it being the main component of connective tissue throughout the body make it a desirable material for alveolar ridge preservation. Another absorbable collagen product, Salvin® OraPlug Absorbable Collagen Sponge (Salvin® Dental Specialties) was used to provide a matrix for cell/tissue ingrowth and material transfer.

As research, surgical procedures, and technology have advanced over time, the next frontier in alveolar ridge preservation is one that is minimally invasive, consistently predictable, affordable, and effective. Various biomaterials have sought to satisfy each of the aforementioned categories. Bone morphogenic protein-2 (BMP-2) and BMP-7 rose in popularity because of its osteoinductive properties where it acts a chemotactic agent, a growth factor, and a differentiation factor (Subach, 2001). RhBMP is relatively expensive and may be cost prohibitive for most pracitioners. Additionally, it has been shown to induce swelling that may be life threatening after spinal surgery (James, 2016).

Leukocyte-platelet rich fibrin may decrease post-operative pain after extraction, but very limited evidence on whether it is as effective as a bone graft substitute in ridge preservation procedure exists (Dragonas, 2018). Platelet derived growth factor is commercially available as GEM-21 and may be able to accelerate implant site development via a system that is completely synthetic, and off the shelf (Geurs, 2014).

1.3. ERYTHROPOIETIN

Erythropoietin, or EPO, is a glycoprotein that weighs 35Kd and is the primary regulator of erythropoiesis in the blood. Prior to birth, the liver produces the majority of EPO (Congote, 1977). Later in life, the adult kidney produces greater than 90% of the body's EPO (Jacobson, 1957), and the remaining (and insufficient amount to maintain erythropoiesis) is produced by the liver and the brain (Koury, 1988; Erslev, 1980;Marti, 1996). Renal cells equipped with oxygen sensors monitor the oxygen content of the blood and regulate the amount of EPO release. Once released, EPO binds to its receptor (Epo-R) on the red cell surface. After EPO engages with Epo-R, the activation of Janus-tyrosine-kinase-2 (JAK2), a tyrosine kinase, takes place. JAK2 then phosphorylates Epo-R, as well as various other proteins (Kubatzky KK 2011; Constantinescu JN 2001). Fu-Kuen Lin and his team successfully established the gene encoding erythropoietin in 1983, and a patent for recombinant human erythropoietin (rHuEPO) was approved in 1989.

The red cell surface is not the only cell surface that expresses Epo-R. In the developing fetus, and in adult tissues, Epo-R is seen on; lymphocytes, myocardial cells, endothelial cells, megakaryocytes, prostatic cells, smooth muscle cells bone marrow cells and even on peripheral and central nerve cells (Lykissas, 2007). In turn, these individual cell types undergo reactions that are specific, and result in the activation of biological pathways within their respective cells. Examples of unique roles, based on cell type include, erythropoietin stimulating the proliferation of epithelial cells in vitro, and the natural formation of new blood vessels in vivo (Jaquet K 2002; Ribatti D 1999; Yasuda Y). The blood vessels in the skeletal system play active roles in controlling multiple aspects of bone formation and its physiological maintenance (Sivaraj & Adams 2016), and it has been said that angiogenesis leads the way for ossification (Bueno, 2011).

To be more specific, Jaquet concluded that erythropoietin has the same angiogenic potential as vascular endothelial growth factor on endothelial cells that were derived from human myocardial tissue, and his studied showed promise that EPO may have the ability to serve as a *direct* angiogenic substance (Jaquet 2002).

Additionally, the hormone elicits a response on red blood cell precursor cells in the bone marrow that ultimately stimulates their proliferation and maturation, which ends in an increase in the number of red blood cells in the peripheral circulation (Foote, 2009). Hamad also demonstrated that erythropoietin has counterparts in skin tissue that aids in the healing of diabetes related skin wounds (Hamad 2014) and reported that topical EPO administration hastens healing in burn victims via an aquaporin-3 dependent

mechanism (Hamad 2017). Erythropoietin's multifaceted abilities may rely on its ability to interact with and direct the extracellular matrix. As mentioned previously, Hamad et al showed improvements in wound healing after topical application of EPO, and identified that EPO played parts in stimulating angiogenesis, enhancing the act of reepithelialization by keratinocytes, improving collagen deposition, and even played a role in downregulating inflammatory signaling and apoptosis (Hamad, 2010).

Due to the fact that an expanded red cell mass enhances performance by increasing the amount of oxygen delivered to muscle (Castle, 1966), erythropoietin became a household name after its abuse was discovered in athletic events. In addition to its performance-enhancing properties in sport, EPO is already being used to offer a myriad of benefits to a wide range of patients in need. The benefits from rHuEPO therapy include increased exercise tolerance, improved central nervous system function, reduced heart enlargement, reduced extreme fatigue, increased ability to perform daily functions of life, improved coagulation and a reduced risk of alloimmunization in transplant recipients (Foote, 2009).

More specifically, patients with chronic kidney disease often end up with anemia because they are unable to produce adequate amounts of endogenous erythropoietin (EPO) to stimulate red blood cell production. Recombinant human erythropoietin(rHuEPO) enabled physicians to ameliorate anemia in this patient population because it mimics the endogenous hormone (Foote, 2009).

Individuals with cancer often have damaged bone marrow, which can be made worse by the insult of chemotherapy. Compromised bone marrow may not completely

respond to endogenous EPO, and these patients also benefit from rHuEPO because the bone marrow is the major therapeutic target of the recombinant hormone (Molineux, 2009).

In a surgical context, Li, Y et al performed a meta- analysis and systematic review investigating if EPO was a substance that could potentially supplant blood transfusions after significant blood loss following orthopedic surgery, specifically total hip and knee arthroplasty. Allogeneic blood transfusions are common after surgery, and trauma but come with inherent risks that include transmission of infective diseases, and immunologic complications, along with a high cost (Marcucci, 2004). Li concluded that when given preoperatively, EPO generally increased hemoglobin levels during the whole perioperative period. Also EPO reduced the need for transfusion significantly in patients undergoing hip or knee surgery, while not increasing the chance of developing thrombotic complications. Therefore, EPO could be a substitute blood management strategy in total hip arthroplasty and total knee arthroplasty.

It has been over one hundred years since Carnot and DeFlandre first announced that a factor, which they named aemopoietin, was responsible for the regulation of the production of red blood cells (1906). Based on EPO's proven record, and ever-expanding list of uses it only seems logical that researchers and clinicians alike have tested its ability to aid in defect regeneration, more specifically- alveolar ridge preservation. Therefore, the aim of our study was to to evaluate the efficacy of erythropoietin's angiogenic and osteogenic potential compared to two popular ridge preservation techniques; anorganic bovine bone mineral (Bio-Oss®) with non-cross

linked collagen membrane (Bio-Gide®), and collagen membrane alone.

2. MATERIALS AND METHODS

2.1. BILATERAL MAXILLARY FIRST MOLAR EXTRACTION

All animal procedures were approved by and were in compliance with the guidelines provided by the Institutional and Animal Care and Use Committee at Texas A & M University Health Science Center (Approval of AUP IACUC 2017-0103-CD), and researching pertaining to biohazards and toxins was carried out in accordance with Texas A&M institutional biosafety standards. Thirty, male, Sprague-Dawley were used in the study. The rats weighed between 400-500 grams and underwent general anesthesia via a mixture of ketamine (100mg/kg)/xylazine (5mg/kg). The right and left maxillary molars were extracted by a single researcher (TD) by careful elevation with a 7/8 Younger-Good Currette (Hu-Friedy®), after sufficient sedation was confirmed. Next, a #703 fissure bur (Brasseler USA®) was used in the extraction socket to create 3mm deep, uniform defects. The rats were randomly assigned to three different groups. The control groups were to receive no additional material in the extraction socket and the test groups received eukaryotic erythropoietin, 100 micrograms/mL (Biomatik® RPU54825) via a saturated, Salvin® OraPlug Absorbable Collagen Sponge (Salvin® Dental Specialties) 1mm wide and 3mm long. The last group's extraction defects were filled with anorganic bovine bone (Bio-Oss®, Geistlich Biomaterials). All sites were covered with a 1x1mm non cross-linked, resorbable membrane (Bio-Gide®, Geistlich Biomaterials) that was secured in

place with Periacryl Purified Cyanoacrylate Dental Adhesive (Salvin® Dental Specialties). Each of the rats was fed a soft food diet of DietGel® Recovery, (ClearH_2O) for the first two days following surgery. The groups of rats were separated into four- and eight-week time points and were sacrificed on their selected dates via carbon dioxide overdose.

After sacrifice, the rats were beheaded, and their maxillae harvested and hemisected at the mid-palatal raphe. The final, trimmed hemi-maxillae were made up of the anterior portion of the maxilla, the extraction site, and the second and third molars. Of the ten hemi-maxillae in each group, four were randomly assigned to be fixated in liquid nitrogen, and six were assigned to be fixed in 10% formalin for further processing.

2.2. ULTRATHIN GROUND SECTIONS OF RAT HEMI-MAXILLAE

Three hemi-maxillae from each of the four and eight-week-old hemi-maxillae of test and control rats that were fixed in 10% formalin underwent processing as per the EXAKT company standard protocol. The protocol includes a series of various gradients of alcohol, in addition to an ethanol/technovit mixture, and when the samples were in 100% light cure technovit (Technovit 7200, EXAKT) they underwent polymerization and embedding. Next the samples were subjected to gross section with a diamond bandsaw, (EXAKT 300 CP), then they were ground and polished into 30 micrometer sections (Liu et al, 2016). Of the three hemi-maxillae for each time point, two underwent alizarin red and the other was subjected to von Kossa staining.

2.3. ALIZARIN RED STAINING

Samples de-paraffinized to distilled water, stained with Alizarin Red Solution, then dehydrated in acetone and then Acetone-Xylene. Finally, sampled cleared in xylene and mounted with mounting medium (Sheehan, 1980).

2.4. VON KOSSA STAINING

The remaining sample underwent von Kossa staining via hydration, and subsequent rinsing with distilled water, followed by incubation in 1% silver nitrate solution in clear glass under UV light. The sample was rinsed in distilled water again, un-reacted silver was removed with 5% sodium thiosulfate for five minutes. Another rinse. Counterstain sample with nuclear fast red for five minutes. Rinse. Dehydrate via increasing alcohol and xylene, and cover with coverslip and mounting medium (Sheehan, 1980).

2.5. RADIOGRAPHS

All samples fixed in 10% formalin were radiographed and analyzed with a Faxitron MX-20 specimen radiography system (Faxitrono X-ray Corp., IL) at 20kV for twenty seconds.

2.6. MICRO-CT ANALYSIS

Two hemi-maxillae from each group underwent individual imaging and analysis with a Micro-CT 20 Scanco Medical Scanner (Zurich, Switzerland). The samples were scanned in standard resolution mode (~ 10 micrometer resolution in the X,Y,Z axis). X-Ray exposure was 55kVp, with an exposure time of 800ms. Following scanning, 3-D data were made by segmenting specimens at a threshold that accurately displays the desired sections for analysis.

2.7. PARAFFIN SECTIONS

Three hemi-maxillae sections per time group that were initially fixed in 10% formalin were then decalcified EDTA, and subjected to microwave heat at forty degrees celcius for four days. After decalcification, the samples were dehydrated via a graded series of ethanol and xylene. Paraffin fixed samples were then sliced in five micrometer thick sections.

2.8. MASSON'S TRICHROME STAINING

Samples deparaffined and rehydrated with decreasing alcohol concentrations, then washed in distilled water. Yellow color removed with running tap water. Samples stained with Weigter's iron hematoxylin solution for ten minutes. Rinsed again in warm

tap water. Washed in distilled water. Stain via fuchsin solution. Differentiation achieved by phosphomolybdic-phosphotungstin acid solution. Samples moved to aniline blue solution, then rinsed with water then acetic acid. Samples then washed in distilled water, and dehydrated quickly before mounting with mounting medium and coverslip. (Sheehan, 1980)

2.9. HEMOTOXYLIN AND EOSIN STAINING

Sections treated with xylene, and rehydrated with alcohol at decreasing concentrations and washed in distilled water. Samples then stained in hematoxylin, and washed with tap water. Differentiation via acid alcohol and another wash in tap water. Bluing achieved with ammonia water, and washed again, then rinsed in 95% alcohol. Eosin used as counter stain, and dehydration with alcohol, then xylene and final mounting with cover slip and mounting medium (Kiernan, 2008).

2.10. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Reverse transcriptase polymerase chain reaction data was obtained with Takara Bio USA RNA to cDNA EcoDry™ Premix (Double Primed) Cat# 639549 Lot# 1805796A and directions for use were followed from takarabio.com.

2.11. IMMUNOHISTOCHEMISTRY

The immunohistochemistry staining protocol for paraffin sections from Abcam® was followed.

3. RESULTS

3.1 RADIOGRAPHS

Figure 1. shows the selected results from radiographic analysis. Based on qualitative observation, erythropoietin promoted radiopaque fill of the extraction socket that was similar to that of the anorganic bovine bone group. The control group appeared to be the most radiolucent of the extraction sockets.

3.2 VON KOSSA STAINING

Mineralization was evaluated via Von Kossa staining, and selected sections can be observed in Figure 2. The sections appear to resemble the results obtained via radiographic analysis. The control groups exhibited a greater extent of soft tissue ingrowth (shown in amber), and less mineralization (shown in black/brown).

3.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed for the sake of comparing fibronectin organization in the extracellular matrix in each of the samples. Figure 1.3 shows that the anti-fibronectin antibody was present in all of the samples, but erythropoietin was unique due to fibronectin organization into sheaths.

3.4. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Figure 4 shows the results of reverse transcriptase polymerase chain reaction, and the following erythropoietin genes were expressed at a significantly higher rate than in the control group; Collagen I (ColI) , Collagen III (ColIII), Vascular Endothelial Growth Factor(VEGF), Osterix (OSX), Runt-related transcription factor 2 (Runx2), CadherinI (CDH1) , and Fibronectin (FN). The relative gene expression of Interleukin 6 (IL6), and Osteocalcin (OCN) was significantly lower in the erythropoietin group compared to the control group.

3.5. MINERALIZATION AROUND NEW BLOOD VESSELS

Figure 5 is provided in order to highlight new bone formation associated with blood capillaries. The arrows point to blood vessels, and the maroon substance is new bone in Masson's Trichrome. In the Von Kossa image, the arrows point to mineralized material around blood capillaries.

4. DISCUSSION

If the success of alveolar ridge preservation is measured by a given material's ability to provide an environment conducive to revascularization and remodeling of the grafted tissue into vital load bearing bone, then a potent angiogenic hormone like erythropoietin may aid in this procedure for the following reasons. Increased blood vessels lead to increased mineral transport and deposition to healing wound sites. An increase in calcium deposition leads to increased calcium and phosphate interaction with collagen fibrils and apatite crystals extracellularly, which leads to calcium hydroxy apatite formation. Additionally, erythropoietin's action on the master regulating transcription factors Osx and Runx2 allow for heightened osteoblast performance compared to no treatment at all.

5. CONCLUSION

A summary diagram can be seen in figure 6. To conclude, our data show that the hormone erythropoietin acts in two different ways to facilitate new bone formation. Erythropoietin increases relative expression of vascular endothelial growth factor, which enhances capillary and blood vessel formation and leads to increased mineral transport. Erythropoietin also increased relative expression key transcriptions factors for osteoblast differentiation and maturation, Osterix (Osx) and Runt-related transcription factor 2 (Runx2). EPO's effects on angiogenesis suggests that EPO treatment might be useful to promote nutrient supply during the regeneration of large-scale bone defects, especially in combination with traditional scaffolds and bone filling materials.

The combination of the two aforementioned pathways is believed to have led to improved new bone formation in the healing extraction sockets of rats compared to no treatment at all. While freeze-dried bovine bone might not be acceptable to some patients and BMP-2 is associated with side effects, EPO as a bioadditive might provide novel treatment strategies for bone defects, especially since its use has already been approved by the FDA.

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APPENDIX

Figure 1. Radiographic Analysis

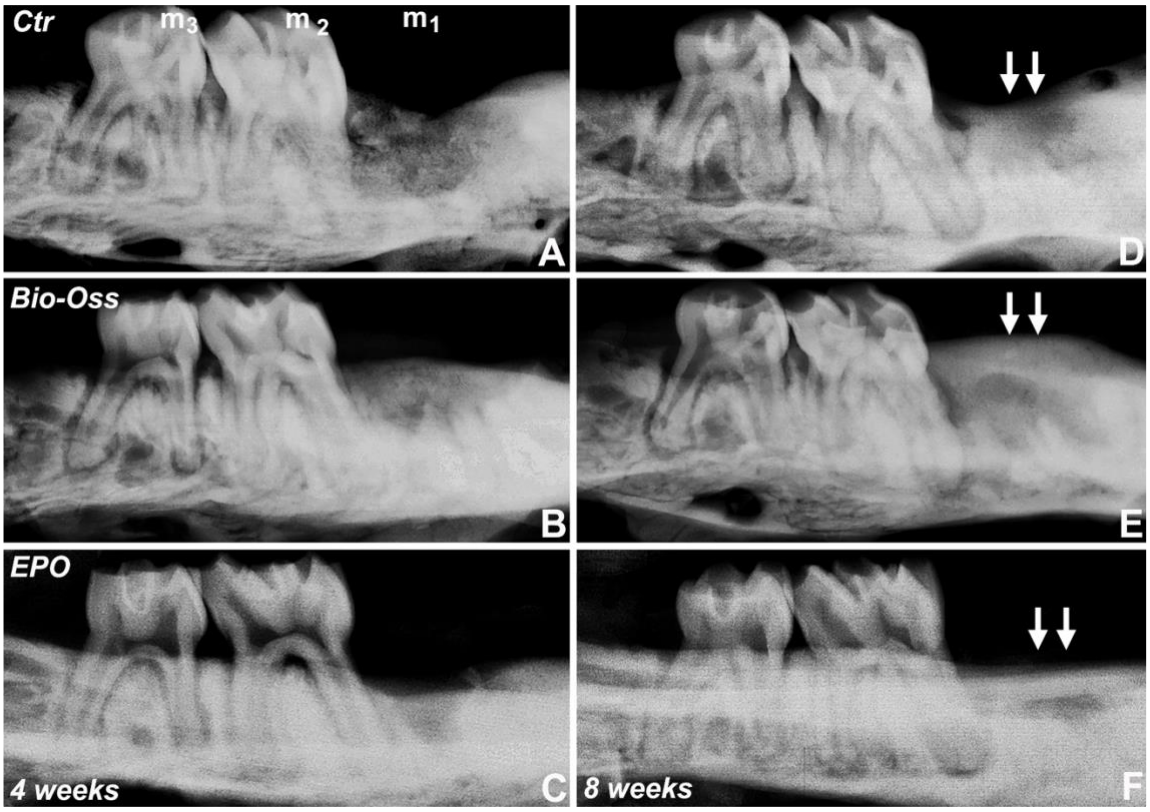

Figure 2. Von Kossa Staining

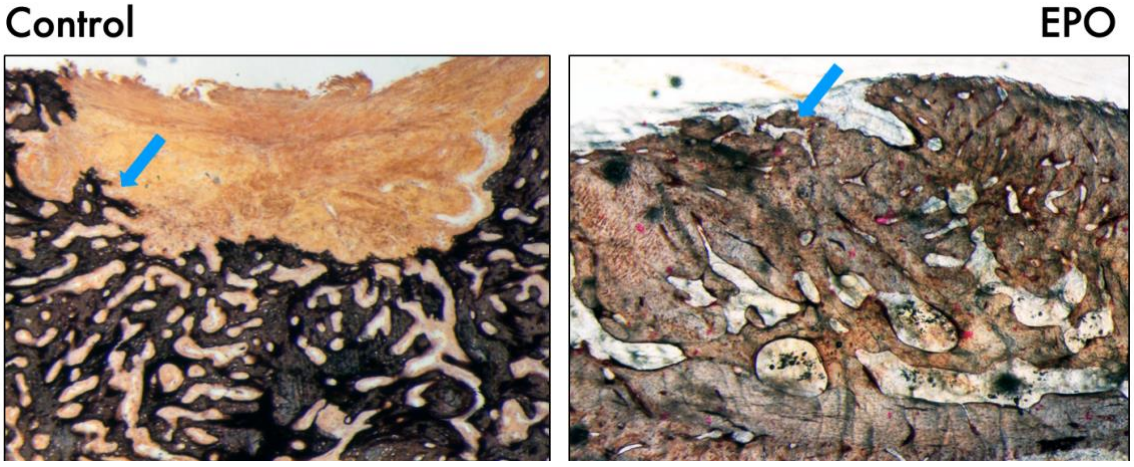

Figure 3. Fibronectin Immunohistochemistry

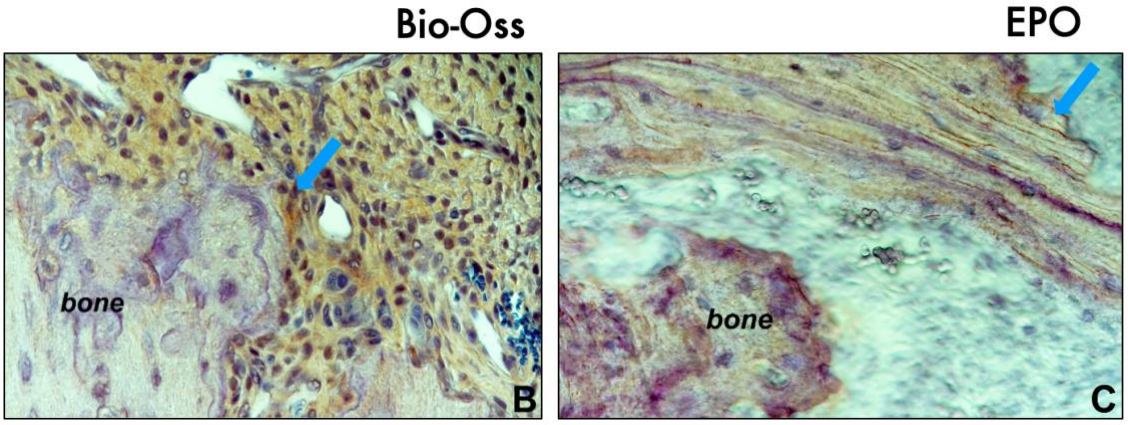

Figure 4. Reverse Transcriptase Polymerase Chain Reaction

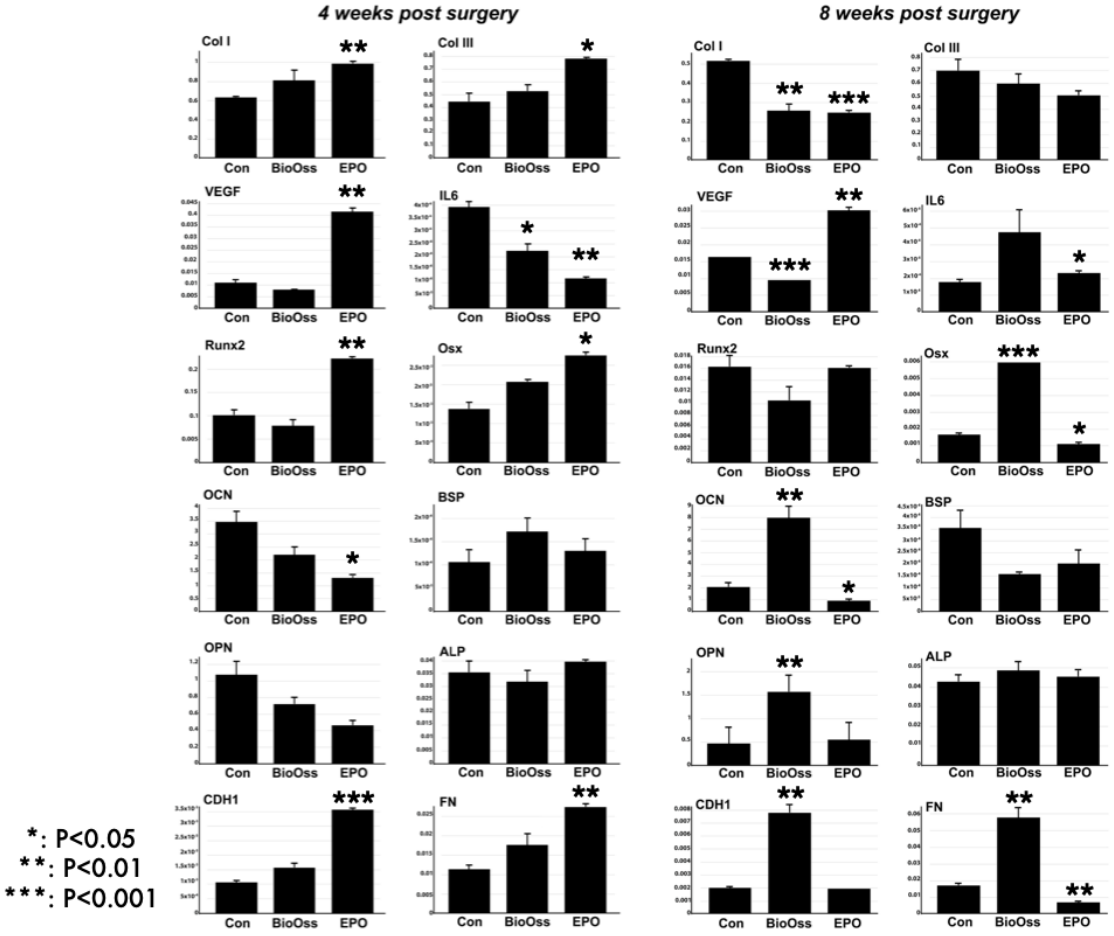

Figure 5. Mineralization Around New Blood Vessels

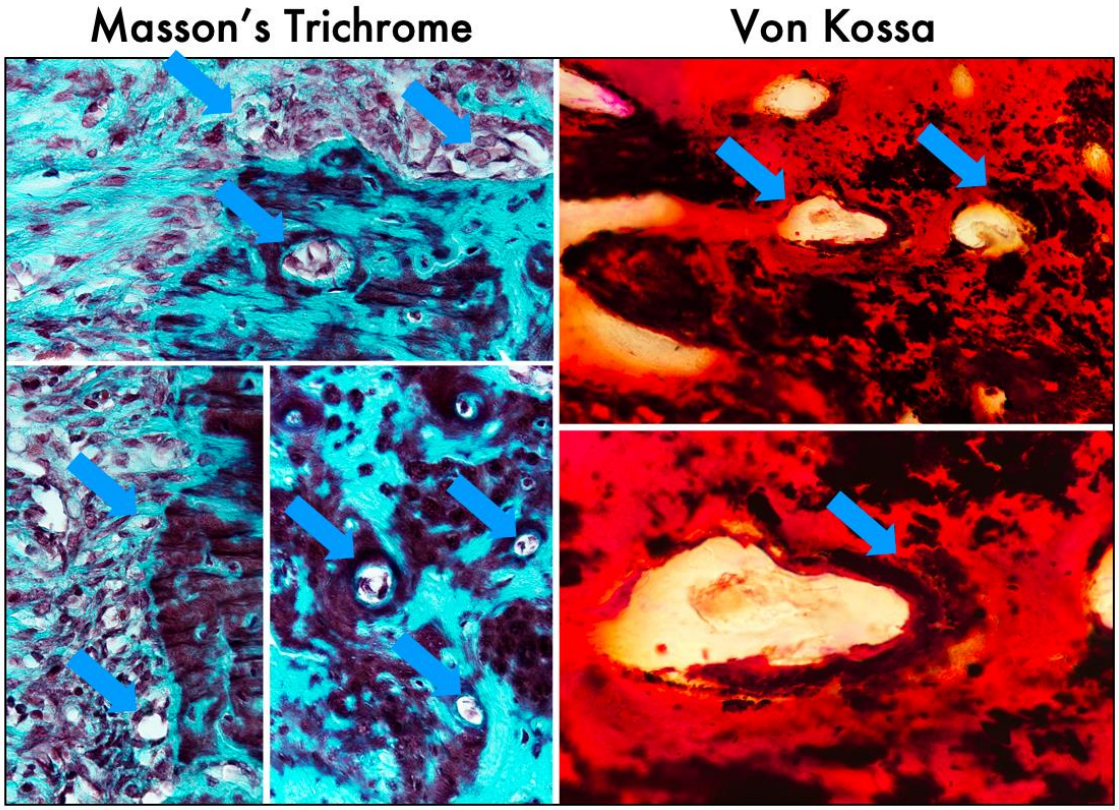

Figure 6. Mechanism For Erythropoietin Induced New Bone Formation