Osseointegration-Pharmacology

By

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This thesis is dedicated to my family for their love, endless support and encouragement

Abstract

Several orthopedic and craniofacial surgical interventions require implant insertion to fix or restore bone functions. The success of these implants relies mainly on osseointegration, a direct functional and structural interlocking between implants and bone. The osseointegration processes around implants are similar to the biological events occurring during bone repair and fracture healing. Dysregulation of any of these biological events is known to have a negative impact on bone healing and implant osseointegration. Some medications are known to interfere with biological processes involved in bone biology. Proton Pump Inhibitors (PPIs) and anti-Vascular Endothelial Growth Factors (Anti-VEGFs) are among these medications.

Proton Pump Inhibitors are over counter drugs taken by millions of patients worldwide for treatment of gastroesophageal diseases. Recent studies have shown that PPIs have a negative impact on bone accrual. Anti-VEGFs are antibodies developed to inhibit angiogenesis in cancer and neo-vascular age related macular degeneration of the eye. Anti-VEGFs inhibit angiogenesis which is an essential process during bone formation, bone healing and osseointegration of implants.

We hypothesized that PPIs and anti-VEGF could have negative effects on bone healing and implant osseointegration. Accordingly, this study was designed to assess the effect of PPIs and anti-VEGFs therapies on bone healing and implant osseointegration in a rat model.

We conducted two *in vivo* experiments to investigate the effects of PPIs (omeprazole) and anti-VEGF therapies on bone healing and implant osseointegration. In both studies,

we followed the same surgical intervention in a rat animal model. Two unicortical bone defects were created in the tibial metaphysis of each rat, in left defect; a custom made titanium implant was placed whereas the right one was left empty.

In the first study, rats were randomly assigned into two groups: omeprazole (n=12) and control (n=12). In the second study, rats were randomly assigned into three groups and received either anti-VEGF neutralizing antibody (n=12), Ranibizumab (n=12), or saline as control (n=12). Findings of the first study revealed that the defect volume was significantly higher (*P=0.009*) in omeprazole treated rats (2.92 $\pm$ 0.62 mm^3) compared to saline treated rats (2.13 $\pm$ 0.32 mm^3). Moreover, the average percentage of osseointegration in omeprazole group (23.3 $\pm$ 10.8 %) were significantly lower ($p<0.0001$) than in the control group (40.2 $\pm$ 13.3 %). Findings of the second study revealed that the mean volumes of the bone defect in the Anti-VEGF (2.48 $\pm$ 0.33 mm^3) and Ranibizumab (2.35 $\pm$ 0.23 mm^3) groups were significantly higher than the controls (2.11$\pm$0.63 mm^3). Furthermore, the average percentages of osseointegration in Anti-VEGF (21.1 $\pm$ 10.2%) and Ranibizumab (18.4 $\pm$ 9.5%) groups were significantly lower than in controls (40.2 $\pm$ 13.3 %).

In conclusion, post-operative administration of omeprazole and anti-VEGFs impaired bone healing and implant osseointegration. Therefore, omeprazole and anti-VEGFs might be potential risk factors for several orthopedic and craniofacial surgical interventions that require implant insertion to fix or replace missing anatomical structures.

Résumé

Plusieurs interventions chirurgicales orthopédiques et cranio-faciales nécessitent une insertion d'implant pour corriger ou rétablir les fonctions osseuses. Le succès de ces interventions repose principalement sur l'ostéointégration, une imbrication fonctionnelle et structurelle directe entre l'implant et l'os. Les processus d'ostéointégration autour des implants sont semblables aux événements biologiques qui se produisent lors de la réparation de l'os et la guérison des fractures. Le dérèglement de l'un de ces événements biologiques est connu pour avoir un impact négatif sur la cicatrisation osseuse et l'ostéointégration de l'implant. Certains agents pharmacologiques peuvent interférer avec des processus biologiques impliqués dans la guérison de l'os. Les inhibiteurs de la pompe à protons (IPP) et les anti-facteur de croissance de l'endothélium vasculaire (Anti-VEGF) font partie de ces médicaments.

Les IPP sont des médicaments sans ordonnance pris par des millions de patients à travers le monde pour le traitement des maladies d'ordre gastro oesophagien. Les études récentes ont montré que les IPP ont un impact négatif sur la croissance de l'os. Les anti-VEGF sont des anticorps développés pour inhiber l'angiogenèse dans le cancer et la dégénérescence maculaire néo- vasculaire de l'oeil liée à l'âge. Cependant, l'angiogenèse est un processus essentiel au cours de la formation des os, la guérison osseuse et l'ostéointégration des implants. Ainsi, l'inhibition de l'angiogenèse par les anti-VEGF peut avoir des impacts négatifs sur la cicatrisation osseuse et l'ostéointégration de l'implant.

Cette étude visait à évaluer l'effet des IPP et des anti-VEGF sur la cicatrisation osseuse et l'ostéointégration de l'implant dans un modèle de rat. Nous émettons l'hypothèse que les

IPP et les anti-VEGF pourraient avoir des effets négatifs sur la cicatrisation osseuse et l'osséointégration de l'implant.

Nous avons effectué deux études in vivo; Nous avons étudié les effets de l' IPP oméprazole et les thérapies anti-VEGF sur la cicatrisation osseuse et l'osséointégration de l'implant. Dans les deux études, nous avons fait suivi la même protocole d'intervention chirurgicale dans un modèle animal de rat. Deux défauts osseux unicorticaux ont été créés dans les métaphyses tibiales de chaque rat. Puis, un implant en titane sur mesure a été placé dans le défaut de gauche alors que le droit a été laissé vide. Dans la première étude, les rats ont été répartis au hasard en deux groupes: l'oméprazole (n = 12) et témoins (n = 12). Dans la seconde étude, les rats ont été répartis au hasard en trois groupes et ont reçu soit de l'anticorps neutralisant l'anti-VEGF (n = 12), ou le ranibizumab (n = 12). Le troisième groupe a servi de témoin (n = 12). Les résultats de la première étude ont révélé que le volume de défauts était significativement plus élevée (P=0,009) chez les rats traités à l'oméprazole (2,92 $\pm$ 0,62 mm^3) par rapport à une solution saline traitée rats (2,13 $\pm$ 0,32 mm^3). En outre, le pourcentage moyen de l'osséointégration dans le groupe d'oméprazole (23,3 $\pm$ 10,8%) étaient significativement plus bas ($p<0,0001$) que dans le groupe témoin (40,2 $\pm$ 13,3%). Les résultats de la seconde étude ont révélé que les volumes moyens de la perte de substance osseuse dans les groupes anti-VEGF (2,48 + 0,33 mm3) et ranibizumab (2,35 $\pm$ 0,23 mm^3) étaient significativement plus élevés que les témoins (2,11 0,63 mm^3). De plus, les pourcentages moyens de l'ostéo-intégration dans Anti-VEGF (21,1 $\pm$ 10,2%) et le ranibizumab (18,4 $\pm$ 9,5%) groupes étaient significativement plus bas que chez les témoins (40,2 $\pm$ 13,3%).

En conclusion, l'administration post-opératoire de d'oméprazole et d’anti-VEGF peut provoquer une altération de la cicatrisation osseuse et de l'osséointégration de l'implant. Par conséquent, l'oméprazole et anti-VEGF peuvent être des facteurs de risque potentiels de plusieurs interventions de chirurgie orthopédique et cranio-faciales qui nécessitent insertion de l'implant pour réparer ou remplacer les structures anatomiques manquantes.

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Authors Contribution and Statement of Originality

This thesis consists of two manuscripts in preparation for publication. The data contained herein was generated from experiments performed by the candidate who also performed the data collection and analysis.

Prof. Faleh Tamimi was the supervisor responsible for the candidate throughout experiment performance and thesis preparation. He provided scientific and technical support, and guidance throughout the time of study.

Prof. Elham Emami was the co-supervisor responsible for the candidate throughout data analysis and thesis preparation. She provided scientific guidance throughout the time of study.

Prof. Jocelyn Feine and Robert Durand provided scientific guidance throughout the time of study.

Hazem Eimar aided in the animal surgeries and micro-CT data analysis.

Mohamed Nur Abdallah aided in animal surgeries.

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Chapter One: Introduction

1.1. Thesis Outline

This thesis includes a literature review introducing the concept of osseointegration pharmacology and two manuscripts addressing this issue, the first one entitled "**Proton Pump Inhibitors Interfere with Bone Healing & Implant Osseointegration"** and the second one entitled "**Emerging risk factors for implant osseointegration: Anti-Vascular Endothelial Growth Factors"**. Both manuscripts are under preparation for publication.

1.2. Research Rationale

Osseointegration and bone healing are crucial factors in the success of several orthopedic and craniofacial surgical interventions (e.g. fixation of orthopedic and craniofacial fractures, bone cancer surgery, joint replacements and dental implant placements). Implant osseointegration and bone healing are strongly influenced by bone metabolism (1-4) and angiogenesis (5). Failures in bone healing and osseointegration can lead to deleterious complications such as pain, infections, functional impairment, implant loss and death (6, 7). Accordingly, pharmacological agents known interfere with bone metabolism could have a negative effect on osseointegration and bone healing. Drugs such as proton pump inhibitors (PPIs) (used to manage gastric acidity) are known to interfere with bone metabolism whereas anti-vascular endothelial growth factors (VEGFs) (used to treat cancer) interfere with angiogenesis (5, 8). Despite distinctive clinical relevance, knowledge about the potential effect PPIs and anti-VEGFs on bone healing and implant osseointegration is scarce.

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Chapter Two: Literature Review

2.1. Bone

Bone is a unique tissue that constitutes the supporting skeletal framework of all higher vertebrates. It is a dynamic structure composed of an organic matrix (30-35%), inorganic calcium phosphate minerals (65-70%) and cells (1). The highly organized structure of bone at many length scales gives rise to diverse mechanical, biological and chemical functions; such as protection to vital organs, structural support and self-repairing properties. Moreover, bone is considered the main reservoir for calcium and phosphate ions and a wide range of cytokines and growth factors. Mechanical strength of bone is maintained throughout life by modeling and remodeling process that undergoes continuously (2).

2.2. Human Skeletal System

The adult human skeletal system is usually composed of 206 bones divided into axial and appendicular skeletons. Generally, bones can be categorized into four groups: long bones (e.g. femur and radius), short bones (e.g. wrist), flat bone (e.g. clavaria) and irregular bone (e.g. mandible and maxillae) (3). The gross structure of long bone can be further subdivided into epiphysis, metaphysis and diaphysis.

- Epiphysis represents the area of bone located between the growth plate and the bone end. Metaphysis is the part located between the growth plates and the diaphysis.
- Diaphysis forms the shaft of long bones and it is composed mainly of cortical bone. This cortical bone encloses marrow and some trabecular bone (4).

At the histological level, bone can be divided into two types of tissues, cortical and trabecular**:**

- Cortical bone, also called compact bone, forms the diaphysis of long bones and the external shell of bone metaphysis and epiphysis (5). Cortical bone is composed of smaller functional sub-units, called the Haversian systems or osteons. Each Haversian system consists of concentric layers, or lamellae, surrounding a central canal known as the Haversian canal. The lamellae contain lacunae occupied by osteocytes, whereas the Haversian canal houses the capillaries and nerves. In addition, there are small canaliculi that connect the osteocytes to each other and large canals (Volkmann's canals) that communicating the Haversian canals to each other (2).
- Trabecular bone, also known as spongy or cancellous bone, is found mostly at the end of long bones and inside the irregular bones (e.g. vertebrae) (5). Microscopically, trabecular bone is composed of tiny small struts that enclose three-dimensional and interconnected pores. These hollow pores provide room for bone marrow that plays an important role in hematopoiesis (the formation of blood cellular components) (6).

2.3. Bone Cells

Bone is composed of three basic cells types: osteoblasts, osteoclasts, and osteocytes:

- Osteoblast is a mononucleated cell of mesenchymal origin that is responsible for new bone formation (7, 8). This cell produces the osteoid (un-mineralized bone matrix) and an enzyme called alkaline phosphatase which facilitates the mineralization process (9).

- Osteoclast is a large, multinucleated, hematopoietically derived cell that is responsible for bone resorption during bone remodeling, growth and healing (10).
- Osteocytes are considered fully differentiated and specialized osteoblasts and represent the most abundant cell type in mature bone (9, 10). Osteocytes are responsible for functional adaptation and maintenance of bone health (9).

2.4. Bone Development

Human skeleton is derived from three different lineages of mesenchymal origin; the paraxial mesoderm, somites and lateral mesoderm. The paraxial mesoderm cells derived from the neural crest are responsible for development of the branchial arches that give rise to the craniofacial skeleton. The somites develop to sclerotomes and become the axial skeleton. The lateral plate mesoderm produces the limb skeleton (11).

During bone development, all the above mentioned changes developed first into initial type of bone called woven bone that is eventually replaced by lamellar bone.

- Woven bone, also called primary bone, forms the fetus skeleton, the growth plates, ear ossicles and ligament attachments. This type of bone is composed of irregular and randomly organized collagen fibers with a relatively high number of osteocytes.
- Lamellar bone, also called secondary or mature bone, forms the majority of human skeleton. This type of bone is composed of regular and densely packed collagen fibers (4).

2.5. Bone Remodeling

2.5.1. Remodeling Mechanism

Bone remodeling can be defined as the dynamic continuous process of bone resorption by osteoclasts followed by new bone formation by osteoblasts (12). Bone remodeling is an essential process for bone maintenance and repair (13, 14). Three different mechanisms are involved in the regulation of bone remodeling: direct interaction between osteoblast and osteoclast (osteoblast-osteoclast coupling), local interaction between immune and bone cells, and systemic control of bone remodeling (14). These mechanisms are discussed in detail underneath:

2.5.1.1. Osteoblast-osteoclast Coupling

This mechanism involves two main mechanisms. First, expression of pro-osteoclastogenic cytokines by the osteoblasts, and second, ephrin ligand and ephrin receptor signaling (15). Two essential pro-osteoclastogenic cytokines are required for differentiation of osteoclasts: the receptor activator of nuclear factor κB ligand (RANKL) and, the macrophages colony stimulating factor. These cytokines are expressed in Osteoblasts and provide the first level of interaction between osteoclast and osteoblast during bone remodeling (15). Ephrin ligands are expressed on the surface of osteoclast progenitors in response to pro-osteoclastogenic signaling. Depending on the ligand involved, ephrin upregulation can stimulate bone formation (e.g. ephrin B2 increases osteoblast differentiation) or bone resorption (e.g. ephrin A2 stimulates osteoclast differentiation and inhibits osteoblast differentiation) (14).

2.5.1.2. Immune System Regulation of Bone Remodeling

Bone remodeling is also influenced by the immune system. It was shown that RANKL, which has a crucial role in osteoclast function, is expressed on several immune cells (e.g. CD8, CD4, T helper (T_H) 1, T_H2) (16, 17). These findings suggested a down regulatory effects of T cells on bone (14). Moreover, T cells can suppress osteoclastogenesis through expression of interferon γ (INF- γ), IL-4 or T lymphocytes protein 4, which in turn suggests a protective effects of T cells on bone (18).

2.5.1.3. Systemic Regulation of bone Remodeling

Many systemic hormonal pathways are involved in the bone remodeling process. Parathyroid hormone, vitamin D, growth hormone, glucocorticoids, thyroid hormone, estrogens, androgen and insulin are hormones known to influence bone metabolism. Parathyroid hormone increases bone turnover and induces bone resorption (19). Vitamin D enhances bone mineralization and suppresses bone resorption (20, 21). Growth hormone stimulates skeletal growth and bone formation directly through stimulation of growth hormone receptors and indirectly through insulin-like growth factor (IGF)-1(22). Glucocorticoids have dual effects on bone; they stimulate bone formation by promoting osteoblast differentiation and maturation. Conversely, they inhibit bone formation by suppressing osteoblast activity (23). Thyroid hormone increases bone turnover and bone loss (24). Estrogen suppresses osteoclast formation and stimulates osteoblast differentiation (25). Androgen maintains skeletal growth, increases bone formation and decreases bone resorption (26). Insulin stimulates bone growth by direct stimulation of osteoblasts and indirect enhancement of estrogen production (27, 28).

2.5.2. Bone Remodeling-Pharmacology

2.5.2.1. Drugs that Negatively Affect Bone Remodeling

Drugs can have adverse effects on bone remodeling which in turn can modify bone accrual. Drugs with negative effects on bone remodeling can induce osteoporosis (29). Five categories of medications are known to induce osteoporosis: drugs targeting hormones, drugs targeting the central nervous system, cardiovascular drugs, drugs targeting the immune system and gastrointestinal drugs (29). Drugs that target VEGFs can interfere with bone remodeling by suppressing angiogenesis.

2.5.2.1.1. Drugs Targeting Hormones

Since bone metabolism is influenced by several hormonal pathways, drugs that interfere with these pathways can have a negative impact on bone homeostasis such as glucocorticoids, thyroid hormones, estrogens, androgens and insulin.

Glucocorticoids are a family of medications used to treat autoimmune diseases. Glucocorticoids affect bone by increasing bone resorption and decreasing bone formation. They also reduce vitamin D plasma level (29-31). Thyroxine is a thyroid hormone used to treat thyroid related conditions such as hypothyroidism and thyroid carcinoma. Thyroid hormones affect bone by increasing bone turnover and decreasing bone mineral density (29). Aromatase inhibitors are used in the estrogen-receptor-positive breast cancer. Estrogen inhibition by aromatase inhibitors increases bone turnover, bone loss and fracture (29). Androgen deprivation therapy is commonly used in the treatment of prostate cancer. This therapy reduces the level testosterone and estradiol which may contribute to increase bone loss (29, 32). Thiazolidinediones are used for

treatment of type II diabetes mellitus. Their use down regulates IGF-1 expression, stimulates osteoclasts and induces bone resorption (29, 33).

2.5.2.1.2. Drugs Targeting the Central Nervous System

The central nervous system is a main regulator of bone metabolism (34). For this reason, neurological drugs such as selective serotonin reuptake inhibitors (SSRIs) and anticonvulsants, can have a negative effect on bone accrual.

SSRIs are widely used in psychiatric conditions, such as depression. Functional serotonin receptors found in osteocytes, osteoblasts and osteoclasts can be activated by SSRIs and alter their function. As a result, SSRI have a negative effect on bone remodeling (35). Anticonvulsants are drugs used to treat epilepsy and other psychiatric conditions. Their use is believed to cause vitamin D deficiency which accelerates bone loss and compromises bone mineralization (36).

2.5.2.1.3. Cardiovascular Drugs

Some of the drugs used for cardiovascular diseases, such as heparin, may have an adverse effect on bone. Heparin is a drug used for treatment of venous embolism and can adversely affect bone by down regulating the expression of osteoprotegerin which leads to decrease bone formation and increases bone resorption (29, 37).

2.5.2.1.4. Drugs Targeting the Immune System

The immune system has an intimate interaction with bone (14, 38). Dysregulation of the immune system by some diseases such as type I diabetes mellitus and inflammatory arthritis, might be associated with bone loss (14, 39). Likewise, drugs affecting the

immune system (e.g. calcineurin inhibitors and antiretroviral drugs) are also associated with bone loss and fracture (29).

Calcineurin inhibitors, such as cyclosporine, are immunosuppressant agents used to reduce the risk of rejection after organ transplantation. Calcineurin inhibitors accelerate bone resorption and increase bone loss (40). Antiretroviral therapies are commonly used to treat (HIV). These drugs increase osteoclastogenesis, induce osteoclastic function and lead to increased bone resorption and loss (29, 41).

2.5.2.1.5. Gastrointestinal Drugs

Proton pump inhibitors (PPIs), are antacid drugs that suppress gastric acidity by inhibiting the proton pump (H+/K+ ATPase) functions. PPIs are the most effective, and the first choice, anti-acid medications for treating gastrointestinal related conditions (42-46). Due to their assumed safety, their use without proper indications has become very popular with about 50-80 % of inappropriate prescriptions (45, 46). However, several adverse effects (e.g. hypomagnesaemia, reduced intestinal absorption of calcium and vitamin B12, community acquired pneumonia, gastrointestinal infections, interference with metabolism of other medications) are associated with PPIs long term use (45, 47, 48). An increased bone fracture risk associated with PPIs use was recently reported by the Food and Drug Administration (FDA) (45). However, the association between increased fracture risk and PPIs use is an area of controversy because the exact underlying mechanism of increased bone fracture risk is still unknown (49).

In humans, PPIs use was shown to be associated with lower bone mineral density (BMD) (50, 51), delay of fracture healing (52) and increased risk of bone fracture (45, 53-55). In

animal models, PPIs decreased bone density, minerals content, cortical thickness, long bones weight and mechanical properties (56). Furthermore, PPIs are associated with reduced bone accrual and expression of bone formation markers such as bone morphogenetic protein (BMP) -2, BMP-4 (57).

The negative effects of PPIs on bone could be caused by the following mechanisms; elevated levels of parathyroid hormone and histamine which may enhance osteoclast differentiation and induce bone loss (58-60).

2.5.2.1.6. Drugs Targeting Angiogenesis

Angiogenesis, the outgrowth of new capillary blood vessels from the pre-existing vessels, is an essential process during bone formation, remodeling, healing and osseointegration of implants (61-64). Angiogenesis is closely controlled by the balance between proangiogenic and antiangiogenic factors and involves the coordination of several growth factors. Vascular endothelial growth factor (VEGF) is considered a key regulator in blood vessels growth (65). During embryogenic development and wound healing process, VEGF has a crucial role in vasculogenesis and restoration of vascular supply (66-68). Blockage of VEGF results in suppression of blood vessels, compromised trabecular formation and growth arrest (66, 69, 70).

Overexpression or up-regulation of VEGFs might enhance bone formation (63, 71). However, up-regulation is also associated with pathologic angiogenesis such as that observed in cancer and chronic intestinal inflammation. In fact, VEGF overexpression was shown to be associated with advanced and distant metastasis of cancer, as well as poor overall survival rate (72-77). For these reasons, anti-VEGFs, antibodies targeting

VEGFs were developed to treat pathological angiogenesis. Anti-VEGFs have proven efficacy in cancer management, and other neovascular diseases such neovascular age related macular degeneration (AMD) as well as resolution of inflammatory conditions (78-80). However, anti-VEGFs not only target the pathological angiogenesis, but they can also affect the physiological one as well. Inhibiting VEGF-dependent angiogenesis and decreasing vascular permeability by some drugs may have a negative impact on bone healing.

Ranibizumab is humanized antibody designed for intraocular use (81). Ranibizumab binds to human vascular endothelial growth factor A (VEGF-A) and inhibits its biological activity (82). Ranibizumab is FDA and EMA (European Medicines Agency) approved for the treatment of neovascular AMD and it has become the standard of care for the therapy of neovascular AMD (83-85). The FDA and the EMA recommend ranibizumab (0.5 mg/0.05 ml) to be administered monthly until maximum visual acuity is achieved (86). Intravitreal anti-VEGF therapies are generally well tolerated, but a variety of side effects may limit treatment outcomes and patient compliance (87). These include inhibition of bone growth, and impairments in wound healing and collateral vessel development, which might be involved in cardiovascular ischemic events, especially in trials using systemic anti-VEGF treatment (88-90). Although intravitreal injections are generally safe, strict adherence to the procedure of injection and standard guidelines are required to ensure a favorable outcome and to minimize the incidence of complications (87). Even though its adverse effects on bone and bone remodeling are still unknown, they should have a negative effect on bone.

2.5.2.2. Drugs that Positively Affect Bone Remodeling

Drugs with positive effects on bone remodeling can improve bone accrual, and are being used to treat osteoporosis. Many medications are approved and known to be effective in treatment of osteoporosis such as bisphosphonates, estrogen replacement therapy, vitamin D, Calcitonin and parathyroid hormone replacements (91).

2.5.2.2.1. Bisphosphonates

Bisphosphonates, bone anti-resorptive therapies, have revolutionized the management of cancer and osteoporosis (a disease characterized by loss of bone mass and impaired bone structure) (92, 93). Bisphosphonates act by inhibiting osteoclastic activity and bone resorption (94). Beside their common side effects (e.g. fatigue, gastrointestinal reaction and mucosal ulceration) atypical bone fractures and osteonecrosis of the jaw are the most significant adverse effects of bisphosphonates on bone (94).

2.5.2.2.2. Estrogen Replacement Therapies

Estrogen replacement therapy is a well-known hormonal medication, used to prevent and treat dementia and osteoporosis in postmenopausal women (95). Estrogen is very effect in reducing age related bone loss and bone fracture risk (96, 97)

2.5.2.2.3. Vitamin D

Vitamin D supplement is also used to treat osteoporosis. Vitamin D increases calcium absorption, improves bone mineral density and may reduce the bone fracture risk (98, 99).

2.5.2.2.4. Calcitonin

Calcitonin is a hormonal therapy, used to treat postmenopausal osteoporosis, hypercalcemia, Paget's disease, and other bone related conditions such as bone metastases. Calcitonin inhibits osteoclasts activity and increases bone menial density (91).

2.5.2.2.5. Parathyroid Hormone Replacement Therapies

Although parathyroid hormone increases bone turnover and induces bone loss, it was shown that intermittent administration of small doses of parathyroid replacement therapy improves bone density (100).

2.6. Bone Healing

2.6.1. Bone Healing Mechanism

Bone healing is the ability of bone to restore its original physical and mechanical properties following fractures, injuries or medical interventions (e.g. bone surgeries) (101). It occurs by primary or secondary bone healing. Primary bone healing is very rare because it requires an intimate contact between broken bone fragments (102). Secondary bone healing, which is the most common healing process, can follow one of the following two possible mechanisms depending on the embryogenic origin of the bone: endochondral ossification (e.g. femoral fracture) or intramembranous ossification (e.g. skull bone defects) (102).

2.6.1.1. Types of Ossifications

2.6.1.1.1. Intramembranous Ossification

In intramembranous ossification, osteoblasts adjacent to the defect area synthesize the bone matrix that later on mineralizes without any intermediate cartilage formation (103, 104).

2.6.1.1.2. Endochondral Ossification

In endochondral ossification, chondrocytes adjacent to the defect area synthesize cartilaginous extracellular matrix that gradually gets calcified and invaded by blood capillaries. Later, osteoblasts start osteoid synthesis and the cartilage is ultimately replaced by bone. This model is characterized by callus formation (104).

2.6.1.2. Stages of Bone Healing

In both types of ossifications, immature woven bone forms first and gets eventually replaced by mature lamellar bone (4). The ossification process occurs in three overlapping stages: inflammatory, repair and remodeling. these stages are detailed underneath (101).

2.6.1.2.1. Inflammatory Stage

Trauma to bone is associated with disruption of the bone cortex, trabeculae and marrow. In addition, it is associated with blood supply interruption leading to extravasation in the defect area (105). However, the extravasation gets confined by the surrounding tissues forming a hematoma. Bone injury activates several pathways of non-specific wound healing (106). Polymorphonuclear neutrophils, macrophages and mast cells are the first cells colonize the healing area (101, 107). Degranulated platelets, lymphocytes,

monocytes and macrophages release different molecules including cytokines and growth factors (108, 109). These factors have a regulatory role on the cellular response during bone healing process which is discussed later on in detail in the **signaling molecules in bone repair** section. Granulation tissues and neo-vascularization (formation of new bold vessels) develops later in the inflammatory stage (1).

2.6.1.2.2. Repair Stage

During this stage, fibroblasts begin secreting stroma that facilitates capillary in-growth. Furthermore, osteoblasts synthesize and release osteoid leading to the formation of a soft callus that gets ossified into a hard callus made of woven bone. The hard callus eventually bridges the fracture (101).

2.6.1.2.3. Remodeling Stage

Bone remodeling is the final stage of the bone healing process in which irregular woven bone is replaced by regular lamellar bone; thus, restoring the original bone anatomy and function. The remodeling stage during bone healing is similar to the ongoing physiological bone remodeling process in the healthy skeleton.

2.6.2. Bone Healing Pharmacology

Even thou the bone healing process requires healthy bone (110) and despite the plenty of facts about the adverse effects of many drugs on bone, only view drugs are considered risk factors for bone healing. Non-steroidal anti-inflammatory drugs NSAID) and corticosteroids are well-known medications that impair bone healing (111).

2.6.2.1. Non-steroidal Anti-inflammatory Drugs

NSAIDs are widely used drugs to relive pain and inflammation especially after bone surgeries. However, many studies have reported their negative effects on bone healing. NSAIDs influence prostaglandin synthesis, and prostaglandin plays an important role in bone metabolism (112).

2.6.2.2. Corticosteroids

Corticosteroids effects on bone were reviewed under drugs targeting hormone system.

2.7. Signaling Molecules in Bone Repair

Three categories of signaling molecules are involved in the bone repair process: Pro-inflammatory cytokines, growth factors and angiogenic factors:

2.7.1. Pro-inflammatory cytokines

Pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have a key role in the initiation of the healing process. These factors that are up regulated during the bone healing process stimulate inflammation, fibrogenic cells chemotaxis, extracellular matrix synthesis and neo-vascularization (103, 113).

2.7.2. Growth Factors

Growth factors have well established roles in the bone repair process. These factors are poly peptides that are usually stored in bone extracellular matrix and are released upon activation by bone injury (114). Bone morphogenetic proteins (BMPs), insulin-like

growth factors (IGFs), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) are the main growth factors involved in bone repair through different pathways (115). Each growth factor has multiple pathways that affect the bone repair process, although often more than one growth factor may be involved in the same pathway. For example, BMPs stimulate chemotaxis, proliferation and differentiation of osteoprogenitor cells, as well as differentiation of osteoblasts (114, 116). IGFs regulate the effect of growth hormones on bone, stimulate osteoblast proliferation, and modulate proliferation and differentiation of osteoprogenitor cells (114). FGFs regulate vascular growth, stimulate chondrocyte maturation, osteoblast proliferation and differentiation, inhibit apoptosis of osteoblasts and induce apoptosis of mature osteocytes (114, 116). PDGFs enhance chemotaxis, proliferation and differentiation of osteoprogenitor cells (114, 116, 117). TGF-βs induce chemotaxis of osteoblast precursors, differentiation of osteoblasts and chondrocytes, and production of bone matrix (114, 117).

2.7.3. Angiogenic Factors

Angiogenic factors, such as vascular endothelial growth factors (VEGFs), VEGFs are key regulators of angiogenesis and play an essential role in the restoration of vascular bone supply during the tissue repair process (67, 68). Following bone injury and blood clot formation, platelets release several cytokines and growth factors, including VEGF-A, that attract inflammatory cells and mediate the chemotactic response. Also, it has been demonstrated that VEGFs stimulate osteoblast chemotaxis and differentiation, and recruit osteoblastic progenitor cells (118). Indeed, several studies showed the effectiveness of VEGFs on bone formation and bone tissue engineering models (119).

2.8. Osseointegration

Osseointegration is defined as a direct functional and structural interlocking between alloplastic materials and bone. This concept was first introduced by Branemark to describe the integration between Ti implants and bone. In 1981, Alberktsson defined osseointegration as an intimate contact between bone and implant without any tissues interposed between them at the light microscope level. Success in implants placed in bone is highly dependent on osseointegration (120-123).

Endo-osseous implants have revolutionized orthopedic and craniofacial reconstructive surgeries. Nowadays, osseointegrated implant devices are being placed for many applications such as replacement of missing teeth, fixation of bone fractures, restoration of missing facial structures, and joint replacements (124). In the United States, about 100,000 to 300,000 dental implants and more than 300,000 prosthetic joints are placed yearly (125-127).

Following their surgical placement, implant osseointegration involves two stages, primary and secondary stability.

i. Primary stability is gained by initial mechanical interlocking between bone and implant.
ii. Secondary stability is gained by bone apposition and remodeling around the implant (120).

Osseointegration of synthetic materials in bone is a very sensitive process that requires several conditions to succeed. Requirements of implant osseointegration include (120):

i. Minimal traumatic surgical procedure.
ii. Initial implant stability and elimination of micro motion.
iii. Infection free environment.
iv. Bio-compatible implant materials (e.g. Ti, Nb).
v. Healthy bone.

During the surgical installment of an implant, the implant screw-form is inserted into a prepared bone bed (cavity). The implant bone bed is similar to a common bone wound, and the mechanisms underlying the healing process around implants are physiologically similar to those occurring during bone fracture healing (128). Distant osteogenesis and contact osteogenesis are two phenomena described by Osborn and Newesley to elucidate the bone healing around implants. In distant osteogenesis, new bone formation starts at the old bone surface and approximates the implants and it occurs at the cortical bone area of bone-implant inter-phase. In contrast to distant osteogenesis, new bone formation in contact osteogenesis starts at the surface of the implant and it occurs at the trabecular part of the inter-phase (3, 128, 129).

Bone healing around implants involves activation of different biological events including, thrombosis, inflammation, granulation tissue formation, neovascularization and bone formation (130). This healing process comprises five overlapping phases (131, 132):

i. Inflammation.
ii. Homeostasis.
iii. Proliferation.
iv. Wound healing

v. Bone remodeling.

Inflammatory and homeostasis phases start immediately following the surgical trauma caused by implant insertion. In the homeostasis phase, matrix protein and growth factors are released. Vasoactive substances such as serotonin and thromboxane play an important role in vasoconstriction. Cytokines, such as interleukin-1, interleukin-6 and tumor necrosis factor, are secreted by lymphocytes to induce the inflammatory process. During the inflammatory phase, macrophages release VEGFs and other growth factors that stimulate angiogenesis and osteogenesis (133). Following resolution of acute inflammation, granulation tissue starts forming during the proliferative phase. This phase is characterized by new extracellular matrix formation and angiogenesis which is a prerequisite for the formation of new bone (132).

The bone wound healing phase starts one to two weeks following implant insertion. In this phase, the woven bone starts forming at the surface of the implant. Subsequently, bridging between implant and the bone bed takes place in about four weeks following implant placement (133).

2.8.1. Osseointegration Risk Factors

Osseointegration could be influenced by systematic and local risk factors (134-140). Several medical conditions and medications are considered risk factors for implant therapy (141). According to the guidelines provided by Buser and coworkers (142) in the second ITI consensus Conferences, the general medical/systemic risk factors for osseointegration include very high and significant risk factors. These risk factors are detailed underneath.

2.8.1.1. Very High Risk Factors

Very high risk factors include:

i. Serious systemic disease such as rheumatoid arthritis, osteomalacia, osteogenesis imperfecta.
ii. Immunosuppressive medications such as corticosteroids, oncologic chemotherapy or other immunosuppressive drugs, and immunosuppressive diseases such as HIV.
iii. Drug and alcohol abuse.
iv. Noncompliance patients.

2.8.1.2. Significant Risk Factors

Significant risk factors include:

i. Irradiated bone.
ii. Severe and uncontrolled diabetes.
iii. Bleeding disorders or drug-induced anticoagulation.
iv. Heavy smoking.

2.8.2. Osseointegration Pharmacology

Although the osseointegration process is dependent on good bone health (110) and despite the abundance of evidence about the adverse effects of many drugs on bone, only some drugs are considered risk factors for implant osseointegration such as corticosteroids and immunosuppressive drugs (143-146). It is apparent from ITI guidelines that many drugs that have been known to affect bone have not been assessed yet as potential risk factors for osseointegration

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Chapter Three: Hypothesis and Objectives

3.1. Hypothesis

The general hypothesis of this thesis was that drugs that interfere with biological processes involved in bone metabolism and angiogenesis could negatively affect bone healing and implant osseointegration. The specific hypotheses of the thesis are detailed underneath:

i. Drugs that interfere with bone metabolism such as PPIs (omeprazole) could have negative effects on bone healing and implant osseointegration.
ii. Drugs that interfere with angiogenesis such as anti-VEGFs could have negative effects on bone healing and implant osseointegration.

3.2. Objectives

This thesis had two main objectives:

i. To investigate the impact of PPIs on bone healing and implant osseointegration.
ii. To investigate the impact of anti-VEGFs on bone healing and implant osseointegration.

Chapter Four: Proton Pump Inhibitors Interfere with Bone Healing & Implant Osseointegration

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4.1. Abstract

Background: Recent studies have shown that proton pump inhibitors (PPIs) (omeprazole), an over counter drugs taken by millions of patients for treatment of gastroesophageal conditions have a negative impact on bone accrual. Accordingly, we hypothesized that omeprazole could has a negative impact on bone healing and implant osseointegration. This study was designed to investigate the effect of the post-operative omeprazole on bone healing and implant osseointegration in a rat model.

Methods: Twenty four Sprague Dawley rats were anesthetised and two unicortical defects were created in both the right and left tibial metaphysis of each rat. A custom made titanium implant was placed in the left metaphysis while the right defect was left empty. After surgery, rats were divided equally into two assigned groups and treated daily by either omeprazole (5 mg/Kg) or saline (0.1 ml). After two weeks of treatment, rats were euthanized and the tibias were assessed for bone healing and osseointegration using micro-CT and histomorphometry, respectively.

Result: Micro-CT analysis revealed that the defect volume was significantly higher (*P=0.009*) in omeprazole (2.92 ± 0.62 mm^3) treated rats compared to saline (control) (2.13 ± 0.32 mm^3) treated rats. Moreover, histology and histomorphometry analysis demonstrated that the average percentage of osseointegration in omeprazole group (23.3 ± 10.8 %) were significantly lower ($p<0.0001$) than in the control group (40.2 ± 13.3 %).

Conclusion: Omeprazole have a negative effect on bone healing and implant osseointegration, and thus omeprazole might be a potential risk factor for implant therapy and bone surgery in craniofacial and orthopedic surgery.

Keywords: proton pump inhibitors, PPIs, osseointegration, bone healing, omeprazole.

4.2. Introduction

Proton pump inhibitors (PPIs) are a group of drugs that suppress gastric acidity by inhibiting functions of the proton pump (H+/K+ ATPase) (1-3). PPIs are the most effective anti-acid medications for upper gastrointestinal acid-related diseases. Accordingly, PPIs are considered the first choice and most commonly prescribed drug for treating peptic ulcer, dyspepsia, Helicobacter Pylori infection, eosinophilic esophagitis gastrinomas, stress gastritis and gastric adverse effects of other medications such as nonsteroidal anti-inflammatory drugs (NSAID) (4, 5). Their extensive use in medicine as well as in surgery (6-8) is spreading fast due to their effectiveness and safety (8). In the United States, more than 113 millions PPI prescriptions are filled per year (9). In Australia, PPIs are the third most commonly prescribed drugs (6). In the Netherlands, around one quarter of population use PPIs (10). Due to their unique effectiveness in preventing gastroesophageal side effects of NSAIDs, PPIs are commonly prescribed after surgeries to overcome the side effects of NSAIDs (11, 12).

Although PPIs are well tolerated, many case-control studies, cohort studies and meta-analysis suggest an association between PPIs use and lower bone minerals density (BMD), delay of fracture healing and increase risk of bone fracture in adults and infants (8, 13-17).

In agreement with clinical studies, recent *in vivo* studies have shown that PPIs decreased bone density, minerals content, cortical thickness, weight and bio-mechanical properties (18). Furthermore, PPIs are associated with reduced bone accrual and expression of bone formation markers such as bone morphogenetic protein (BMP) -2 and BMP-4 (19).

Several orthopedic and craniofacial surgical interventions (e.g. fixation of orthopedic and craniofacial fractures, bone cancer surgery, joint replacements and dental implant placements) require implant insertion to fix or replace missing anatomical structures. Success of these interventions relies mainly on the integration between bone and implant (osseointegration), which is strongly influenced by bone metabolism (20-23). Failures in osseointegration and bone healing can lead to deleterious complications such as pain, infections, functional impairment, implant loss and even death (24, 25).

Despite the negative effects of PPIs on bone metabolism, no studies assessed their potential negative effects on bone and implant surgery. Since bone healing and implant osseointegration are strongly dependant on bone metabolism, we hypothesized that post-operative administration of PPIs might have negative effects on bone healing and implant osseointegration. Accordingly, this study was designed to assess the effect of post-operative administration of omeprazole (a commonly prescribed PPI drug (26)) on bone healing and implant osseointegration in a rat model.

4.3. Materials and Methods

After obtaining the ethical approval from McGill Ethics Board Committee (# 2012-7269), this experiment was conducted on a group of twenty four female, 10 week old Sprague-Dawley rats (Charles River Laboratories, Montreal, QC) weighing between 200 to 250 g. The rats were caged in a controlled environment at 22 C with 12-hour light/dark cycles. A rodent breeding diet and water were provided ad libitum. All rats were allowed to acclimatize to this environment for 2 weeks prior to experimentation.

4.3.1. Surgical Procedure

The rats were anesthetized with isoflurane (3-5% at induction time and 2-2.5% during the maintenance period); the limbs were shaved, disinfected with chlorhexidine scrub and covered with a sterile drape. A longitudinal skin incision was made over the proximal medial diaphysis, and the anterior tibial muscle was divided. The medial surface of the tibia was exposed, and the periosteum was preserved. A unicortical defect was created using a cylindrical bur (1.5 mm ø) adapted to a handpiece drill (Stryker, Hamilton, ON) under constant saline irrigation. A custom made titanium implant (1.5 mm ø x 2.0 mm in depth) was placed in the left metaphysis defect (Fig. 4.1,A). The same procedure was repeated in the right tibia but the defect was 2.5 mm in diameter and was left empty (Fig. 4.1,B). Incisions were closed using 5-0 monocryl sutures.

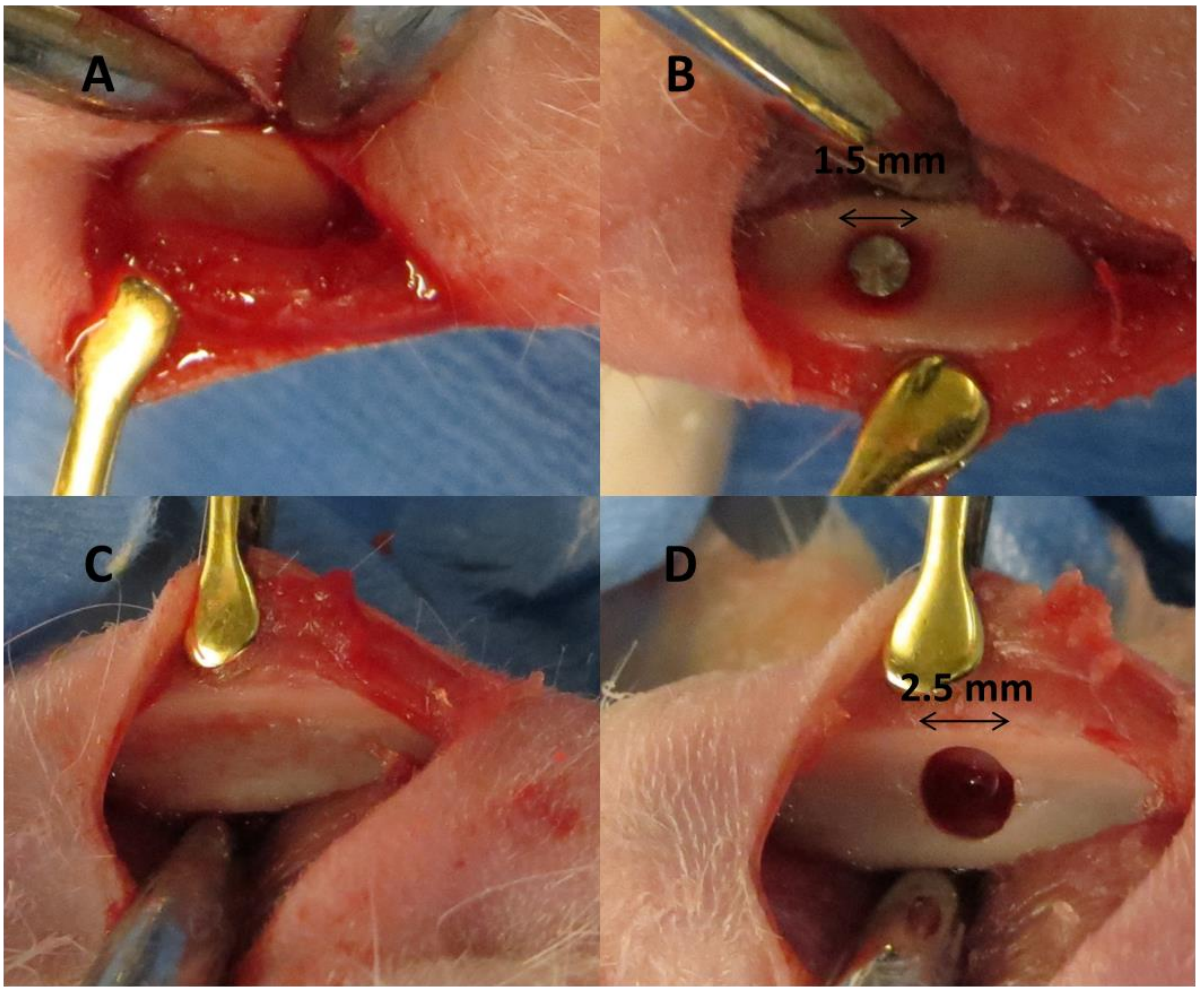

Figure 4.1: A: Rat left tibia metaphysis exposed before implant placement. B: 1.5 mm ø x 2 mm Ti implant placed in tibia metaphysis. C: Rat right tibia metaphysis exposed before creating the bone defect. D: 2.5 mm ø unicortical bone defect.

4.3.2. Post-Operative Care

For analgesia, Carprofen (Pfizer Animal Health, Montréal, QC) was injected (5-10 mg/kg) subcutaneously (SC) to the rats 30 minutes prior to surgery and 24 hours post operatively for 3 days. After the intervention, the rats were randomly assigned into two groups. The first group received a daily injection of omeprazole (10 ml/kg, 5 mg/Kg)

while the second group received a daily injection of saline (0.1 ml) to serve as control. Rats were euthanized at day 14 using CO_2 asphyxiation, and the tibias were retrieved and preserved in 10% neutral buffered formalin (Richard Allan Scientific, Kalamazoo, MI).

4.3.3. Micro-CT Analysis

Right tibias (with empty defect) were scanned using a micro-CT (SkyScan1172; SkyScan; Kontich, Belgium) set at a resolution of 12.7 µm, a voltage of 50 Kv, a rotation step of 0.5, a random movement of 10 and an Aluminum filter of 0.5 mm. The region of interest (ROI) was determined by including all cortical bone of the original defect area. The volume of the defect was determined by subtracting the bone volume from total volume of the ROI. Additional parameters such as trabecular pattern factor, number and separation were calculated.

4.3.4. Histology & Histomorphometry

All left tibia samples (tibias with implants) were dehydrated in ascending concentrations of ethanol (70%,80%, 85%, 90%, 95% and 100%) and infiltrated with polymethyl methacrylate histological resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Germany). After polymerization, samples were sectioned into histological slides using a diamond saw (SP1600, Leica Microsystems GmbH, Wetzlar, Germany). Histological sections were stained using basic fuchsine and methylene blue for histological analysis under an optical micro-scope (Carl Zeiss Microscopy, GmbH, Germany). Histomorphometry was performed using ImageJ software (Wayne Rasband; National Institute of Health,

Bethesda, Maryland). Implant osseointegration was calculated by dividing the bone-covered implant perimeter by the total implant perimeter.

4.3.5. Statistical Analysis

Descriptive statistics were conducted and the normal distribution of the data was tested using the Kolmogorov–Smirnov test. Data were analyzed for significant differences using Student's *t* test. Data analyses were carried out using Origin 8 software (Origin Lab Co., Northampton, MA). Statistical significance was set at $P < 0.05$.

4.4. Results

Micro-CT analysis revealed that the volume of the bone defect was higher (*p=0.*009) in the omeprazole treated rats (2.13 $\pm$ 0.32 mm^3) compared to the saline treated group (2.13 $\pm$ 0.32 mm^3) Trabecular number was lower (*P=0.034*) in the omeprazole treated rats (0.343 $\pm$ 0.094 1/mm) than in the saline treated rats (1.26 $\pm$ 1.21 1/mm). Moreover, trabecular separation was higher (*P=0.030*) in the omeprazole treated rats (1.09 $\pm$ 0.033 mm) compared to the control (0.6 mm $\pm$ 0.420 mm) (Fig. 4.2 & 4.3). Trabecular pattern factor was higher (*P=0.0076*) in the omeprazole group (0.483 $\pm$ 2.01 1/mm) compared to control group (-0.605 $\pm$ 6.87 1/mm). Histology and histomorphometry revealed a significant reduction (*p<0.0001*) in osseointegration among omeprazole group (23.3 $\pm$ 10.8 %) compared to the control group (41.8 $\pm$ 12.4 %) (Fig. 4. 4).

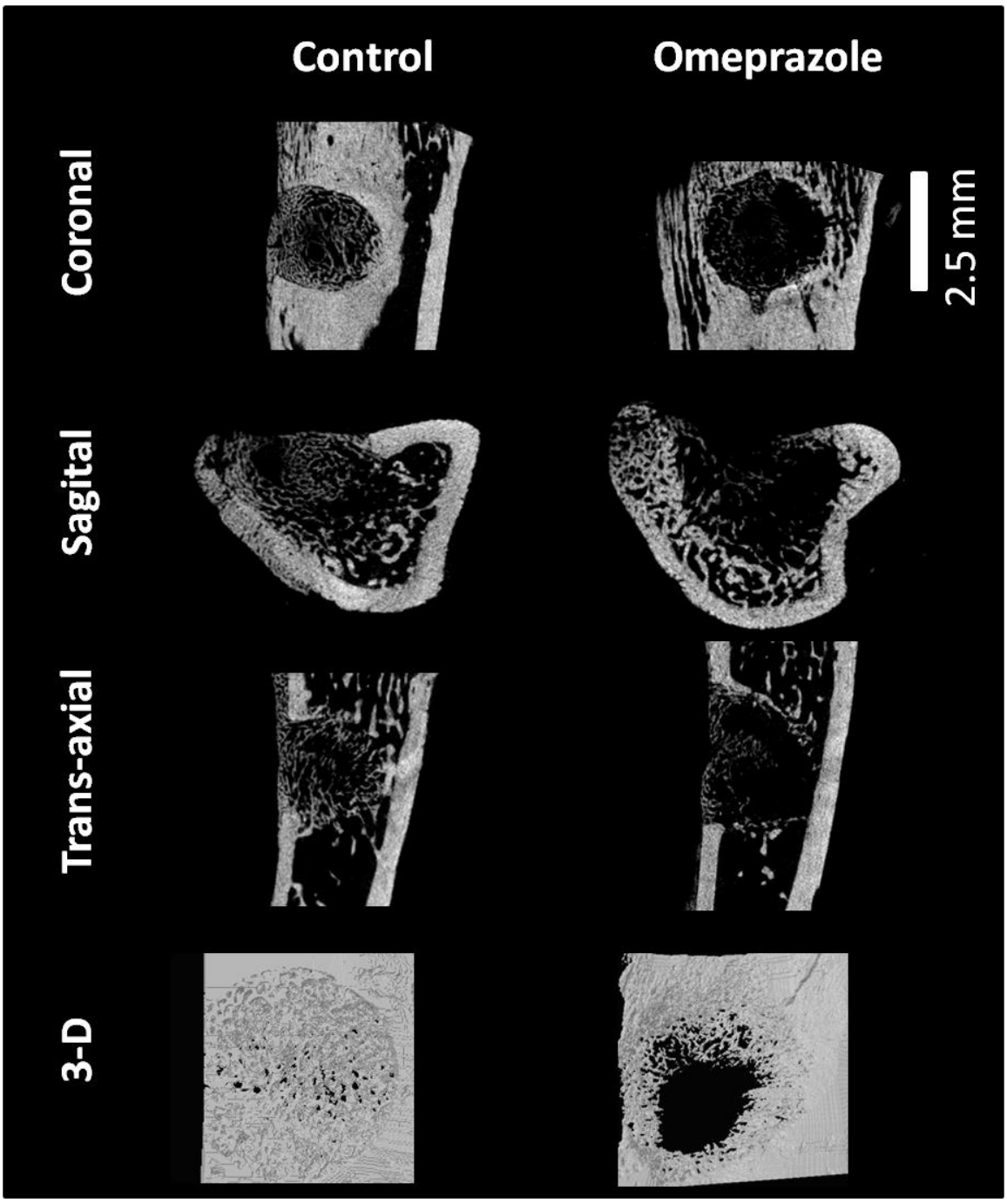

Figure 4.2: Coronal, sagittal, trans-axial and 3-D reconstruction of the micro-CT of bone defects show compromised healing in omeprazole treated rats compared to the controls.

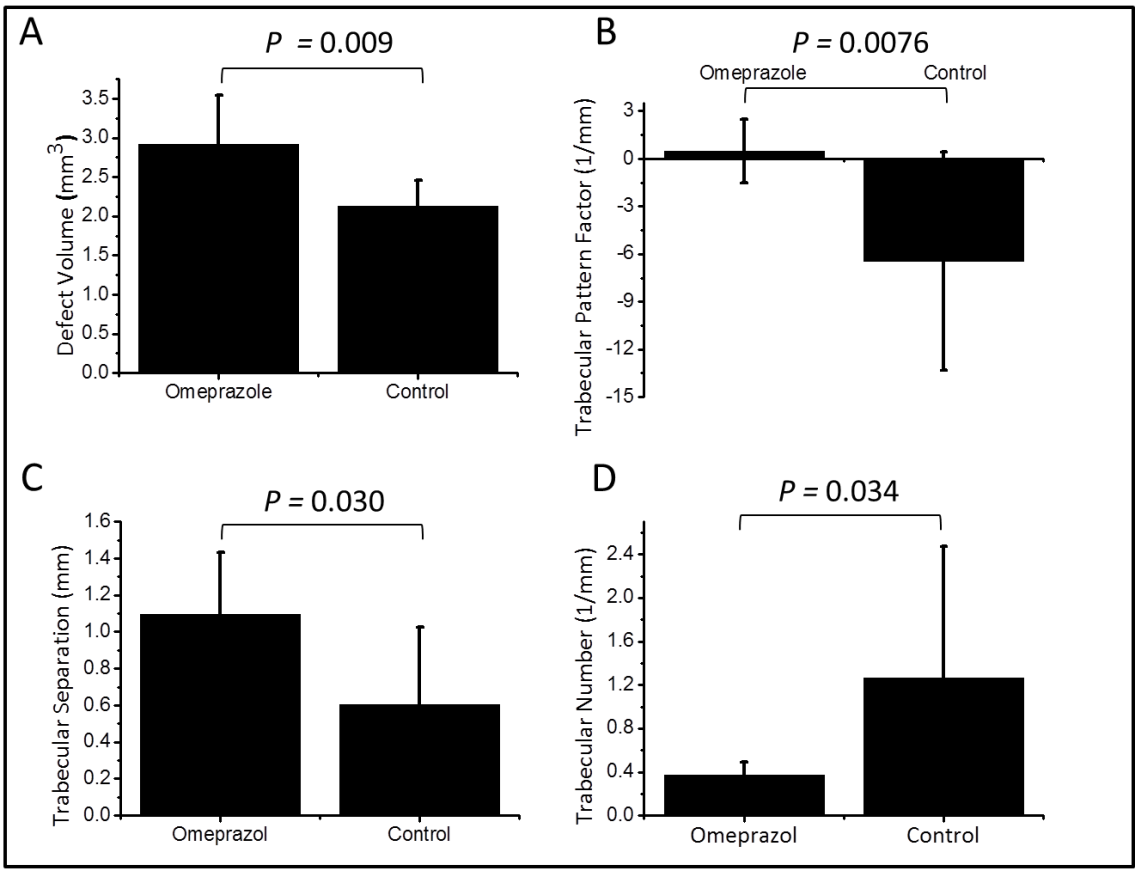

Figure 4.3: Micro-CT data analysis of bone defects in omeprazole treated rats compared to control group: A. Defect volume, B. Trabecular number, C. Trabecular separation D. Statistical analysis by Student's *t*-test, $n_{(omeprazole)} = 12$, $n_{(control)} = 12$.

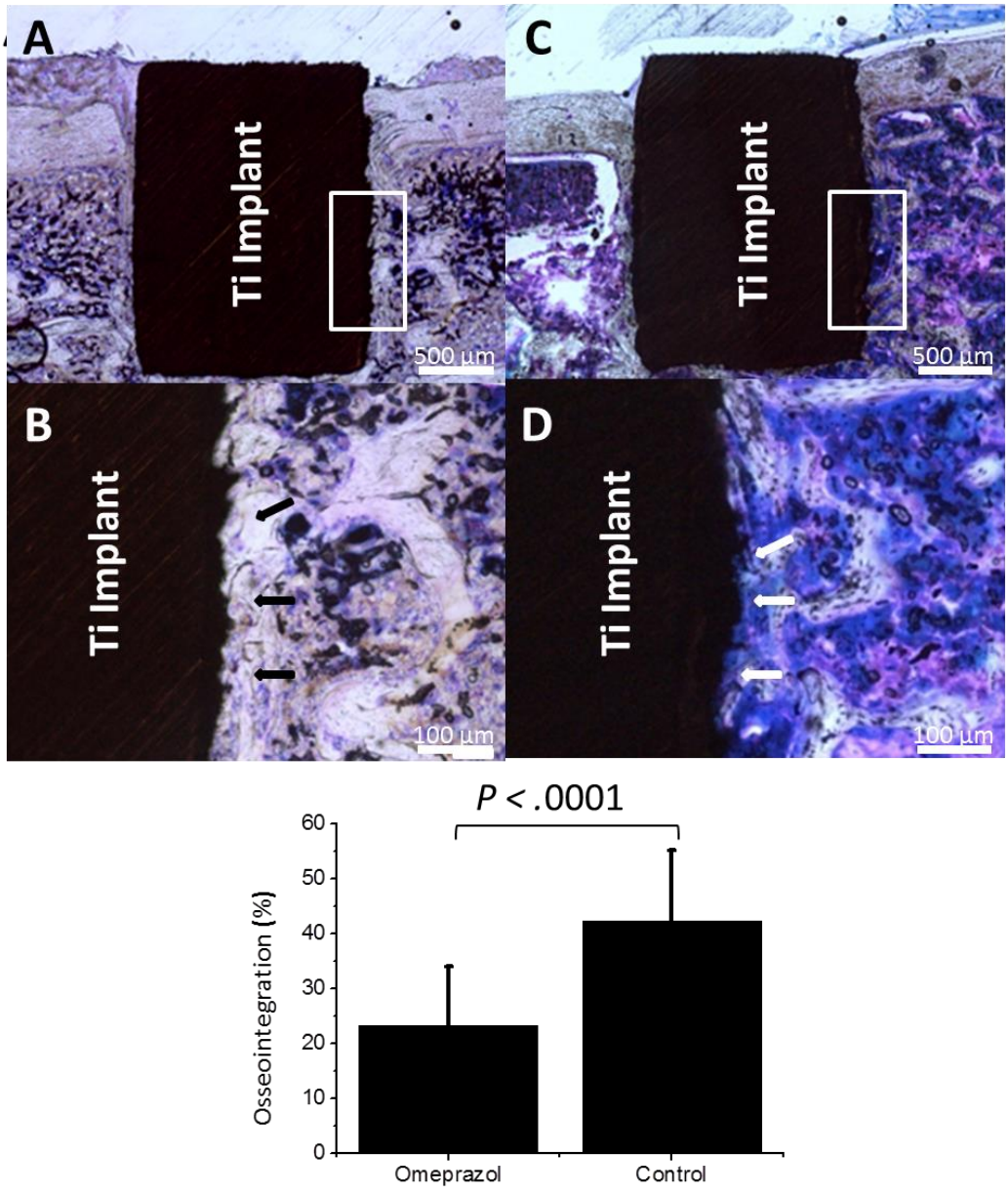

Figure 4.4: A: Sagittal histological section of Ti implant in tibia of control rat. B: Higher magnification (10 X) of Ti implant in tibia of control rat, black arrow indicates bone grown on the implant surface. C: Sagittal histological section of Ti implant in tibia of omeprazole treated rat. D: Higher magnification (10 X) of Ti implant in tibia of omeprazole treated rat, white arrow indicates soft tissue forming on surface of the implant. E: histomorphometric analysis of osseointegration was presented as mean ± standard deviation. Statistical analysis by Student's *t*-test, $n_{(omeprazole)}$ =12, $n_{(control)}$ =12.

4.5. Discussion

In this study we provide evidence of the previously unexplored effects of omeprazole on bone healing and implant osseointegration. We showed that post-operative omeprazole impairs bone healing and implant osseointegration in rats.

Micro-CT analysis of bone defect indicated increased trabecular pattern factor and trabecular separation and decreased trabecular number among omeprazole-treated rats compared to saline-treated rats. This indicated that omeprazole impairs bone quality and delays bone maturation during bone healing in the bone defect. The negative effects of omeprazole may be related to decreased expression of growth factors (BMP-2 and BMP-4) (19, 27, 28), increased histamine level (29), or reduced calcium absorption (30-32). Although future studies would be needed to understand the mechanism behind this phenomenon.

Our study showed that omeprazole had a negative effect on osseointegration. The process of osseointegration around implants is very similar to those occurring during bone repair and fracture healing remodeling. Since we observed a delayed healing, it was expected to have lower osseointegration (33), probably because omeprazole had negative effects on bone healing.

In our study, we used machined Ti implants and we found an average of 41.8 % of osseointegration among control group that is consistent with other studies using similar materials (34). However, we found significant reduction in osseointegration of about 45 % in rats treated with omeprazole. A reduction in osseointegration of this magnitude is known to have a significant effect on mechanical fixation of Ti implants (34). Although

the clinical relevance of this impairment in osseointegration should be further investigated, it is well known that this amount of reduction in osseointegration could increase the risk of failure of osseointegrated implants by up to three folds (35).

Orthopedic and craniofacial interventions that require implant or bone surgeries are very common. In the US, about 100,000 to 300,000 dental implants and more than 300,000 joints are replaced yearly (36-38).The number of these surgical interventions are increasing dramatically, e.g. in the United States, the number of hip and knee replacement surgeries will exceed the 4 million annually by 2030 (39). NSAIDs are frequently prescribed following bone surgeries and implant placements (11). NSAIDs expose patients to higher risk of developing gastric adverse effects; for instance, around one quarter of NSAID chronic users develop peptic ulcer. PPIs can be used to avoid or treat these adverse effects (12); therefore, they are frequently prescribed along with NSAID following bone and implant surgeries. Our results clearly suggest that future clinical studies are needed to further investigate the effects PPIs on bone healing and implant osseointegration in humans, also alternatives to PPIs (e.g. histamine receptor-2 blockers) might have to be considered in patients treated with implants (40, 41).

4.6. Limitations and Future Directions

In this study, we assessed the effect of omeprazole on bone healing and implant osseointegration on tibias in rats. Further investigations will have to be performed to extrapolate our results to other bones (e.g. jaw, femur) and to human, although we expect similar effects due to generalized effects of PPIs on bone metabolism (41).

One limitation in this study was that the effects of omeprazole on bone healing and implant osseointegration were assessed at single time point following post-operative administration of omeprazole. Further studies are needed to assess the effects of omeprazole pre-operative and longer term administration on bone healing and implant osseointegration.

4.7. Conclusions

Post-operative administration of omeprazole has a negative effect on bone healing and implant osseointegration. Therefore, omeprazole might be a potential risk factor for bone surgery and implant therapy.

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Chapter Five: Emerging risk factors for implant osseointegration: Anti-Vascular Endothelial Growth Factors

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5.1. Abstract

Background: Angiogenesis is an essential process during bone formation, bone healing and osseointegration of implants. Angiogenesis involves the coordination of several growth factors including vascular endothelial growth factors (VEGFs). We hypothesized that drugs interfere with angiogenesis such as anti-VEGFs have a negative impact on bone healing and implant osseointegration.

Objective: To investigate the effect of anti-VEGF therapies on bone healing and implant osseointegration in a rat model.

Methods: In a group of 36 Sprague Dawley rats, two unicortical defects were created in both right and left tibias. In the left tibia, a custom-made titanium implant (1.5 mm ø x 2.0 mm in depth) was placed whereas the contralateral defect was left empty. Rats were randomly assigned into 3 groups and received either anti-VEGF neutralizing antibody (4 µg, i.p. twice a wk, n=12), Ranibizumab (a humanized anti-VEGF medication, 0.5 mg/0.05ml; n=12) or saline (n=12) as control. After a healing period of two weeks, animals were sacrificed and tibias were assessed for bone healing and implant osseointegration using micro CT, and histomorphometry, respectively.

Result: Micro-CT analysis revealed that the volumes of the bone defect in anti-VEGF neutralizing antibody and Ranibizumab treated rats were significantly higher (p= 0.026) than the controls. Moreover, Histology and histomorphometry revealed significant reduction (p< 0.001) in osseointegration among treatment groups compared to the controls.

Conclusion: drugs that inhibit the activity of vascular endothelial growth factors may have a negative impact on bone healing and implant osseointegration. These medications should be considered as potential risk factors for bone and implant surgeries.

Keywords: osseointegration**,** bone healing, angiogenesis, VEGF, Anti-VEGF, Ranibizumab, Lucentis.

5.2. Introduction

Endosseous implants are widely used in the rehabilitation of pathologic bone impairments, such as fractures, and as a substitute for lost anatomical structure such as tooth loss (1, 2). Success in implant therapy relies on osseointegration, which is defined as the process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading (3). However, despite extensive research on critical and key factors in osseointegration, the level of evidence indicating absolute and relative contraindications for implant therapy due to systemic conditions and use of medications is low (4).

The implant bone cavity is similar to a common bone wound, and the mechanisms underlying the osseointegration process around implants are very similar to those occurring during bone repair and fracture healing. Bone healing depends on a cascade of different biological events provoked by traumatic insults that include, thrombosis, inflammatory reaction, neovascularization, granulation tissue formation and bone remodeling (5, 6). Therefore, drugs targeting any of these events may have an effect on bone healing and implant osseointegration.

Following fracture fixation and installment of implants in bone, at the initiation of the clotting process, platelets will release several cytokines and growth factors. These factors, such as VEGF-A, will attract the inflammatory cells and will mediate the chemotactic response. VEGFs have been demonstrated to attract osteoblasts, to stimulate osteoblast chemotaxis and differentiation, as well as to recruit osteoblastic progenitor cells (7). The secretion of VEGF is regulated by factors that stimulate osteogenesis, including 1,25

dihydroxyvitamin D_3, 17β-estradiol, and bone morphogenetic protein-2. In fact, those factors that enhance osteoblast differentiation may also enhance their ability to promote angiogenesis.

Nowadays, millions of people all around the world are using drugs that target vascular endothelial growth factors (VEGF), and this number is expected to be over 500 millions in the next decades (8, 9). It is a growth factor involved in many human physiologic processes. VEGF is a key component of neovascularization and plays a crucial role in the restoration of vascular bone supply during the bone healing process (10, 11). However, up-regulation of VEGFs is associated with pathologic angiogenesis such as cancer and age related macular degeneration. Therefore, anti-VEGF therapies have been proved valuable in clinical oncology and are used as a first-line treatment of patients with various types of cancer such as breast cancer, colorectal cancer and malignant glioma (12, 13). Furthermore, they are used to control aberrant angiogenesis in several debilitating and neovascular eye diseases, such as age-related macular degeneration (AMD) (14, 15), for example Ranibizumab, an antibody that inhibits the biologic activity of human VEGF-A, has become the standard of care for the treatment of AMD (16-18). Since VEGFs are crucial factors in angiogenesis and angiogenesis is important during bone repair process, VEGFs inhibition by some medications may have a negative impact on bone healing and implant osseointegration.

Despite its importance, clinical and theoretical knowledge about the potential effect of inhibiting VEGF-dependent angiogenesis in implant osseointegration is scarce. This lack of knowledge may expose susceptible patients to the increased risks of failures in implant rehabilitation. In an effort to contribute towards developing understanding on this topic

and fill the gap in knowledge, we aimed to investigate the effect of anti-VEGF therapies on bone healing and titanium implant osseointegration in a rat model.

The primary hypothesis was that the administration of anti-VEGFs will not affect the healing of bone defect as defined by the change in the volume of bone defect, the trabecular number, thickness and separation in a rat model. The secondary hypothesis was that anti-VEGFs therapies will not affect implant osseointegration as defined by a bone-implant contact area.

5.3. Materials and Methods

The ethical approval for this experimental study was obtained from McGill Animal Ethics Board (# 2012-7269). The study was conducted on a group of 36 female, 10 to 12-week-old (growing rats) Sprague-Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing between 200 to 250 g. The rats were kept in a standard environmental 22° C with 12-hour light/dark cycles. A rodent breeding diet and water were provided ad libitum. Animals were allowed to acclimatize to this environment for at least 1 week prior to surgical intervention.

5.3.1. Surgical Intervention

The animals were anesthetized with isoflurane (3-5% at induction time and 2-2.5% during the maintenance period). After the animal showed signs of being fully anesthetized, the legs were shaved and disinfected with chlorhexidine scrub. Then, animals were covered with a sterile drape. A longitudinal skin incision was created over the proximal medial diaphysis, and the anterior tibial muscle was dissected. The medial plane of the tibia was exposed, and the periosteum was conserved. A unicortical defect

was produced using a cylindrical bur (1.5 mm ø) adapted to a handpiece drill (Stryker, Hamilton, ON) under constant saline irrigation. A custom-made (1.5 mm x 2.0 mm in depth) titanium implant was placed in left defect (Fig. 5.1, A). The incision was closed using 5-0 monocryl sutures. The same procedure was done on the contralateral tibia but the defect was 2.5 mm in diameter and was left empty (Fig. 5.1, B). Carprofen (Pfizer Animal Health, Montréal, QC) was injected (5-10 mg/kg) subcutaneously (SC) to the rats 30 minutes prior to surgery and 24 hours after surgery for two days in order to provide analgesia to the rat. After surgical intervention, the animals were randomly assigned into three groups. The first group received an anti-VEGF neutralizing antibody (4 ug, i.p. twice a wk, n=12). The second group revived a Ranibizumab (Lucentis; Genentech Inc., San Francisco, Calif., USA, single intra-peritoneal injection 0.5 mg/0.05ml; n=12). The third group (n=12) served as control. After the intervention, the rats were left to heal for a period of two weeks, and were assessed daily for signs of toxicity. Rats were euthanized at day 14 using CO_2 overdose, and the tibias were retrieved and preserved in 10% neutral buffered formalin (Richard Allan Scientific, Kalamazoo, MI).

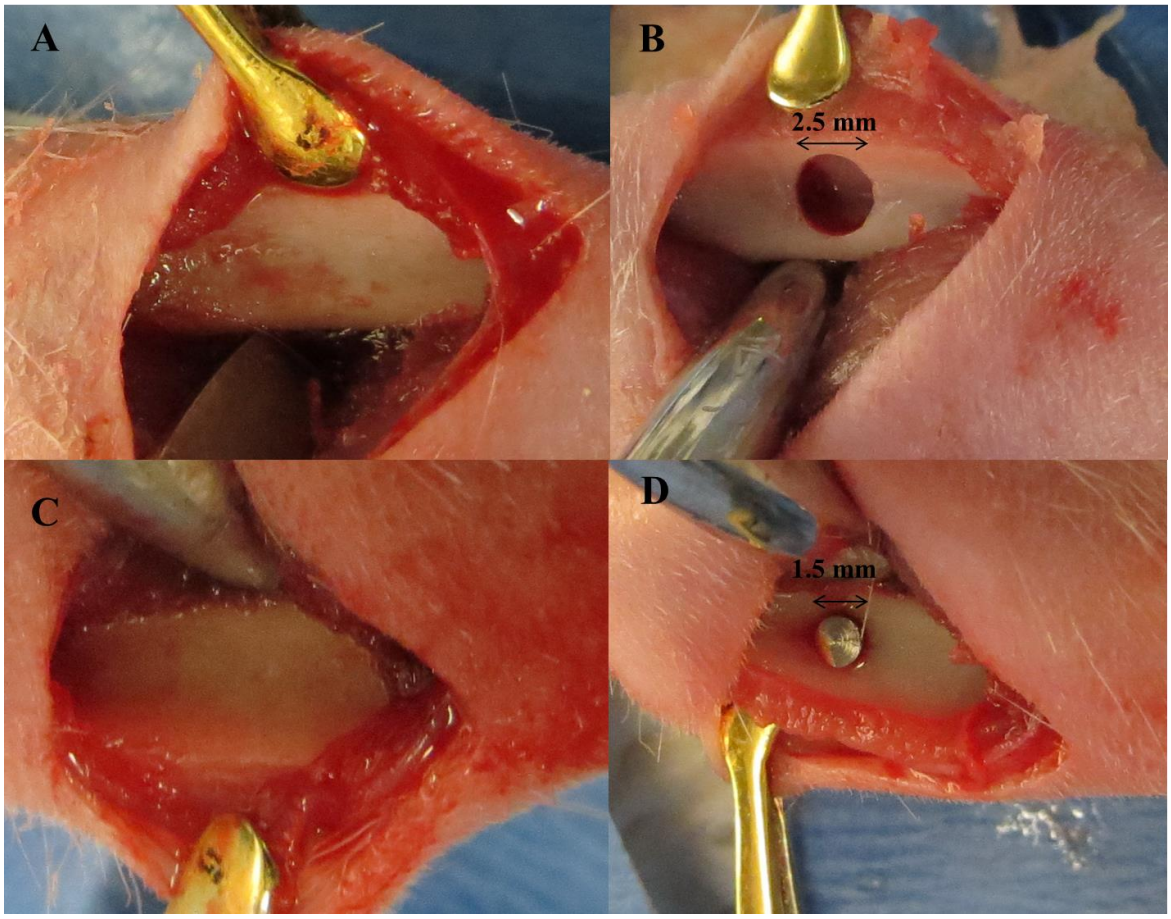

Figure 5.1. A: Rat right tibia metaphysis exposed before creating the bone defect. B: 2.5 mm ø unicortical bone defect. C: Rat left tibia metaphysis exposed before Ti implant insertion. D: 1.5 mm ø x 2 mm Ti implant placed in tibia metaphysis.

5.3.2. Assessment of bone healing

A micro-CT analysis was conducted to assess healing of bone defect. All tibias with empty defect were scanned using a micro-CT (SkyScan1172; SkyScan; Kontich, Belgium) set at a 12.7 µm resolution, a 50 Kv voltage, a 0.5 rotation step, a 10 random movement and a 0.5 mm Aluminum filter. The area of bone defect was determined and included in the region of interest (ROI). The ROI was reconstructed and analyzed by Skyscan CT-analyzer. Three dimensional parameters quantified in each defect including

tissue volume, bone volume, trabecular thickness, trabecular number and trabecular separation. The volume of the defect was determined manually by subtracting the bone volume from the total tissue volume.

5.3.3. Assessment of Osseointegration

Histology and histomorphometry were conducted to assess osseointegration. All left tibias with implants were dehydrated in ascending concentrations of ethanol (Range 70% - 100%) before embedding in polymethyl methacrylate histological resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Germany). After polymerization, the osseointegrated implants were sectioned into histological slides using a diamond saw (SP1600, Leica Microsystems GmbH, Wetzlar, Germany) and stained using basic fuchsine and methylene blue. Digital optical micrographs of the histological sections were recorded with an optical micro-scope (Carl Zeiss Microscopy, GmbH, Germany). Histomorphometrical measurements were performed using ImageJ software (Wayne Rasband; National Institute of Health, Bethesda, Maryland). Implant osseointegration was defined as bone implant contact area (BIC) and was calculated by dividing the bone-covered implant perimeter (BIP) by the total implant perimeter (TIP) as showed in this Equation: BIC= BIP/TIP %. All histomorphometric measurements were calculated as percentage values ± the standard deviation.

5.3.4. Assessment of Angiogenesis and Osteoclastogenesis

All right tibia samples (tibias with bone defect) were rinsed in 50% alcohol for 5 hours, (70% - 90 %) alcohol for 2 hours each, 100% alcohol for 1 hour, and 100% alcohol for 30 minutes (twice), respectively. Then, samples were rinsed in xylene for 1 hour, xylene for

30 minutes, respectively, before embedding in paraffin. Von Willebrand Factor or Factor VIII Related Antigen (Millipore's Blood Vessels Staining Kit, Millipore, Billerica, MA) staining was performed in order to assess the angiogenesis and neovascularization in bone defect area. Digital optical micrographs of the histological sections were recorded with an optical micro-scope (Carl Zeiss Microscopy, GmbH, Germany). Five different areas were randomly selected from each histological section of the bone defect to assess the angiogenesis activity. Angiogenesis activity measurements were performed using ImageJ software (Wayne Rasband; National Institute of Health, Bethesda, Maryland). Angiogenesis activity was defined as the total surface area positive stain and was calculated by dividing the positively stained surface area by the total histological section area. All angiogenesis measurements were calculated as percentage values ± the standard deviation. Other sections of bone defects were stained using Tartrate Resistance Acid Phosphatase (TRAP) staining to assess the osteoclastogenesis activity. The total numbers of osteoclasts were quantified using Zeiss imaging software (ZEN 2012 SP2, GmbH, Germany). The osteoclastogenesis activity was calculated as total number of osteoclasts in the bone defect area.

5.3.5. Statistical Analysis

Sample size for this experimentation was determined by a power analysis (based on pilot data, p=0.05, 1-β =0.80) taking into account potential animal mortality. Descriptive statistics were conducted and the normal distribution of the data was tested using the Shapiro-Wilk test. The square root was used to normalize non-normally distributed variables. The Levene statistic was used to assess homogeneity of variance. In case of homogeneity of variance, one-way ANOVA followed by the Tukey test for paired

comparisons were used. When homogeneity of variance was rejected, the Welch F test followed by Games-Howell test for paired comparisons was used. Data analyses were carried out using SPSS 17 (SPSS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

5.4. Results

Surgical part of the study proceeded without complications or any animal drop-out. However, one rat from Ranibizumab group and one from Control group were excluded because of post-operative bone fracture.

5.4.1. Micro-CT: Bone Healing

There were statistically significant differences between group means for bone defect volume as determined by one-way ANOVA (F (2.31) = 4.137, p = 0.026). Post hoc analyses using Tukey's test indicated that the mean of bone defect volume was significantly higher (F (2.31) = 4.137, p = 0.022) in the anti-VEGF neutralizing antibody ($M_{\text{anti-VEGF neutralizing antibody}}$ =2.48 mm^3, SD = 0.33 mm^3) than in the other two groups ($M_{\text{Ranibizumab}}$ = 2.35 mm^3, SD =0.23 mm^3, M_{Control} = 2.11 mm^3, SD =0.36 mm^3), (Table 5.1, Fig 5.3 A).

Table 5.1: Multiple comparisons within rat groups (Anti-VEGF neutralizing antibody), Ranibizumab and control in regard to the mean volume of created bone defect

Group	Group	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	Ranibizumab	-0.25	0.152	-0.57	0.072
	Anti-VEGF neutralizing antibody	-0.36*	0.022	-0.68	-0.047
Ranibizumab	Control	0.25	0.152	-0.07	0.57
	Anti-VEGF neutralizing antibody	-0.11	0.663	-0.43	0.20
Anti-VEGF	Control	0.36*	0.022	0.04	0.68
	Ranibizumab	0.11	0.663	-0.20	0.43

*.Tukey's test,

Moreover, a statistically significant difference was observed among group means in term of trabecular thickness as determined by Welch F test (F (2.31) = 5.13, p = 0.024). Post hoc analyses using Games Howell test indicated that the mean was significantly lower (F (2.31) = 4.137, p= 0.041) in the anti-VEGF neutralizing antibody ($M_{\text{anti-VEGF neutralizing antibody}}$=0.204 mm, SD=0.035 mm) than in the other two groups ($M_{\text{Ranibizumab}}$=0.23 mm, SD=0.045 mm, M_{Control}=0.288 mm, SD=0.096 mm, table 5.2, Fig 5.3. B).

Furthermore, there was a statistically difference among group means in term of trabecular number as determined by Welch F test (F (2.31) = 11.53, p < 0.001). Post hoc analyses using Games Howell test indicated that the mean trabecular number was significantly higher (F (2.31) = 11.52, p = 0.007) in the anti-VEGF neutralizing antibody ($M_{\text{anti-VEGF neutralizing antibody}}$=2.60 1/mm, SD=0.394 1/mm) than in the other two groups ($M_{\text{Ranibizumab}}$=2.23 1/mm SD=0.477 mm, M_{Control}=1.26 1/mm SD=1.21 1/mm, Table 5.2, Fig 5.3. C).

Also, there was a statistically difference among group means in term of trabecular separation as determined by Welch F test (F (2.31) = 11.52, $p < 0.001$). Post hoc analysis using Games Howell test indicated that the mean trabecular separation was significantly lower (F (2.31) = 11.52, p = 0. 013) in the anti-VEGF neutralizing antibody ($M_{\text{anti-VEGF neutralizing antibody}}$ =0.148 mm SD=0.039 mm) than in the other two groups ($M_{\text{Ranibizumab}}$=0.215 mm SD=0.074 mm, M=0.6 mm SD=0.420 mm, Table 5.2, Fig 5.3.D).

Table 5.2: Multiple comparisons within rat groups (Anti-VEGF neutralizing antibody, Ranibizumab and control) in regard to Trabecular Thickness, Separation and Number

Dependent Variable	Group	Group	Mean Difference	Sig.
Trabecular thickness	Control	Ranibizumab	0.053	0.253
		Anti-VEGF	0.084*	0.041
	Ranibizumab	Control	-0.053	0.253
		Anti-VEGF	0.031	0.184
	Anti-VEGF	Control	-0.0841*	0.041
		Ranibizumab	-0.031	0.184
Trabecular Separation	Control	Ranibizumab	0.396*	0.027
		Anti-VEGF	0.453*	0.013
	Ranibizumab	. Control	-0.396*	0.027
		Anti-VEGF	0.056	0.097
	Anti-VEGF	Control	-0.453*	0.013
		Ranibizumab	-0.0561	0.097
Trabecular Number	Control	Ranibizumab	-0.454*	0.035
		Anti-VEGF	-0.592*	0.007
	Ranibizumab	Control	0.454*	0.035
		Anti-VEGF	-0.138	0.073

Games-Howell test

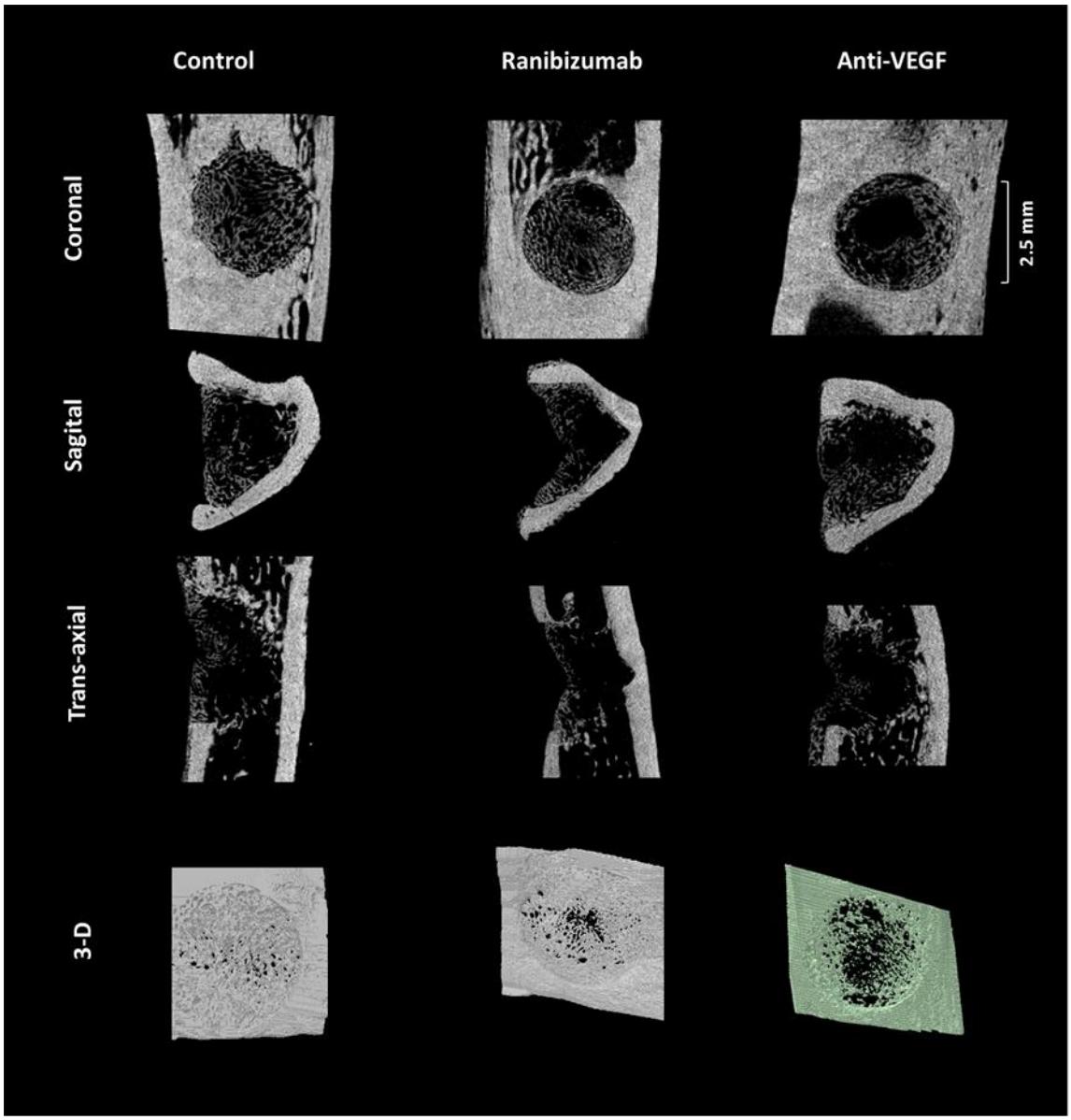

Figure 5.2. Coronal, sagittal, trans-axial and 3-D reconstruction of the micro-CT scans of bone defects shows compromised healing in ranibizumab and anti-VEGF treated rats compared to the controls group.

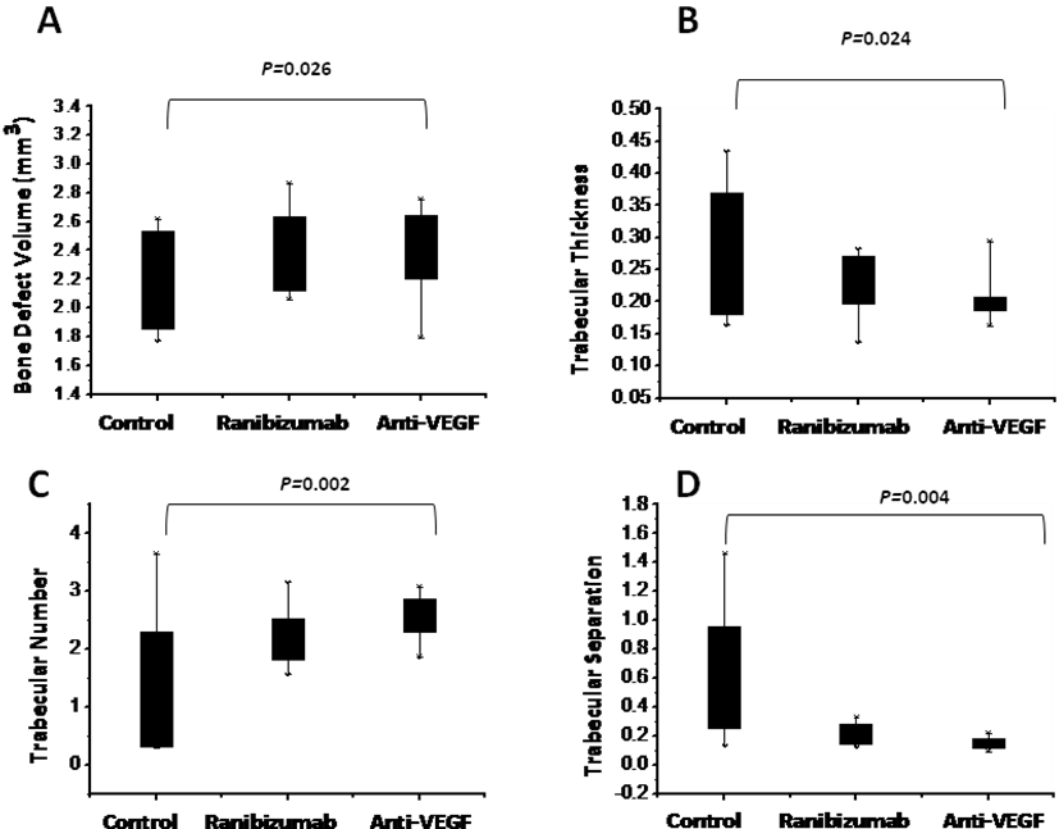

Figure 5.3. Micro-CT data analysis of bone defects in omeprazole treated rats compared to control group: A. Defect volume, B. Trabecular thickness, C. Trabecular number D. Trabecular separation. Statistical analysis by one-way ANOVA, $n_{(control)}$ =11 $n_{(Ranibizumab)}$ =11, $n_{(Anti\text{-}VEGF}$ =12.

5.4.2. Histology and Histomorphometry: Osseointegration

The anti-VEGFs group presented the lowest bone contact ratio, with BIC of 19.9 %, followed by Ranibizumab group with 21.7 % and control with 41.8 %. There were a statistically significant differences between group means for BIC % as determined by one-way ANOVA (F (2.31) = 14.1, p < 0.001). Post hoc analyses using Tukey's test indicated that the BIC % was significantly lower (F (2.31) = 14.1, p < 0.001) in the anti-VEGF neutralizing antibody (M =19.9 %, SD = 9.40 %,) and Ranibizumab ($M_{\text{Ranibizumab}}$ =21.7%, SD=9.18 %,), than in the control (M_{Control} =41.8%, SD=12.4 %, Table 5.3, Fig

4).

Table 5.3: Multiple comparisons within rat groups (Anti-VEGF neutralizing antibody, Ranibizumab and Control) in regard to Implant-bone contact area.

		Mean Difference		95% Confidence Interval	
Group	Group	(I-J)	Sig.	Lower Bound	Upper Bound
Control	Ranibizumab	19.58*	0.00	8.40	30.76
	Anti-VEGF	21.62*	0.00	10.68	32.57
Ranibizumab	Control	-19.58*	0.00	-30.76	-8.40
	Anti-VEGF	2.03	0.89	-8.90	12.98
Anti-VEGF	Control	-21.62*	0.00	-32.57	-10.68
	Ranibizumab	-2.03	0.89	-12.98	8.90

Tukey's test

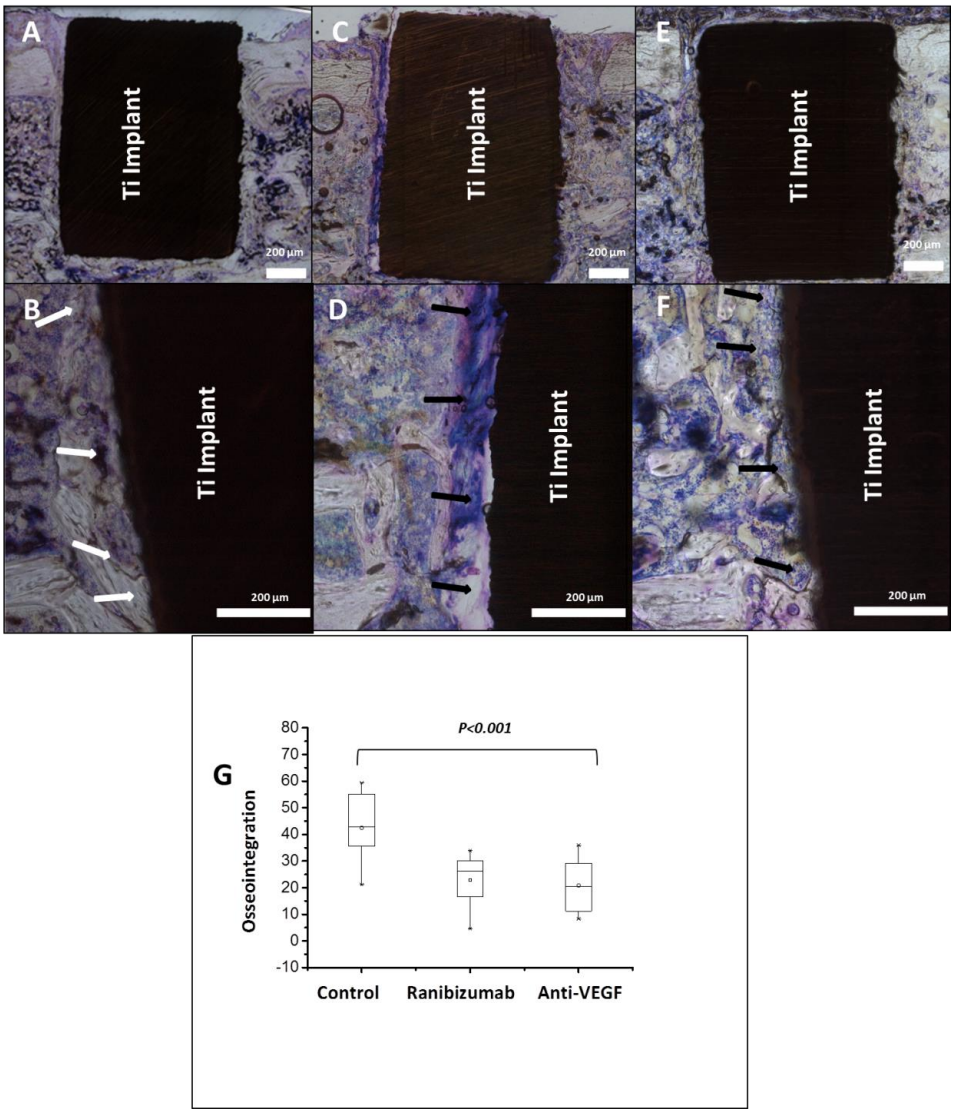

Figure 5.4. A: Sagittal histological section of Ti implant in tibia of control rat. B: Higher magnification (20 X) of Ti implant in tibia of control rat, black arrow indicates bone growing around implant. C: Sagittal histological section of Ti implant in tibia of ranibizumab treated rat. D: Higher magnification (20 X) of Ti implant in tibia of ranibizumab treated rat, white arrow indicates soft tissue forming around implant. E: Sagittal histological section of Ti implant in tibia of anti-VEGF neutralizing antibody treated rat. F: Higher magnification (20 X) of Ti implant in tibia of anti-VEGF neutralizing antibody treated rat, white arrow indicates soft tissue forming around implant. G: histomorphometric analysis of osseointegration was presented as mean $\pm$ standard deviation. Statistical analysis by one-way ANOVA, $n_{(control)}=11$ $n_{(Ranibizumab)}=11$, $n_{(Anti\text{-}VEGF)}=12$.

5.4.3. Histology and Histomorphometry: Angiogenesis and Osteoclastogenesis

The anti-VEGFs group presented the lowest angiogenesis activity, with angiogenesis average of 2.96 %, followed by Ranibizumab group with 3.11 % and control with 8.66 %. There were a statistically significant differences between group means for angiogenesis % as calculated by one-way ANOVA (F (2.26) = 30.61, $p < 0.001$). Post hoc analyses using Tukey's test indicated that the angiogenesis % was significantly lower (F (2.26) = 30.61, $p < 0.001$) in the anti-VEGF neutralizing antibody (M =2.96 %, SD = 0.79 %,) and Ranibizumab ($M_{\text{Ranibizumab}}$ =3.11%, SD=0.95 %), than in the control (M_{Control} =8.66%, SD=3.11 %, Table 5.5, Fig 5.5.A, B, C and G). One-way ANOVA revealed not statistically difference (F (2.26) = 1.72, p = 0.198) in the osteoclastogenesis activity among the three groups (Table 5.5, Fig 5.5.D, F, E and H).

Table: 5.4: Multiple comparisons within rat groups, Anti-VEGF neutralizing antibody, Ranibizumab and control in regard to the mean neovascularization of created bone defect

		Mean Difference		95% Confidence Interval	
Group	Group	(I-J)	Sig.	Lower Bound	Upper Bound
Control	Ranibizumab	5.55*	.000	3.55	7.56
	Anti-VEGF	5.82*	.000	3.85	7.81
Ranibizumab	Control	-5.55*	.000	-7.56	-3.55
	Anti-VEGF	0.276	.909	-1.37	1.93
Anti-VEGF	Control	-5.82*	.000	-7.81	-3.85
	Ranibizumab	-.276	.909	-1.93	1.37

Tukey's test

Table 5.5: Multiple comparisons within rat groups, Anti-VEGF neutralizing antibody, Ranibizumab and control in regard to the mean number of osteoclasts in the created bone defect

		Mean Difference		95% Confidence Interval	
Group	Group	(I-J)	Sig.	Lower Bound	Upper Bound
Control	Ranibizumab	-2.394	.997	-84.34	79.55
	Anti-VEGF	-47.000	.333	-127.73	33.73
Ranibizumab	Control	2.394	.997	-79.55	84.34
	Anti-VEGF	-44.606	.246	-112.01	22.79
Anti-VEGF	Control	47.000	.333	-33.73	127.73
	Ranibizumab	44.606	.246	-22.79	112.01

Tukey's test

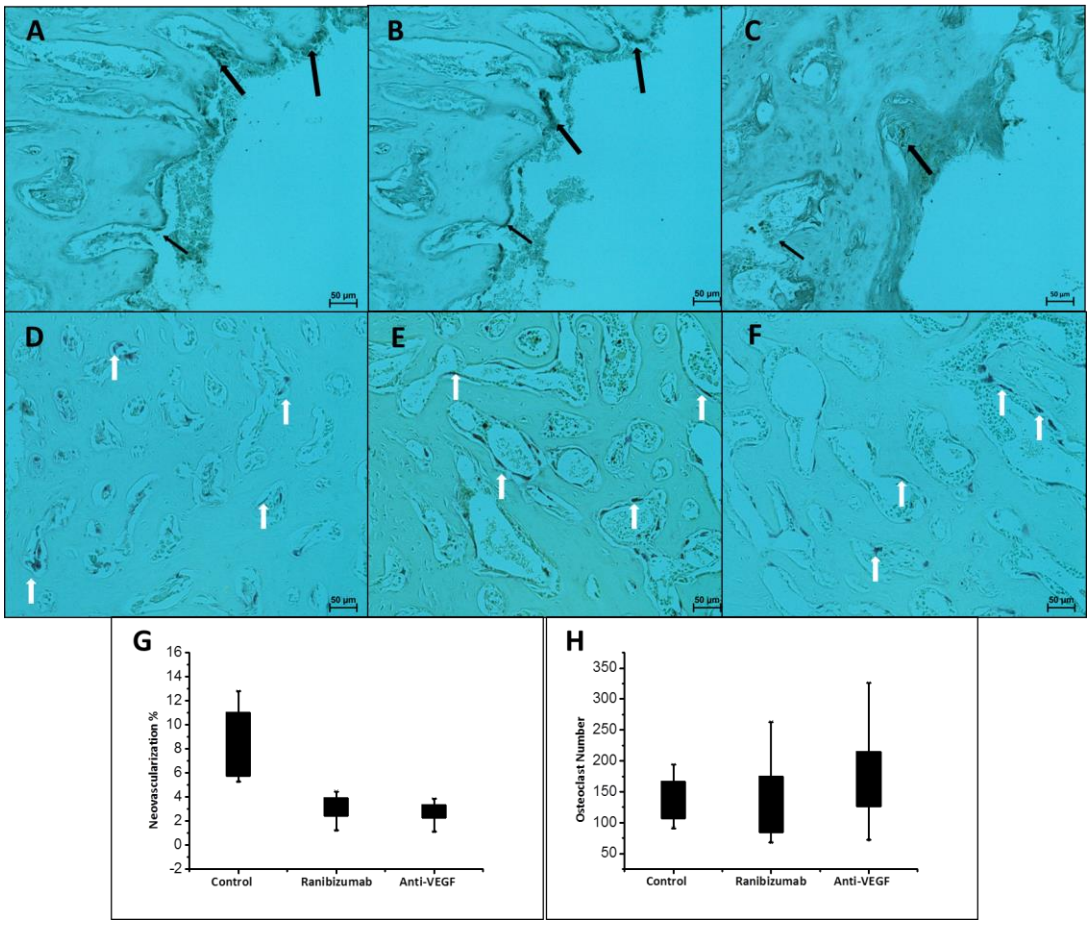

Figure 5.5. A: Coronal histological section of bone defect in tibia of control rat, black arrow indicates neovascularization. B: Coronal histological section of bone defect in tibia of Ranibizumab treated rat, black arrow indicates neovascularization. C: Coronal histological section of bone defect in tibia of anti-VEGF treated rat, black arrow indicates neovascularization. D: Coronal histological section of bone defect in tibia of control rat, white arrow indicates osteoclasts. E: Coronal histological section of bone defect in tibia of Ranibizumab treated rat, white arrow indicates osteoclasts. F: Coronal histological section of bone defect in tibia of anti-VEGF treated rat, white arrow indicates osteoclasts. G: histomorphometric analysis of neovascularization. H: histomorphometric analysis of osteoclastogenesis. Statistical analysis by one-way ANOVA, $n_{(control)} = 11$ $n_{(Ranibizumab)} = 11$, $n_{(Anti\text{-}VEGF} = 12$.

5.5. Discussion

The present study, for the first time provides evidence that anti-vascular endothelial growth factors (VEGFs) impair bone healing and implant osseointegration in a rat model. Our clinical experience on this issue supports these findings. Recently, we encountered total implant failure in a patient who received intravitreal injections of Ranibizumab (Lucentis, Genentech Inc.) for macular degeneration (20, 21). These pilot data

determined that there is a need for bench side investigation on angiogenesis inhibitor medications as potential risk factors for implant therapy. The use of animal model is justified by the novelty of this research for better understanding the mechanism without the added of harmful risks to humans. In this study, we performed an in vivo experiment because there is no computer program available which can simulate the process of bone healing and titanium implant osseointegration. Although cell culture based in vitro mineralization assays are available, these assays often use non-physiologic conditions and are unable to fully recapitulate the complex process of bone maturation and osseointegration in vivo. The choice of a rat model is considered a suitable model to evaluate the bone-implant interface (22).

Many systemic/medical risk factors could influence osseointegration (23-30). These risk factors can be categorized into: very high and significant risk factors. Very high risk factors include serious systemic diseases including (rheumatoid arthritis, osteomalacia, osteogenesis imperfect), immunosuppressive medications including (corticosteroids, chemotherapy or immunosuppressive drugs), and immunosuppressive diseases such as HIV, drug and alcohol abuse. Significant risk factors include irradiated bone, severe and uncontrolled diabetes, bleeding disorders or drug-induced anticoagulation and heavy smoking (31). However, a recent systematic review on this topic concluded that the level of evidence indicating absolute and relative contraindications for oral implant therapy due to systemic conditions and medications is low and that no data exist for all medical conditions (28).

Mair et al. (2007) have recently studied the negative impact of TNP-470, an inhibitor of angiogenesis, on the peri-implant bone formation and osseointegration. This research

group has suggested testing the effect of anti-VGEF on osseointegration to provide further insights on this topic (32).

Two mechanisms can explain the impact of anti-VGEFs on bone healing and osseointegration. Firstly, anti-VGEFs suppress angiogenesis (33); secondly, they inhibit bone formation by suppressing osteoblast differentiation (34, 35), and osteoblasts chemotaxis (36).

Several studies have evaluated the possible mechanisms of the impact of vascular endothelial growth factors on bone healing, and their results are in line with our findings. Gao et al. (2013), had observed that the bone regeneration were enhanced by VEGFs (37). Joce et al. (2014) concluded that application of Simvastatin, a cholesterol lowering-drug, stimulates bone regeneration through the up-regulation of VEGFs (38). Street et al. (2002) showed that VEGF activity is important for normal endochondral and intramembranous ossification during the healing process. Moreover, they demonstrated that VEGF inhibition by anti-VEGFs impaired bone remodeling (39). Raines et al. (2010) suggested that VEGF is of crucial importance for vasculogenesis, and therefore the absence of neovascularization could be a risk factor for implant failure (40). Our findings provide evidence that anti-VEGFs therapies compromise bone healing and implant osseointegration by reducing angiogenesis.

In this experiment, we used machined Ti implants and we found an average of 42 % of bone-implant contact ratio among control rats. This finding is consistent with previous studies using similar materials (41). However, a statistically significant decrease in BIC ratio was observed among anti-VEGF neutralizing antibody and Ranibizumab groups.

This high magnitude of effect could be deleterious for mechanical fixation and clinical success of Ti implant (41).

Surprisingly, we found that the trabecular number was higher and trabecular separation was lower in anti-VEGF neutralizing antibody and Ranibizumab groups compared to the controls. However, the trabecular thickness and volume was lower in the treatment groups compared to the control. These observations indicate to the formation of higher number of small or less developed trabeculae in the treatment groups. Several cytokines and growth factors such as interleukin-1 (IL-1), Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) (42, 43), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and VEGF (44) are involved in osteoblast chemotaxis, differentiation and proliferation. However, VEGFs are the key regulators of angiogenesis during the bone growth process. VEGF inhibition during bone formation suppresses angiogenesis and consequently reduces trabecular bone volume, probably because of insufficient oxygen and nutrients (45). Gerber et al. (1999) found that VEGFs inhibition suppresses capillaries invasion, reduces chondroclasts recruitment and increases the hypertrophic cartilaginous zone in the growth plate, whereas, the bone mineralization process was normal in the area surrounding the cartilaginous zone (46). These key observations along with our findings suggest that bone formation process had started but not continued due to inadequate nutrients and oxygen caused by deficient blood supplies (46, 47).

Our results showed that the negative effect of anti-VEGF neutralizing antibody on bone healing was higher than those of Ranibizumab. This observation was expected because

the anti-VEGF neutralizing antibody used in this study was rat specific. However, Ranibizumab (Lucentis; Genentech Inc., San Francisco, Calif., USA) is a fragment of a recombinant, humanized, monoclonal antibody designed for intraocular use (48). Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A) in vitro and in vivo (15-17, 49).

As with any animal model, there are some limitations to be acknowledged. These include inherent variation in any in vivo model, potential difference with human conditions (e.g. bone pattern and bone quality), and the sensitivity of the histomorphology analysis. Furthermore, the effects of anti-VEGFs on bone healing and implant osseointegration were assessed only at one time point after post-operative administration of medications, thus did not allowed to evaluate the long term effects of these anti-bodies on bone healing and implant osseointegration. Future clinical studies are needed to support these findings

5.6. Conclusions

The results of this study suggest that drugs that inhibit the activity of vascular endothelial growth factors may have a negative impact on bone healing and implant osseointegration. These medications should be considered as potential risk factors for implant therapy and orthopedic/craniofacial surgical procedures. Future investigations are needed to provide further insights on this topic.

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Chapter Six: General Discussions and Conclusions

Osseointegration could be influenced by systematic and local risk factors (1-8). Several medical conditions and medications are considered risk factors for implant therapy (9). According to the guidelines provided by Buser and coworkers (10) in the second ITI consensus Conferences, the general medical/systemic risk factors for osseointegration are classified into very high and significant risk factors. Very high risk factors include serious systemic diseases including (rheumatoid arthritis, osteomalacia, osteogenesis imperfect), immunosuppressive medications including (corticosteroids, chemotherapy or immunosuppressive drugs), and immunosuppressive diseases such as HIV, drug and alcohol abuse. Significant risk factors include irradiated bone, severe and uncontrolled diabetes, bleeding disorders or drug-induced anticoagulation and heavy smoking (11). However, a recent study concluded that the level of evidence indicating contraindications for oral implant therapy due to systemic conditions and medications is low and that no data exist for all medical conditions (12).

Proton pump inhibitors and anti-VEGF therapies are two types of medications known to interfere with bone metabolism (13, 14). Implant osseointegration is regulated by bone metabolism; therefore, PPIs and anti-VEGFs might have negative effects on bone healing and implant osseointegration. However their potential negative effects on osseointegration have not been previously explored.

In this *in vivo* study, we provide evidence that post-operative administration of PPIs (omeprazole), and anti-VEGFs, have negative effects on bone healing and implant osseointegration.

Omeprazole had a negative effect on osseointegration probably because it hindered bone healing. The negative effects of omeprazole on bone healing may be related to decreased expression of growth factors (BMP-2 and BMP-4), increased histamine level, or reduced calcium absorption (15-17). Although future studies would be needed to understand the mechanism behind this phenomenon.

VEGFs are of crucial importance for vasculogenesis, an important physiological process for bone healing (6). Therefore, absence of neovascularization could be a risk factor for bone healing and implant osseointegration. Our findings provide evidence that anti-VEGFs therapies compromise bone healing and implant osseointegration by reducing angiogenesis.

In this thesis, the machined Ti implants used and showed an average of around 42 % of osseointegration among control rats. This finding is consistent with previous studies using similar materials (18). However, osseointegration was significantly lower among ($p<0.001$) anti-VEGF, Ranibizumab and Omeprazole treated rats. This high magnitude of effect could be deleterious for mechanical fixation and clinical success of Ti implants. Although the clinical relevance of this impairment in osseointegration should be further investigated, it is well known that this amount of reduction in osseointegration could increase the risk of failure of osseointegrated implants by up to three folds (7).

Orthopedic and craniofacial interventions that require implant and bone surgeries are very common. In the US, about 100,000 to 300,000 dental implants and more than 300,000 joints are replaced yearly. The number of these surgical interventions are increasing dramatically, e.g. in the United States, the number of hip and knee replacement surgeries will exceed the 4 million annually by 2030 (19). PPIs are extensively used by millions of patients (20-22) for instance, in the United States, more than 113 millions PPI prescriptions are filled per year (23). Anti-VEGF therapies are being used by millions of patients all around the world and their use is expected to be over 500 millions in the next decades (24, 25). Therefore, the risk of failures in bone and implant surgeries among PPIs and anti-VEGFs users could have a significant impact due the huge portion of the population that are being treated with these drugs.

Our results clearly suggest that future clinical studies are needed to further investigate the effects PPIs and anti-VEGFs on bone healing and implant osseointegration in humans. Moreover, patients on PPIs or anti-VEGFs should be carefully evaluated before considering any intervention that involves bone or implant surgeries.

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Appendices

I. List of Abbreviations

- AMD: Age related macular degeneration.
- BIC: Bone Implant contact area.
- BIP: Bone-covered implant perimeter.
- BMD: Bone mineral density.
- BMP: Bone morphogenetic protein.
- EMA: European Medicines Agency
- FDA: Food and Drug Administration.
- FGF: Fibroblast growth factor.
- HIV: Human immunodeficiency virus.
- IGFs: Insulin-like growth factors.
- IL-1: Interleukin-1.
- IL-6: Interleukin-6.
- INF- γ: Interferon γ.
- Nb: Niobium.
- NSAID: Non-steroidal anti-inflammatory drugs.
- PDGF: Platelet-derived growth factor.
- PPIs: Proton pump inhibitors.
- RANKL: Receptor activator of nuclear factor κB ligand.
- SSRIs: Selective serotonin reuptake inhibitors.

- TGF-β: Transforming growth factor-β.
- Ti: Titanium.
- TIP: total implant perimeter.
- TNF-α: Tumor necrosis factor-alpha.
- VEGF: Vascular endothelial growth factor.