The Effect of Histamine-2 Receptor Antagonist on Bone Healing and Implant Osseointegration

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Dedication

This thesis is dedicated to my parents, to my wife, Arwa, and our little son Saeed. Thank you for your unconditional love and support.

Acknowledgment

First and foremost, I praise Allah for his endless gifts, and for blessing me with a supportive and understanding family.

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Abstract

Bone injuries represent a significant health problem globally. The management of such conditions could cause a loss of function or morbidity, and their management can sometimes be considered challenging for orthopedic and maxillofacial surgeons. After such injuries occur, the bone healing process is initiated, this involves reactive, reparative, and remodelling phases that can be influenced positively or negatively by multiple local and systemic (endogenous and exogenous, e.g. medications) factors. In some cases, such injuries cannot resolve spontaneously; orthopedic and craniofacial surgeries are needed to restore function. This is often done with the aid of implantable devices, and their success depends on osseointegration, which is the functional and biological interlocking between the implant and the living bone. Both bone healing and osseointegration processes follow the same biological sequence of events, and multiple studies found that medications (e.g. proton pump inhibitors) that affected bone healing could also affect osseointegration.

Histamine H_2-receptor antagonist is a selective over the counter drug that has been widely used since the 1980s as a potent gastric acid-suppressing drug. It interferes with the bone remodelling process by impairing the osteoclastogenesis via suppressing the receptor activator of nuclear factor $_k$B ligand (RANKL) that is important in the differentiation of precursors cells into osteoclasts. This could influence bone healing and implant osseointegration by hindering the osteoclasts-osteoblasts coupling mechanism.

We hypothesize that H_2-receptor antagonists could have a negative effect on bone healing and osseointegration. We designed this study to assess the effect of ranitidine (an H_2-receptor antagonist) on bone healing and implant osseointegration in a rat model.

An *in vivo* study was conducted to assess this hypothesis in a rat model. A unicortical defect on the left rat tibiae was created to assess bone healing, and an implant was placed on the right tibiae to assess osseointegration.

Post-operatively, rats were assigned randomly to two groups: Ranitidine (n=11), and control (n=11). Euthanasia was done on day 14, and bone samples were collected for analysis. Micro-computed tomography (Micro-CT) analysis of the bone defect revealed a larger bone defect volume in the ranitidine group (0.82 ± 0.13 mm^3 vs 0.66 ± 0.16 mm^3; p=0.034), thinner cortical thickness (0.54 ± 0.07 mm vs 0.63 ± 0.11 mm; p=0.026) and less bone regeneration at defect site ($40 \pm 12\%$ vs $57 \pm 11\%$; p=0.003) in comparison to the saline group. Implant site micro-CT analysis showed less osseointegration in the ranitidine group ($34.1 \pm 2.7\%$ vs. $43.5 \pm 2.1\%$; p=0.014), and implant site histological analysis showed less medullary (p=0.021), cortical (p=0.001) and total (p=0.003) bone-implant contact, and less peri-implant BV/TV (p=0.002) in the ranitidine group in comparison to the control group. Histological analysis for osteoclastic activity (TRAP staining) showed a lower number of osteoclasts in the ranitidine group (4.8 ± 2.4 mm^{-2} vs. 9.1 ± 2.1 mm^{-2}; p=0.026).

We concluded that the post-operative use of ranitidine impaired bone healing and osseointegration.

Résumé

La guérison osseuse est complexe et implique de multiples facteurs physiologiques et mécaniques permettant à l'os de récupérer son état physiologique et fonctionnel. Elle peut être influencé par plusieurs facteurs, notamment des facteurs locaux, systémiques et exogènes (par exemple, les médicaments). Les prothèses implanto-portées ont été largement utilisées dans les chirurgies orthopédiques et cranofaciales pour restaurer ou améliorer de la fonctionnalité. Ils dépendent principalement sur du succès de l'ostéointégration, qui est le verrouillage mutuel entre le matériel des implants et l'os fonctionnellement et biologiquement. La guérison osseuse et le processus d'ostéointégration suivent la même séquence biologique d'événements. Certaines études ont montré que les facteurs qui affectaient la cicatrisation des os pouvaient également affecter l'ostéointégration. L'utilisation d'antagonistes des récepteurs d'histamine H2 a été associée à une interférence dans le processus du remodelage osseux.

Les antagonistes du récepteur d'histamine H2 sont un médicament sélectif en vente libre qui est largement utilisé depuis les années 1980 en tant que médicament puissant pour supprimer l'acide gastrique. Les antagonistes des récepteurs de l'histamine H2 affectent également négativement l'ostéoclastogenèse en supprimant l'activateur du récepteur du ligand du facteur kB du facteur nucléaire (ARLKN), qui joue un rôle important dans la différenciation des cellules précurseurs en ostéoclastes. Ce qui pourrait influencer la guérison osseuse et le processus d'ostéointégration des implants.

Nous émettons l'hypothèse que les antagonistes des récepteurs H2 pourraient avoir un effet négatif sur la cicatrisation osseuse et l'ostéointégration. Nous avons conçu cette étude pour évaluer l'effet de la ranitidine (un antagoniste des récepteurs H2) sur la guérison osseuse et l'ostéointégration des implants chez le modèle du rat.

Une étude *in vivo* a été menée pour évaluer cette hypothèse sur un modèle de rat. Un défaut unicortical sur le tibia gauche du rat a été créé pour évaluer la guérison osseuse et un implant a été placé sur le tibia droit pour évaluer l'ostéointégration.

Dans nos études, 22 rats ont été répartis de manière aléatoire dans deux groupes: ranitidine (n = 11) et contrôle (n = 11). La euthanasie a été faite au jour 14 et des échantillons d'os ont été recueillis pour analyse. L'analyse par micro-scanner du défaut osseux a révélé un volume de défaut plus important dans le groupe ranitidine (0.82 ± 0.13 mm^3 vs 0.66 ± 0.16 mm^3; p=0.034), une épaisseur corticale plus fine (0.54 ± 0.1mm versus 0.63 ± 0.1 mm; p=0.026) et moins. régénération osseuse au site du défaut ($40 \pm 12\%$ vs $57 \pm 11\%$; p=0.003) par rapport au groupe solution saline. La micro-TDM du site implantaire a révélé une moindre ostéointégration dans le groupe ranitidine ($34.1 \pm 2.7\%$ vs. $43.5 \pm 2.1\%$; p=0.014), et une analyse histologique du site implantaire a montré une diminution de la médullarité (p=0.021), de la corticale (p=0.001) et total (p= 0.003) contact os-implant, et moins péri-implants BV / TV (*p*=0.002) dans le groupe ranitidine par rapport au groupe témoin. L'analyse histologique de l'activité ostéoclastique (coloration TRAP) a montré une diminution du nombre d'ostéoclastes dans le groupe ranitidine (4.8 ± 2.4 mm^{-2} vs. 9.1 ± 2.1 mm^{-2}; p=0.026).

Nous avons conclu que l'utilisation de la ranitidine après une opération chirurgicale altère la cicatrisation osseuse et l'ostéointégration.

Contribution of Authors

This thesis includes one manuscript entitled **"Ranitidine Impairs Bone Healing and Implant Osseointegration in Rats' Tibiae"**

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Contribution: N.A. wrote the manuscript, performed the animal surgeries, performed micro-CT analysis, performed histomorphometric analysis, collected and analyzed the data and performed statistical analysis. A.M., F.A., and A.A. helped in animal surgeries and sample collection. L.A. helped in histological analysis, J.L.R.A. and J.E.H helped with mast cell analysis. N.M reviewed the manuscript, F.T. is the primary supervisor, he designed the experiment, supervised the work, provided scientific guidance and reviewed the manuscript.

Originality: This is the first study to investigate the effect of an H2 antagonist, Ranitidine, on bone healing and implant osseointegration. This study concluded that the post-operative use of ranitidine has a negative effect on bone healing and implant osseointegration.

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Chapter 1: Introduction and Research Rationale

1.1 Thesis outline

This thesis includes 4 chapters: 1. Introduction, 2. Literature review, 3. Methods and techniques, and 4. One manuscript entitled **"Ranitidine Impairs Bone Healing and Implant Osseointegration in Rats' Tibiae".**

1.2 Research rationale

Bone healing is a process that aims to restore the shape, structure, and function of bone in response to injury. The bone healing process consists of 4 phases starting with hemostasis, inflammation, proliferation, and finally, bone remodelling(1). Intraosseous implants have been used for decades in surgeries to restore lost anatomical structures. (e.g. hip replacement, dental implants). The success of these titanium implants depends mainly on the process of osseointegration, a functional mechanical interlocking between implants and living bone at a microscopic level(2). Implant osseointegration follows the same principles of bone healing and undergoes the same phases(3). These phases (hemostasis, inflammation, proliferation, remodelling) are influenced by multiple factors, including drugs that could either promote or hinder healing and osseointegration. Bisphosphonates, estrogens, calcitonin, and vitamin D could have a positive effect on bone healing, whereas glucocorticoids, proton pump inhibitors, and selective serotonin-reuptake inhibitors are known to hinder osseointegration(4).

A class of drugs that have been linked with a negative effect on bone accrual are H_2-receptor antagonists (H2RAs), such as ranitidine(5). Ranitidine is extensively used as a first-line treatment for gastroesophageal reflux disease (GERD) and peptic ulcers(6). However, there are studies that

link the use of ranitidine to decreased osteoclasts proliferation and impaired osteoclasts-osteoblasts coupling, which in turn could hinder the bone remodelling cycle(7).

1.3 Hypothesis and objective

The main hypothesis of this thesis is that drugs that have been proven to affect bone metabolism and osteoclastogenesis could influence bone healing and implant osseointegration.

Working hypothesis

H_2-receptor antagonist, Ranitidine, could have a negative effect on bone healing and implant osseointegration.

Objectives

1. To investigate the impact of ranitidine on bone healing.
2. To investigate the impact of ranitidine on implant osseointegration.

We achieved our objectives by using a rats model to investigate the effect of post-operative administration of ranitidine on bone healing and osseointegration.

Chapter 2: Literature review

2.1 Bone

Bone is a hard, firm, and dynamic connective tissue that consists of extracellular matrix (ECM) and multiple cells in a constant active state. Bone ECM is divided into two main components: i) An organic phase (30%), which consists mainly of type 1 collagen (90%) and non-collagenous proteins (e.g. osteopontin, osteocalcin), ii) An inorganic phase (70%), which consists mainly of calcium-deficient carbonated apatite, a mineral composed of calcium, phosphate, and carbonates, as well as traces of other inorganic elements(8).

Anatomically, bones are divided into four groups: Long bones, short bones, flat bones, and irregular bones (9), while histologically, bone exists mainly in two forms: Cortical (compact) and trabecular (Cancellous). Both types share the same substance but differ in structure, amount and density. Cortical bones have a higher matrix density and less porosity than trabecular bones. These differences in density and porosity are essential for the functionality of each bone type(10).

2.1.1 Bone cells

The three main cells responsible for bone formation and remodelling are: Osteoblasts, osteoclasts, and osteocytes.

Osteoblasts

Osteoblasts are derived from the mesenchymal stem cells, which first differentiate to osteoprogenitor and then preosteoblasts before finally becoming osteoblasts. Osteoblasts are the main cells responsible for bone formation. First, they produce the extracellular matrix of bone by secreting collagen proteins (mainly type-1 collagen), non-collagen proteins (osteopontin, c), and proteoglycans. The product of this step is called osteoid, and it is a non-mineralized matrix. Second, osteoblasts facilitate the process of mineralization by releasing the enzyme alkaline phosphatase and phosphate. Osteoblasts could undergo both inactive and active phases depending on the need of forming new bone(11).

Osteoclasts

They are specialized multinucleated cells derived from the monocyte/macrophage lineage of hematopoietic stem cells. They are responsible for bone resorption as part of the bone remodelling cycle. Osteoclasts precursors are present in blood and bone marrow, and they are activated when the receptor activator of nuclear factor-κB ligand (RANKL) released from osteoblasts acts on the RANK receptors on their surfaces. Osteoclasts act by secreting lysosomal enzymes that can resorb mineralized bone(11).

Osteocytes

When osteoblasts become trapped in the calcified matrix, they transform into osteocytes. Osteocytes have a different function from osteoblasts, as they maintain bone matrix and guide any potential bone remodelling. In adults, osteocytes are the primary cells found in bone, and they account for 90% of all bone cells(11, 12).

2.1.2 Bone Development

Bone development starts at the embryogenic stage, as cells derived from the mesodermal origin at the lateral plate of the mesoderm initiate the formation of the axial skeleton and the long bones(11). Bone formation occurs by two mechanisms, intramembranous ossification and endochondral ossification. Intramembranous ossification starts at the eighth week of the embryo while the endochondral process starts around the twelfth week(13).

Intramembranous ossification begins with the accumulation of mesenchymal cells at the area where the bone is to be formed, causing an increased vascularization. The mesenchymal cells differentiate into osteoblasts that would form the bone extracellular matrix. Flat bones are formed via intramembranous ossification, including the skull, maxilla, and clavicles (13).

Endochondral ossification begins as mesenchymal cells accumulate and differentiate into chondroblasts instead of osteoblasts to form cartilage resembling the shape of the intended bone. The mesenchymal cells then continue to differentiate into osteoblasts that will initiate the bone formation and replace the cartilaginous matrix. Long bones formation and fracture healing are controlled via endochondral ossification(14).

Two types of bone are seen in the bone development stage, woven bone and lamellar bone.

Woven bone (primary bone) is comprised of irregular collagen fibres. It is mainly found during embryonic development, and it is also the first type of bone to form at a fracture site(15). At a later stage, woven bone is eventually replaced by a more mature bone, made of regular and dense collagen fibres called ***lamellar bone (secondary bone)*** (11).

2.1.3 Bone Remodelling

Bone modelling refers to the mechanically mediated changes in bone shape, size and position(16). It is adaptive and can be influenced by long term loading and stress. Bones that are subjected to more loading tend to be more dense and stronger than bones with minimum or no load at all(17). Bone remodelling is a process in which old bone is resorbed and replaced by new bone periodically while maintaining the bone mass through a balanced relationship between the amount of resorbed and formed bones. Bone remodelling is a lifetime process, and it is responsible for the complete skeletal regeneration every ten years. It is controlled by osteoblasts and osteoclasts, which comprise the bone multicellular unit (BMU) in a harmonized and well-maintained relationship between both cell types. Osteoclasts begin to resorb the targeted bone and will undergo apoptosis once they reach a certain distance, eventually osteoblasts will fill the empty site and begin the new bone formation process through osteoids secretion, and as the process advances further, the osteoids will be mineralized to complete the cycle. Most osteoblasts (50-70%) will undergo apoptosis once they complete their task, the remaining will either become bone lining cells that cover the new bone surface or become entrapped in the mineralized matrix in the form of osteocytes. Osteocytes function as a mediator of the bone remodelling cycle, by transmitting signals that trigger bone formation and resorption in response to micro-damage and functional demand(18).

There are three mechanisms that are involved in bone remodelling: Osteoblast-osteoclast coupling, local immune regulation of bone remodelling, and systemic regulation of bone remodelling.

Osteoblast-osteoclast coupling

This mechanism refers to the activation of both bone resorption and formation through two distinct mechanisms. The first one is dependent on the expression of pro-osteoclastogenic cytokines by osteoblasts, and the second one is dependent on the signalling of the ephrin ligands and their ephrin receptors.

In the first mechanism, pre-osteoblasts are the main pro-osteoclastogenic cells, and they secrete two cytokines: the receptor activator of nuclear factor-κB ligand (RANKL), and the macrophages colony-stimulating factor (MCS-F), both cytokines will induce the differentiation of monocytic progenitors to osteoclasts(19). On the other hand, pre-osteoblasts that mature and slowly become more mineralized in response to Wnt signalling, finally become osteocytes. Osteocytes negatively regulate osteoclastogenic differentiation by secreting the RANKL decoy receptor osteoprotegerin (OPG) that binds to the RANK receptor, osteocytes also secret inhibitors of Wnt signalling such as sclerostin, dickkopf-related protein 1 and the secreted frizzled-related protein 1 that can also bind to and block RANKL(19, 20).

The second mechanism involves the interaction between osteoblasts and osteoclasts through the expression of ephrin ligands, which are found on osteoclast progenitors and can bind to ephrin receptors and activate their tyrosine kinase. There are two ephrin ligands that can regulate bone remodelling, ephrin B2 that binds to ephrin type-B receptor 4 on the osteoblast progenitors promoting their differentiation, and ephrin A2, which binds to ephrin type-A receptor 2 on osteoclasts to increase their differentiation in an autocrine manner, while its paracrine effect on the osteoblasts inhibits their differentiation(21).

Local immune regulation of bone remodelling

The immune system is essential for bone maintenance, and it can act as an activator/inhibitor of the bone remodelling process. This can be evident in autoimmune diseases such as rheumatoid arthritis and also in postmenopausal osteoporosis(22).

Just as osteoclasts, activated T cells also express RANKL, which was found in multiple subsets of proliferative T cells (CD4, CD8, T helper (T_h1) and (T_h2), suggesting that activated T cells are pro-osteoclastogenic, and could regulate inflammation-induced bone loss. Furthermore, T_h17 cells indirectly stimulate osteoclasts differentiation via IL-17, which triggers the production of pro-osteoclastogenic molecules such as RANKL and the macrophage colony-stimulating factor (M-CSF) by the mesenchymal cells(19, 23, 24).

T cells also express or secrete inhibitors which directly repress osteoclastogenesis such as the decoy receptor osteoprotegerin (OPG) and the cytotoxic T lymphocyte protein 4 which are secreted by T_{REG} cells, interferon-gamma (IFN-γ), which is secreted by T_H1, and interleukin 4 (IL-4) which is secreted by T_H2. CD40, which is expressed by activated $CD4^+$ and $CD8^+$, can both inhibit osteoclast proliferation by stimulating the production of OPG by B cells and promote osteoclast proliferation through increasing RANKL production by mesenchymal cells. Activated T cells role as pro-osteoclastogenic or anti-osteoclastogenic cells might depend on the local environment(25, 26).

Systemic regulation of bone remodelling

Systemic regulators can also influence bone remodelling. Two main systemic neuroendocrine pathways can regulate bone remodelling. The first one involves the co-regulation of bone, adipose

tissues, and energy metabolism, and this pathway is mainly controlled by leptin, a peptide hormone produced by the adipocytes of the white adipose tissues that decrease bone mass and obesity(27). Leptin regulates bone formation through two hypothalamic relays, Neuropeptide Y (NPY) and neuromedin-U, that inhibit osteoblasts proliferation and bone formation.(28-30) Leptin also impairs bone formation through the β–adrenergic sympathetic nervous system pathway, which inhibits osteoblast proliferation(31). Also, leptin promotes bone healing by maintaining the pool of undifferentiated mesenchymal precursors, and by stimulating the mineralization of osteoblasts(32).

The second neuroendocrine pathway that regulates bone is the hypothalamic-pituitary-thyroid axis(33). The hypothalamus secrets thyrotropin-releasing hormone (TRH), which induces thyroid stimulating hormone (TSH) by the pituitary gland, TSH stimulates the production of thyroid hormone T_3 and prothyroid hormone T_4(34-36). *In vivo* T_3 binds to TR-α1 in osteoblasts to increase their proliferation, while promoting osteoclasts differentiation directly or indirectly through osteoblasts(37). In addition, Parathyroid hormone (PTH) has a pivotal role in regulating calcium and bone physiology. PTH maintains calcium concentration by three pathways: Triggering bone resorption, stimulating renal calcium reabsorptions, and increasing calcitriol production(38). Calcitriol decreases bone resorption and increases the intestinal absorption of calcium(39). PTH also increases the proliferation of bone cells and matrix by increasing the production of insulin growth factor 1 (IGF-1) (40).

2.1.4 Bone healing

Bone has a unique ability that differs from other tissues in the body. It is capable of healing without a scar at the injury site. This healing process is complex, and it involves multiple mechanisms for the bone to return to its physiological and functional status(41). Bone healing is either ***primary (direct)*** or ***secondary (indirect)***. Primary bone healing is defined as the healing by a union of cortex to cortex in close proximity without motion between fragments(42). It requires absolute stability and rigid fixation along with a gap of less than 200 μm between the fractured fragments. Secondary bone healing refers to the bone formation that is preceded by an intermittent cartilaginous or fibrocartilaginous stage(43). It does not require optimum stability, and it is the most common form of bone healing(1).

Healing occurs in three stages: reactive stage, reparative stage, and remodelling stage(1).

1- Reactive stage (Formation of clot and granulation tissues):

When a fracture occurs, blood starts filling the fracture site, forming a clot. Followed by a rapid accumulation of inflammatory cells (e.g. macrophages, mast cells, neutrophils lymphocytes, monocytes) resulting in inflammation. The inflammatory cells then release multiple cytokines IL-1, -6, -11, -18, and TNF-α which are known to facilitate the formation of both capillary and bone cell formation. This process starts within the first hours of bone fracture and lasts for a few days, and it results in granulation tissues occupying the fracture site due to this inflammatory migration(1, 8, 44).

2- Reparative stage:

This stage follows the inflammatory process of the reactive stage. The reparative callus will be formed in this stage, and it is regulated mainly by the transforming growth factor-beta (TGF-β) superfamily, mainly BMP-5, BMP-6, and GDF10(45). The reparative stage starts with the vascular ingrowth, supported by a stroma produced by fibroblasts; this facilitates the recruitment of mesenchymal stem cells (MSCs) from the surrounding tissues and circulating blood. MSCs will differentiate into chondroblasts that will form a soft callus, which then undergoes mineralization by the collagen matrix osteoids. The formed callus is initially weak in the first 4-6 weeks and will require a form of support and protection (e.g. braces or internal fixation) in order to facilitate the ossification process. The reparative stage will eventually conclude when the chondrocytes are removed by osteoclasts, and MSCs differentiate into osteoblasts forming connecting bridges of woven bone between the fracture fragments(8, 10). In the case of improper immobilization of the fracture site, callus ossification could be impaired resulting in an unstable fibrous union between the bone segments(1).

3- **Remodelling stage**:

This stage involves the replacement of the woven bone with lamellar bone through a process that will take a minimum of 3-6 months(1). The objective of this stage is to restore the bone to its original status in terms of shape, functionality and adequate strength. This process is controlled by osteoclast/osteoblast interaction that starts with the resorption of existing bone and ends with the deposition of new bone. This remodelling cycle consists of three phases: initiation, transition, and termination(46).

a. Initiation phase:

The initiation phase of the remodelling cycle involves osteoclastic resorption of old bone, and it is mainly dependent on the interaction between osteoclast precursors and osteoblasts, mainly regulated through RANKL and M-CSF secreted by preosteoblasts(46). In addition, the release of cytokines such as tumour necrosis factor-alpha TNF-α and IL-1β from the mesenchymal and inflammatory cells and growth factor such transforming growth factor-beta (TGF-β) from the platelets degradation and from osteoblasts and chondrocytes at the fracture site help in promoting osteoclastogenesis(44).

b. Transition phase:

This is a period in which the osteoclast-osteoblast coupling process is switched from bone resorption to bone formation. Osteoblastic activities are increased and osteoclastic ones are reduced, as osteoclastic resorption of the bone matrix may liberate TGF-β, bone morphogenetic proteins (BMPs) and insulin-like growth factor II (IGF-II) that act on the osteoblast precursors to stimulate bone formation(46).

c. Termination phase:

Once an optimal bone formation is achieved. Osteoblasts differentiate into osteocytes, which will release a suppressing molecule called sclerostin, an inhibitor of osteoblastic activities that prevent further formation of excess bone. Also at this point, osteoclasts differentiation is also inhibited through OPG produced by osteoblasts(46, 47).

2.1.5 Molecular signals in fracture healing

Repair of a fracture site is influenced by three types of molecular signals: Inflammatory cytokines, growth factors, and angiogenic factors.

Inflammatory cytokines

The role of inflammatory cytokines in bone healing has been well documented in the literature. Mesenchymal cells and inflammatory cells release pro-inflammatory cytokines such as interleukin-1 (IL-1), (IL-6), and TNF-α at the injury site. These cytokines play a role in extracellular matrix (ECM) synthesis, angiogenesis, and recruitment of endogenous fibrogenic cells. IL-1 and IL6 expression levels peak during the first 24 hours of injury and elevate again during bone remodelling, they enhance extracellular matrix synthesis, promote angiogenesis, induce the recruitment and chemotaxis of endogenous fibrogenic cells and inflammatory cells to the injury site, and facilitate endochondral bone resorption(48, 49). While tumour necrosis factor-alpha (TNF-α) peaks during the first 1-3 days and also on day 14, and induces the recruitment of mesenchymal stem cells, the proliferation of osteoclasts, and is associated with removal of mineralized cartilages in endochondral ossification(50, 51).

On the other hand, multiple anti-inflammatory cytokines are also involved in the bone healing process. IL-4 and IL-13, cytokines mainly produced by the T_h2 of the $CD4^+$ T cells and by mast cells and basophils, are important in inflammation-induced bone loss.(52) They inhibit osteoclast formation and bone resorption by suppressing osteoblast RANKL and enhancing OPG

expressions, and by down-regulation of RANK expression on osteoclasts(53, 54). IL-10, which is produced by multiple immune cells such as macrophages and T cells, down-regulates the early phase of osteoclastogenesis by inhibiting RANKL induced Nfatc1, indicating that it acts in early RANK expression(55), IL-10 also inhibits $1\alpha,25(OH)_2D_3$ stimulated osteoclast formation without affecting mature osteoclasts(56). IL-17, produced mainly by the T_h17, coordinates local inflammation and regulates pro-inflammatory cytokines(57). IL-17 induces osteoclasts proliferation by its up-regulation of osteoblast RANKL expression, and by its action on osteoclasts progenitors to induce osteoclasts formation(58). IL-17 also promotes neutrophils recruitment to the inflamed site, linking it with inflammatory bone destruction(59).

Growth factors

Growth factors such as transforming growth factor-beta (TGF-β), morphogenetic proteins (BMPs), insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), and platelet driven growth factor (PDGF) are essential for bone fracture repair(49).

Transforming growth factor-beta (TGF-β)

TGF-β is a family of growth factors that during fracture healing are secreted by platelets after clot formation, chondrocytes, and osteoblasts in the initial inflammatory phase(60). They have an important role in endochondral bone formation by promoting the proliferation of mesenchymal stem cells, preosteoblasts, osteoblasts, and chondrocytes. They are also involved in the production of extracellular matrix by promoting the production of extracellular proteins (Collagen, proteoglycans,) and alkaline phosphatase(61).

Bone morphogenetic proteins (BMPs)

BMPs are members of the TGF-β superfamily, and they are produced by mesenchymal cells. They have a critical role in the bone healing process, specifically BMP-2, -4, and -7, which have been strongly associated with osteoblasts differentiation and bone healing. They are known to mediate angiogenesis by stimulating the expression of vascular endothelial growth factor (VEGF), as well as chemotaxis, proliferation and differentiation of mesenchymal and osteoprogenitor cells, and the formation of extracellular matrix(50, 62).

Growth hormones and insulin-like growth factor 1 (IGF-1)

Growth hormone releasing hormones (GHRH) produced by the hypothalamus stimulate the release of growth hormone by the anterior lobe of the pituitary gland. The growth hormone then stimulates the production, expression, and release of insulin-like growth factor (IGF-1) from the liver, developing periosteum, growth plates, and healing fracture callus(60, 61). IGFs produced by bone cells act as autocrine and paracrine regulators of bone healing. They stimulate the replication of pre-osteoblastic cells, and increase osteoclasts formation, which enhances the intramembranous bone formation, and improve the bone matrix formation. IGFs could get incorporated within the bone matrix and later released during bone resorption, leading to increased osteoblasts precursors proliferation(63). IGFs may also be secreted by chondrocytes, which increase cartilage matrix synthesis, decrease collagen degradation, and stimulate the clonal expansion of chondrocytes during the proliferative phase of bone healing(64).

Fibroblast growth factors (FGFs)

FGFs are produced mainly by osteoblasts, chondrocytes, macrophages, and monocytes. In the initial stages of fracture, FGFs induce angiogenesis and play a role in the proliferation and maturation of multiple cells such as osteoblasts, fibroblasts, and chondrocytes(49).

Angiogenetic factors

Adequate blood supply at the fracture site is essential for optimal healing; hence, the proliferation of blood vessels is of great importance. Angiogenesis is regulated by one of two pathways: the vascular endothelial growth factor (VEGF)-dependent pathway and the angiopoietin-dependent pathway(65). Osteoblasts express high amounts of VEGF and are its main regulator at the fracture healing site(50). Also, macrophages stimulate the expression and release of VEGF under hypoxic conditions via the intracellular transcription factor called hypoxia inducible factor (HIF-1) (66). VEGF acts as a neo-angiogenesis mediator that induces endothelial mitogenesis(67), and it also induces osteoblast's differentiation and promotes ossification(68). On the other hand, the expression of vascular morphogenetic proteins angiopoietin-1 and angiopoietin-2, is evident at the beginning of the healing process, and they initiate vascular in-growth from existing vessels into the periosteum by inducing the remodelling of the capillary basal lamina and promoting endothelial cells growth and migration(65, 69, 70).

2.1.6 Factors affecting bone healing

There are local and systemic factors that affect bone healing. Local factors that impair healing include the presence of bone pathologies such as malignancies, mobility at the fracture site, which can interfere with vascularization and bridging of the callus, presence of soft tissues at the junction

of the bony segments, and compromise blood supply (e.g. intracapsular fracture at the femoral neck). Fracture type also affects healing, as comminuted or displaced fractures will suffer delayed healing in comparison to non-displaced fractures. Additionally, infections at bone fracture sites can also compromise healing, and if left untreated, delayed union or non-union could develop(1). Systemic factors include age, systemic conditions (i.e. diabetes and smoking), nutrition and the use of medications. Age can affect healing capacity, as children heal better than adults, and the more mature the skeletal system, the slower the healing. Nutrition and use of medication also affect bone healing, poor bone healing correlates with inadequate nutrition, as a single long bone fracture could increase the nutritional demand up to 25%, while multiple injuries or the presence of infection could increase this demand up to 55%. Furthermore, the use of medications (e.g. non-steroidal anti-inflammatory drugs) could delay healing(71).

2.2 Osseointegration

Osseointegration describes the relationship between living bone and implant in direct contact at a microscope level. It was first described by Branemark as "the direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant". (2, 72)
Successful implant osseointegration depends on multiple factors involving the implant, the bone, and the surgical technique. These are:

a. Implant biocompatibility
b. Favorable bone quality
c. Sterile surgical environment
d. Minimally traumatic surgical technique

e. Achievement of initial implant stability. (73)

When the surgical placement is done, a key to successful osseointegration is ensuring that the implant is mechanically stable. Stability can be achieved in two stages; the first stage is primary stability, which is a result of a good initial mechanical locking between the bone and the implant surface. The second stage is secondary stability, which is gained during the period following the surgery by process of bone remodelling at the peri-implant area. The second stage follows the principles of bone remodelling during fracture healing(3).

Osseointegration of titanium implants occurs in four phases: Hemostasis, inflammation, proliferation, and remodelling(74).

Hemostasis begins immediately upon bone bed preparation by the implant drill for implant insertion, and it lasts from minutes to hours post-surgery. Mechanical crushing of bone during the implant procedure facilitates the release and activation of matrix proteins and growth factors and bleeding from the vessels facilitate polymerization of fibrinogen to form the initial extracellular matrix(75). Immediately after implant insertion, plasma proteins such as albumin, fibrin, and globulin will start adhering to the implant surface, increasing the protein concentration on the surface. Proteins with high concentration in the blood will be the first to attach to the surface of implants, these will later be replaced slowly by proteins with higher affinity such as fibronectin and vitronectin, this blood protein coating of titanium will facilitate subsequent cell attachment to the implant surface(76). Molecules such as fibrinogen, thrombin, thrombospondin, adenosine diphosphate (ADP) and collagen from a thrombus at the injury site will stop the vascular bleeding, resulting in platelet activation and aggregation, enhancing its ligand-binding activity onto the implant surface. Upon activation, platelets release growth factors TGF-β, PDGF, and FGF, as well

as serotonin and thromboxane, which will cause vasoconstriction, and supports the hemostasis phase(74).

Histamine, along with bradykinin released from the platelets will cause vasodilation, increasing the blood flow and vascular permeability, leading to increased serum proteins, fluids, and white blood cells, marking the beginning of the inflammatory phase(77). During this phase, the innate host defence system that consists of molecular (glycoproteins) and cellular (macrophages and neutrophils) elements will be activated in response to unspecific molecules of bacterial origin. When bacterial contamination is high, the neutrophils concentration will increase, causing a toxic effect that could lead to the loss of surrounding healthy tissues. This can be minimized by ensuring sterile surgical procedures and by using local antibacterial measures. Macrophage secretion of angiogenic and fibrogenic factors that are necessary for fibroblast activation, as well as increasing the concentration of growth factors will facilitate the transition to the proliferative phase(78).

The proliferative phase is characterized by the formation of a new cellular matrix, and by angiogenesis. FGF, PDGF, TGF-β, and connective tissue growth factor (CTGF) will stimulate and direct the migration of fibroblasts from the surrounding healthy tissues. Angiogenesis is stimulated by macrophages under hypoxic conditions; macrophages contain an intracellular transcription factor called hypoxia inducible factor (HIF-1) that stimulates the expression and release of VEGF, leading to the production of endothelial cell precursors(66). Angiogenesis controlled by VEGF, PDGF, and FGF will lead to the formation of new blood vessels that will connect with the surrounding blood vessels(79). The bone formation will follow the formation of new blood vessels, osteoprogenitor cells attach to the implant surface via cellular adhesion proteins (integrins) which attach to the ECM's fibronectin(80). Osteoprogenitor cells will eventually become active

osteoblasts, the main cells responsible for bone matrix formation and mineralization. The initially formed bone is randomly organized woven bone that is oriented parallel to the implant surface. During the remodelling phase, osteoclastic resorption and osteoblastic bone formation are coupled and balanced evenly in both the cortical and the trabecular bone(81). In cortical bone, osteoclasts resorb the woven bone in cylindrically shaped canals with a diameter of 150-200 μm each, which is equal to the diameter of an osteon, with an average speed of 50 μm per day. Supported by a vascular loop and perivascular progenitor cells, osteoblasts will first appear in the resorption canal 100 μm behind the osteoclasts, initiating the lamellar bone formation. Trabecular bone remodelling also follows the same coupling mechanism, proliferation and accumulation of osteoclasts will create resorption cavities that osteoblasts will refill with newly formed lamellar bone, the result of each resorption and bone formation is called a lamellar packet. The remodelling phase continues through life, and the resulted lamellar bone will attach to the tips of the implant macro-threads, leading to an even distribution of the occlusal load to the surrounding bone, improving the longevity of these implants(74, 81-83).

2.2.1 Osseointegration risk factors

Failure of implant osseointegration can be caused by multiple factors, generally categorized as endogenous and exogenous factors. Endogenous factors are divided into systemic and local ones. Systemic factors include conditions such as age and genetic diseases, osteoporosis, smoking and systemic medications. Local factors include bone quality, quantity, implant site, bone grafting, and bacterial infection. Exogenous factors include operator experience and the surgical technique(84).

Systemic risk factors are divided into two groups, high risk factors and significant risk factors. High risk factors include serious systemic conditions such as rheumatoid arthritis, immunologic impairment (e.g. HIV, patients on immunosuppressive medications), and alcohol/drug abuse. Significant risk factors include radiotherapy, uncontrolled diabetes, bleeding disorders, and heavy smokers(85).

2.3 Impact of drugs on bone physiology

Some medications used for the prevention of various medical disorders can alter the balance of the molecular regulators of bone metabolism, and thus could have a negative or a positive impact on bone accrual.(86) Underneath we discuss some examples of such medications.

2.3.1 Medications associated with negative effects on bone physiology

Thyroid hormones

Excessive levels of thyroid hormones can have a negative effect on bone by increasing bone turnover and decreasing bone mineral density (BMD). Indeed, thyrotoxicosis, a condition characterized by an increase in thyroid hormone T3, has been linked with an abnormal increase in bone turnover resulting in an overall increased bone loss and risk of fracture(87, 88).

Glucocorticoids

Glucocorticoids are used to treat autoimmune diseases, and they have been associated with decreased bone mineral density and increased risk of osteoporotic fractures. This is mainly due to their negative effects on osteoblastic function and osteoclast differentiation(89-91).

Thiazolidinedione

Thiazolidinedione is a glucose-lowering agent used for the treatment of diabetes mellitus type 2 that acts by targeting peroxisome proliferator-activated receptor-γ (PPAR-γ) and. PPAR-γ is known to control the differentiation of mesenchymal and hematopoietic cells; thus the use thiazolidinedione has been shown to act on the bone remodelling cycle by activating osteoclast differentiation and inhibiting osteoblast differentiation, resulting in increased bone loss and decreased bone mineral density(92-94).

Anti-epileptic drugs

There is an association between long term use of anti-epileptic drugs (AEDs) and bone disorders such as rickets and osteomalacia(95). Patients on long term AED (e.g. gabapentin, phenobarbital, carbamazepine) have shown signs of disrupted bone remodelling, lower bone mineral density (BMD), decreased calcium levels, and increased risk of fracture(95-97).

Selective serotonin-reuptake inhibitors (SSRIs)

SSRIs such as Prozac, Celexa, Paxil, and Lexapro are used to treat depression by inhibiting the reuptake of serotonin and increasing its level. SSRIs alter bone remodelling by inhibiting osteoblasts proliferation and increasing osteoclasts differentiation, which leads to increased bone

loss. Thus, studies have shown that patients using SSRIs had a reduced BMD when compared to non-users(98-100). This deteriorating effect has been linked to the inhibition of functional serotonin receptors found on both osteoblasts and osteoclasts.(101)

Proton pump inhibitors (PPI)

Proton pump inhibitors are widely used for the prevention and treatment of gastroesophageal reflux disease (GERD) and peptic ulcer disease. Proton pump inhibitors act on the membranes of parietal gastric cells by inhibiting the function of proton pump H^+/K^+-ATPases resulting in decreased H^+ secretion, thus suppressing gastric acidity. Proton pumps are also found in bones, and multiple studies have linked the use of PPI with increased risk of fracture, lower BMD, poor bone mechanical properties, and improper bone turnover(102, 103). PPIs impair bone directly by decreasing osteoclastic and osteoblastic cell density, decreasing osteoclastic resorption activity, and impairing osteoblastic mineralization(104). PPIs also indirectly affect bone metabolism by altering calcium homeostasis by decreasing the intestinal absorption of calcium(102, 105).

Histamine H2 Blockers

Histamine 2 blockers are used to treat gastric ulcers, and they have been associated with impaired bone metabolism(7). This can be linked to their effect on the histamine h_2-receptors on the surface of both osteoblasts and osteoclasts, and on the effect of histamine on osteoclast differentiation(106-108). A further detailed section on H2RA is included in this review.

2.3.2 Medications associated with positive effects on bone physiology

Bisphosphonate

Bisphosphonates are used in the treatment of Paget's disease, osteoporosis, mainly in postmenopausal women, bone cancer metastasis (breast, lung, and prostate), and to prevent pathologic fractures. These drugs have anti-resorptive effects on bone by binding to hydroxyapatite, preventing crystal dissolution(109). Also, Bisphosphonates, especially nitrogen-containing bisphosphonates, cause inhibition of farnesyl pyrophosphate synthase (FPP synthase), which causes accumulation of isopentenyl pyrophosphate (IPP). IPP accumulation results in the production of ApppI, which inhibits the mitochondrial adenine nucleotide translocase and ultimately causes osteoclast apoptosis (110, 111). Clinical studies showed increased bone mineral density and decreased bone loss in patients treated with bisphosphonates(112). On the other hand, medication related osteonecrosis of the jaw (MRONJ) is a rare complication associated with the use of anti-resorptive drugs, especially bisphosphonate(113). MRONJ is a result of suppressed cell proliferation, increased apoptosis, and decreased cell migration, leading to a necrotic center formation(114).

Estrogen

There is an association between estrogen deficiency and decreased BMD, increased bone remodelling, and increased risk of bone fracture(115). Estrogen deficiency causes an increase in bone resorption coupled with increase bone formation, although the bone resorption outweighs the bone formation leading to an overall bone loss. Estrogen replacement therapy inhibits bone resorption through its effect on the RANKL/RANK/OPG system, it suppresses RANKL

production and increases OPG production by osteoblasts, eventually leading to improved BMD and decreased risk of fracture(116-119).

Calcitonin

Calcitonin is a polypeptide hormone that is primarily synthesized in the thyroid gland. It is known to regulate the serum levels of calcium by inhibiting bone resorption and enhancing calcium excretion. It regulates bone remodelling by acting directly on the calcitonin receptors of osteoclast precursors preventing their differentiation to osteoclasts, and by inhibiting the release of acid phosphatase by osteoclasts(120). Treatment with calcitonin in postmenopausal women improves BMD, decreases bone loss, and decreases the risk of fracture, especially when combined with high calcium intake(121).

Dickkopf-1 (DKK1) antibody

Dickkopf-1 (DKK1) is a glycoprotein that inhibits Wnt signalling. The Wnt/β-catenin pathway promotes bone formation by increasing the renewal of stem cells, osteoblasts differentiation, and inhibition of osteoblasts and osteocytes apoptosis. DKK1 antibody is used to increase the differentiation of osteoblasts and osteoclasts, and thus increasing bone formation and improving the BMD(122, 123).

Vitamin D

Vitamin D plays an integral role in maintaining bone health. Its active form, $1\alpha,25(OH)_2D_3$, binds to the vitamin D receptor (VDR) and prevents calcium release from bone, increases both intestinal calcium absorption, and renal calcium reabsorption. Also, $1\alpha,25(OH)_2D_3$ affects bone remodelling

directly by binding to VDR receptors on osteoblasts, stimulating RANKL production. Vitamin D insufficiency decreases intestinal calcium absorption, resulting in a decrease in ionized calcium concentration, which increases parathyroid hormone (PTH) levels. The increase in PTH secretions causes increased osteoclastic bone resorption, which results in decreased bone mineral density (BMD), and increased risk of fracture(124-126). Vitamin D supplements given to elderly patients increase BMD and decrease bone loss, by increasing calcium and phosphate intestinal absorption and stimulating RANKL production(127).

Parathyroid Hormones (PTH 1-34)

PTH stimulates bone resorption and formation, depending on the blood serum level. An in vivo study found that rats given continuous PTH infusion showed an increased bone formation and bone resorption with a net decrease of bone volume, while daily injections of PTH resulted in an increase in bone formation without an increase in resorption, causing a net increase in bone volume.(128) Parathyroid hormones supplements given to patients with postmenopausal osteoporosis resulted in increased bone formation and improved bone mineral density (BMD)(129)

Sclerostin antibody

Sclerostin is a glycoprotein expressed by osteocytes that inhibits osteoblast differentiation and bone remodelling through the Wnt signalling pathway. Sclerotin antibody treatment has been shown to increase bone formation and strength in rat models.(130)

OBG-Fc

Osteoprotegerin (OPG) acts as a natural antagonist of RANKL signalling. It suppresses the formation of osteoclasts by binding to its precursors' receptors. Studies in rats using OPG-Fc showed a reduction in bone resorption(131).

Beta-blockers

Beta-blockers are one of the most commonly prescribed drugs to treat heart failure and hypertension. They act on β–adrenergic receptors, antagonizing their sympathetic action and resulting in decreased blood pressure. β–adrenergic receptors are also expressed in osteoblasts and osteoclasts; their activation leads to increased bone resorption and decreased bone formation. Thus, the use of beta-blockers has been associated with increased bone mineral density (BMD) and decreased risk of fracture in postmenopausal women(132-135).

2.3.3 Effect of systemic drugs on osseointegration

2.3.3.1 Systemic drugs associated with positive effects on osseointegration

Systemic drugs that have a positive effect on implant osseointegration include vitamin D, calcitonin, sclerotin antibody, DKK1 antibody, Parathyroid Hormones (1-34 PTH), Bisphosphonates, OBG-Fc, selective estrogen receptor modulator (SERM), simvastatin, and beta-blockers. **Vitamin D** supplements improve bone density around implants and improve overall stability by inhibiting bone resorption and promoting new bone formation(136). A study published recently linked the use of **calcitonin** in ovariectomized rats with an increase in bone mass around implants(137). Studies on the effect of **sclerostin antibody** administration in ovariectomized rats

resulted in an increase in bone formation, decreased bone resorption, and improved implant stability(138). **DKK1 antibody** in rat model study showed an increase in bone formation around implants facilitating bone-implant osseointegration(139). Studies done on ovariectomized rats to assess the effect of systemic administration of **PTH 1-34** showed an increase in bone formation around implants compared to the control group(140). **Alendronate (bisphosphonate)** administration in ovariectomized rats showed an improvement in titanium implants osseointegration and prevention of bone loss around inserted implants compared to control group(141). Rats treated with **OBG-Fc** showed an increase in bone density around titanium implants, and increased pull-out force, indicating a positive effect of OBG-Fc on implant osseointegration(142). A study done on ovariectomized rats assessing the use of **raloxifene, a selective estrogen receptor modulator,** on osseointegration showed an improved peri-implant bone healing(143). **Simvastatin**, a lipid-lowering agent which is known to regulate bone remodelling and osteoclastogenesis, has been shown to improve bone density around implants in rats(144). Post-operative use of **propranolol (Beta-blocker)** improved bone healing and osseointegration in rats(145).

2.3.3.2 Systemic drugs associated with negative effects on osseointegration

Systemic drugs that have a negative effect on implant osseointegration include, among others: Non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI), and selective serotonin receptor inhibitors. Reviews on the use of **NSAIDs** and its effect on implant osseointegration reported a decrease in peri-implant bone density and formation through their inhibitory effect on either cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes(146, 147). An *in vivo* study showed impaired bone healing and implant osseointegration

in rats that received **omeprazole (PPI)** (148). Furthermore, a retrospective cohort study suggested that the use of PPIs could be associated with an increased risk of failure of implant osseointegration(149). **Selective serotonin receptor inhibitors** use has been associated with increased risk of failure of implants osseointegration(150).

2.4 Histamine and histamine receptors

Histamine (2-[4-imodazole]-ethylamine) is an amine that can be found naturally in the body. It is produced by gastric enterochromaffin-like cells, neurons, lymphocytes, basophils, and mast cells (151) in response to various signals such as interleukin-1 (IL-1), IL-3, IL-12, IL-18, macrophage-colony stimulating factor (M-CSF), and tumour necrosis factor-alpha (TNF-a) (152).

Histamine was first discovered in 1910 by Sir Henry Dale and his colleagues. Not long after, it was found that it influenced the smooth muscle fibres of the lungs and intestines, stimulated cardiac contraction, and induced vasodilation and the shock syndrome.(153-155) Also, mast cells activated in reaction to implanted biomaterials release histamine results in a pro-inflammatory effect that increases the recruitment of phagocytes at the implant site(156).

There are four types of histamine receptors, named H_1-receptors, H_2-receptors, H_3-receptors, and H_4-receptors(155).

Histamine H_1-Receptors

H_1-receptors are found on multiple tissues and systems, including endothelial cells, lymphocytes, brain, smooth muscles of the respiratory and cardiovascular systems, gastrointestinal tract, genitourinary system, and the adrenal medulla. The primary role of H_1-receptors is modulating the

allergic reaction by increasing histamine release, expression and chemotaxis of eosinophils and neutrophils, and antigen-presenting cell capacity(157).

Histamine H_2-Receptors

H_2-receptors are present mainly in the gastric system and they have a strong effect on gastric acid secretion. They are also found in brain tissues, cardiac muscles, smooth muscles of the vascular tissues, the uterine, and the respiratory tract(155). They are also found on basophils and mast cells, which link histamine H_2-receptors to various immune functions. H_2-receptors on lymphocytes inhibit cytokine production, T-cell proliferation, cell-mediated cytolysis, and antibody synthesis(158).

In bone, H_2-receptors induce osteoclastogenesis by signalling the receptor activator of nuclear factor-$_k$B ligand (RANKL). Suppressing these signals has the potential to hinder the osteoblast-osteoclast differentiation(108).

Histamine H_3-Receptors

H_3-receptors are presynaptic receptors found in both the central and peripheral nervous systems, and they mainly control the release of histamine by mast cells(159).

Histamine H_4-Receptors

H_4-receptors are expressed on immune cells such as mast cells, monocytes, and T-cells. They are also found in the bone marrow, spleen, colon, and thymus. H_4-receptors mediate chemotaxis of eosinophils and mast cells, and they control dendritic cells activation and T cells differentiation(160).

2.4.1 Antihistamines

H_1-receptor antagonists (H1RA)

H1RAs are divided into first generation and second generation. The first generation H1RAs such as diphenhydramine, phenbenzamine, hydroxyzine, and promethazine are non-selective H1RA, and due to their high lipid solubility, can cross the central nervous system (CNS) blood-brain barrier, bind to central H_1 receptors, and alter the histamine action on them. Second generation H1RAs such as loratadine, cetirizine, and desloratadine have low lipid solubility, and thus their ability to penetrate the CNS is surely limited(161).

Both the first and second generation of H1RA are used as anti-inflammatory and anti-allergic medications due to their action on the peripheral H_1 receptors. They are used to manage acute and chronic urticaria and allergic rhinitis. First generation H1RAs, such as promethazine, are also used as sleeping aids due to their sedative effects. Adverse effects of H1RAs include impaired cognitive and psychomotor performance, hallucination, blurred vision, and dry mouth(162).

H_2-receptor antagonists (H2RA)

There are four H2RAs approved for clinical use, cimetidine, famotidine, ranitidine, and nizatidine. They bind to H_2-receptors and inhibit the vasodilatory effects of histamine, and suppress the parietal cells acid secretion(163).

Ranitidine was discovered in 1979 by Bradshaw and his colleagues.(6) Two years later, ranitidine was marketed, and it has been widely used as an over the counter acid-suppressive drug ever since then. It is five to twelve times more potent than cimetidine in inhibition of gastric acid secretion

but similar to cimetidine in ulcer healing and relapse prevention.(164) It has some common adverse effects such as headache, dizziness, and mild gastrointestinal disturbances, and rarer adverse effects such as sinus bradycardia, atrioventricular blockade, confusion and hallucination, and agranulocytosis (white blood cells injury). It also interacts with multiple drugs such as anticoagulants, anticonvulsants, beta-blockers, and calcium channel blockers(165). In recent years, more studies are focusing on the relationship between ranitidine and bone metabolism. There are reports that ranitidine down-regulate osteoclastogenesis by inhibiting the signalling of RANKL in osteoclasts and osteoblasts precursors blocking their differentiation(7).

H_3-receptor antagonists (H3RA)

The majority of H3RAs are still in clinical trials and not yet approved for clinical practice, but they have shown to counteract the effect of histamine on presynaptic H_3-receptors, leading to an increase in the release of multiple neurotransmitters such as histamine, dopamine, and serotonin. H3RAs can be beneficial in the treatment of narcolepsy (a rare long-term sleeping disorder characterized by excessive daytime sleeping), Parkinson's disease, depression, and Alzheimer's disease. In fact, Pitolisant, the first marketed H3RA, has been successful in the treatment of all narcolepsy syndromes.(166, 167)

H_4-receptor antagonists (H4RA)

H4RAs are relatively new, they are still in clinical trials and not yet approved for clinical practice. The use of an H4RA in animal models showed promising results in the management of asthma, pruritus, and colitis. In clinical trials, the use of H4RA (JNJ 39758979), reduced histamine-mediated pruritus, showing that *H_4-receptor* is involved in the pruritus response in humans(168).

2.4.2 Role of histamine in bone remodelling

Histamine has an influence on the overall health of bone. It is involved in bone remodelling by promoting osteoclastogenesis. In fact, patients suffering from systemic mastocytosis, a disease that causes excessive release of histamine by an increased population of mast cells, are known to suffer from osteoporosis and increased bone resorption(169). Histamine promotes osteoclastogenesis through two pathways, directly through autocrine/paracrine action on osteoclast precursors, and indirectly by increasing osteoblasts expression of RANKL/OPG. H_1R regulates osteoblast distribution in the presence of 1,25-$(OH)_2VitD_3$, whereas H_2R is expressed in osteoclasts precursors, and the use of histamine receptors antagonists decreases precursors availability and impairs osteoclastogenesis(108, 170). Studies done on ovariectomized rats showed decreased osteoclastogenesis and bone resorption when H_1-receptor and H_2-receptor antagonists were used (171, 172). Another study found that H_2-receptor antagonists affected osteoclastogenesis in the early bone remodelling phase in rats(173). A recent study in young rats showed an increase in bone resorption and a decrease in bone formation, leading to low bone mineral density (BMD) when using an H_2-receptor antagonist (ranitidine) (7). In humans, a study conducted on a US-based population linked the use of H_2-receptor antagonists with lower bone mineral density (BMD) (174).

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Chapter 3: Methods and Techniques

3.1 Micro-computed Tomography (μ-CT)

Micro-Computed tomography (μ-CT) is a non-destructive radiological imaging technique that produces three-dimensional (3D) information from two-dimensional trans-axial projections of a specimen. μ-CT functions by taking multiple, consecutive, high-magnification images at different viewing angles, which allows for an accurate three-dimensional model to be reconstructed.(1) The majority of μ-CT systems consist of a tungsten-anode x-ray tube, a radiation filter, a specimen stand, and an x-ray detector combined with a capture camera, and the reconstruction of 3D images by digital processing is done after obtaining multiple two-dimensional images by rotating the sample in its stand or rotating the emitter and detector for live animal images. (2)

The first μ-CT scanner was developed by Feldkamp and his colleagues at Ford Motor Company in the early 1980s to examine the structural defects in ceramic automotive materials, and soon after, he collaborated with the University of Michigan and published the first article on the use of μ-CT in the analysis of bone.(3) As a new non-destructive method of examining and analyzing three-dimensional (3-D) bone architecture in small animals, this technique offered a better alternative to the two-dimensional (2-D) X-ray technique that was used before. Quantitative histomorphometry was used previously, but it had limitations in terms of microstructure assessment as an analysis done in a two-dimensional state could lack the representation of the full status of analyzed bone.(4, 5)

μ-CT X-rays attenuation depends on the source of energy and the type of sample material in order to measure the density of the tissue. As X-rays pass through the target tissues, the intensity of the x-ray beam is reduced depending on the density of the tissue being imaged, this is explained in the

equation: $Ix=I_0e^{-\mu x}$ (x: the distance from the source, Ix: the intensity of the beam at x distance from the source, I_0: the intensity of the incident beam, μ: the linear attenuation coefficient).(6)

Advantages of using micro-CT

The use of μ-CT presents multiple advantages regarding the assessment of bone morphology:

1. Direct 3-D measurement of trabecular thickness and separation instead of extracting these values from a 2-D model.
2. Histologic evaluation or mechanical testing can be done after scanning as this technique is non-destructive.
3. Compared with both 2-D X-ray and 2-D histology, a larger volume can be analyzed using the μ-CT.

Practical uses of micro-CT

μ-CT has been extensively used in many studies in the industrial material analysis and in the medical research, such as those that involves bone morphological analysis, including: bone growth and development, disease models such as osteoporosis in animals, assessment of mechanical loading, assessment of pharmacological effects on bone, fracture healing and implant osseointegration in animals.(7, 8)

Standardization of μ-CT use and analysis have been established in the literature. This was done based on four main components: Image acquisition, image processing, image analysis, and reporting the results.(7)

Sample preparation is the first step. A sample should ideally be placed vertically along the axis of the scanner. A standardized medium should be used in all specimens, as scanning medium (e.g. saline, ethanol, or air) could affect x-ray attenuation. μ-CT systems have a range of 20 to 100

kilovolts (kVp) in terms of X-ray energy. Differentiation between bone and marrow can be best obtained in lower energies, while high-density materials may require the use of higher energy. Beam hardening is a phenomenon that occurs when a low energy x-ray is stopped, and high energy beam passes through the object, thus increasing the average energy of the x-ray beam. Filters can be used (e.g. aluminum foil) to reduce the effect of beam hardening. In terms of image processing, methods used for filtration or segmentation (e.g. Separating cortical and trabecular bones) should be described. Analysis of trabecular and cortical bones could include key parameters such as bone volume to total volume (BV/TV), trabecular thickness (Tb.Th), Trabecular separation (Tb.Sp), and trabecular number (Tb.N), and cortical thickness. These variables are also key components of the report of results when a test is concluded.(4, 5, 7)

Implant osseointegration assessment using μ-CT requires multiple steps to ensure proper calibration and avoidance of metal artifacts. Marco Laurenti et al., at McGill University, published a technique to optimize scanning of implants in bone using μ-CT by beam hardening minimization through using copper and aluminum foil filters, scanning with 100 kVp voltage and 100 μA current along with 4.5 um resolution. The second step was defining the correct grayscale threshold levels. The recommended minimal threshold for the implant was 130, while the threshold for the bone was 6 to 255. Minimizing dimensional errors and avoiding scattering artifacts is the last step, and it was done by setting a region of interest ranged 50-70 μm from the implant surface to be the peri-implant region.(9)

3.2 Histology and histomorphometry

Bone histomorphometry is a commonly used technique in animal and human studies. It is used to study, among other things, the effect of biomaterials, drugs, and diseases on fracture healing and bone metabolism. It is also used in pathology as a diagnostic tool for diseases.(10)

Frost, in 1958, was the first to describe a method to cut undecalcified bone samples. By using a diamond saw to cut the samples to 200-500 μm and then using wet sandpaper to create 100 μm thick slides.(11) Nowadays, bone samples are embedded in a transparent resin or plastic material that has similar mechanical properties as those of bone, this technique allows cutting the bone samples in sections less than 30 μm thick.(10)

Multiple stains are used for bone histomorphometry, the main stains used in animal bone analysis are von Kossa, tartrate-resistant acid phosphate (TRAP), and alkaline phosphate (ALP).

Von Kossa

Originally published by Von Kossa in 1901,(12) this stain uses silver nitrate to stain calcium deposits by replacing calcium ions with silver that binds to phosphate and degrades to form gray silver metallic deposits. This type of stain is used to quantify and measure the amount of mineralization, calcium deposition, and abnormalities in bone histology.(13)

Tartrate-resistant acid phosphatase (TRAP)

Tartrate-resistant acid phosphatase (TRAP) is a common enzyme found in immune and bone cells. It can be found in macrophages, osteoclasts, and dendritic cells. TRAP is also foubd in multiple tissues such as liver, lung, spleen, skin, and gastrointestinal tract. The TRAP enzyme plays a role in both immune and skeletal systems. It affects cytokine production, recruitment of macrophages,

production of collagen, and stimulation of osteoclasts proliferation. Hydrochloric acid and proteases secreted by osteoclasts degrade bone matrix, and the product of this degradation is transported intracellularly by intracytoplasmic vesicles that contain TRAP. Therefore, TRAP staining is used as a marker for osteoclasts activities.(14, 15) Acid phosphate activities are detrmined by *p*-nitrophenyl phosphate as a substrate in acetate buffer at pH 4.8, and the tartrate-resistant activities are determined by the addition of sodium tartrate. The samples are then incubated in a temperature 60-70 for a 30 minutes reaction time before analysis, allowing for activated osteoclasts to be stained red while bone is stained blue.(16, 17)

Alkaline Phosphatase (ALP)

Alkaline Phosphatase (ALP) is a membrane-bound metalloenzyme. It plays a role in bone formation and calcification. It is used as a marker for osteoblastic activities, tissue development, and as a diagnostic tool for bone diseases.(15) To measure ALP activities, bones are incubated in alkaline medium with calcium ions and organic phosphate ester. Inorganic phosphate ions are freed by the phosphate esters at sites with phosphatase activities and immediately precipitated by calcium ions to form an insoluble salt, which is then transformed into black silver or cobalt sulphide deposits for histomorphometric analysis.(18, 19)

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Chapter 4: Ranitidine Impairs Bone Healing and Implant Osseointegration in Rats' Tibiae

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Abstract

Background: Ranitidine is an H_2-receptor antagonist, extensively used as an over the counter gastric acid-suppressive drug. It has been found to have an impact on bone metabolism by suppressing osteoclastogenesis through the inhibition of the RANKL signaling in osteoclast and osteoblast precursors, blocking their differentiation. Thus, we hypothesized that the use of ranitidine would have a negative effect on bone healing and implant osseointegration. This study investigated the effect of post-operative administration of ranitidine on bone healing and osseointegration in rats.

Methods: Twenty-two Sprague Dawley rats underwent surgery to create a unicortical bone defect in each tibia. A titanium implant was placed on the right tibia defect, while the contralateral defect was left unfilled. After surgery, the rats were randomly divided into two groups receiving a daily dose of either ranitidine 30mg/kg (n=11) or 0.1 ml saline (n=11) for 14 days and then euthanized for assessment of bone healing and osseointegration using micro-CT and histomorphometry.

Results: Micro-CT analysis of the bone defect revealed a larger bone defect volume in the ranitidine group (0.82 ± 0.13 mm^3 vs 0.66 ± 0.16 mm^3; p=0.034), thinner cortical thickness (0.54 ± 0.07 mm vs 0.63 ± 0.11 mm; p=0.026) and less bone regeneration at defect site ($40 \pm 12\%$ vs $57 \pm 11\%$; p=0.003) in comparison to the saline group. Implant site micro-CT analysis showed less osseointegration in the ranitidine group ($34.1 \pm 2.7\%$ vs. $43.5 \pm 2.1\%$; p=0.014), and implant site histological analysis showed less medullary (p=0.021), cortical (p=0.001) and total (p=0.003) bone-implant contact, and less peri-implant BV/TV (p=0.002) in the ranitidine group in comparison to the control group. Histological analysis for osteoclastic activity (TRAP staining)

showed a lower number of osteoclasts in the ranitidine group (4.8 ± 2.4 mm^{-2} vs. 9.1 ± 2.1 mm^{-2}; p=0.026).

Conclusion: We concluded that the post-operative use of ranitidine impaired bone healing and osseointegration.

Keywords: Histamine-2 receptor antagonist, H2RA, bone healing, osseointegration, ranitidine.

Introduction:

Histamine is an immunological mediator and neurotransmitter that has a major physiological role in the body(1, 2). It is produced by gastric enterochromaffin-like cells, neurons, lymphocytes, basophils, and mast cells(3). It influences the smooth muscles fibers of the lungs and the intestines, it stimulates cardiac contraction, and it can induce vasodilation and the shock syndrome. Also, histamine has an influence on the overall health of bone. It affects bone remodeling by promoting osteoclastogenesis. This can occur directly through autocrine/paracrine action on osteoclast precursors, and indirectly by increasing osteoblast expression of the receptor activator of nuclear factor κB ligand (RANKL) and the decoy receptor osteoprotegerin (OPG) (4, 5). Thus excessive histamine release in patients suffering systemic mastocytosis (increase in mast cells, which produce histamine) causes increased bone resorption and osteoporosis(6).

There are four types of histamine receptors H_1, H_2, H_3, and H_4(7), which could be found on multiple sites, such as smooth and cardiac muscles, blood vessels, the gastrointestinal system, and the immune system(1). H_2-receptors are present mainly in the gastric system, but they are also found in brain tissues, cardiac muscles, smooth muscles of the vascular tissues, uterus, and the respiratory tract(7), as well as basophils and mast cells, which link histamine H_2-receptors with various immune functions. H_2-receptors on lymphocytes causes inhibition of cytokine production, T-cell proliferation, cell-mediated cytolysis, and antibody synthesis(8). In addition, the H_2-receptor is expressed in osteoclasts precursors, and the use of H_2-receptor antagonist decreases osteoclasts precursors availability and osteoclastogenesis(4, 5).

Ranitidine is a selective H_2-receptor antagonist (H2RA) that is widely used as an over the counter acid-suppressive drug(9). It is the second most commonly used drug for inhibition of gastric acid secretion, ulcer healing and relapse prevention(10). H2RAs act by inhibiting histamine-induced acid secretion that follows food ingestion, thereby acting as a prophylaxis or treatment for such conditions (11).

Studies have shown a possible relationship between H2RA and bone metabolism. H2RA suppresses osteoclastogenesis by inhibiting the signaling of RANKL in osteoclasts and osteoblasts precursors blocking their differentiation (12, 13). Ranitidine was also found to decrease osteoclastogenesis and bone resorption in ovariectomized rats (14, 15), and increase bone resorption and decrease bone formation and bone mineral density (BMD) in young rats (16). In humans, a study conducted in a US-based population linked the use of H2RA with low bone mineral density (BMD) (17).

Titanium implants are extensively used in orthopedic and craniofacial surgeries to support prosthetic replacements of missing structures such as hip and knee joint replacements and dental prostheses. These treatments require osseointegration, a direct contact between the implants and supporting bone to fulfil both functional and esthetic proposes (18). Osseointegration is a process that relies on proper bone metabolism. Thus, any condition that would hinder bone metabolism could, in turn, increase the risk of complications such as pain, infection, implant failure and mortality (19, 20).

Despite the well-established association between antihistamines and decreased bone turnover and bone quality, the effect of these medications on bone healing and bone-implant osseointegration remains unknown. Our hypothesis is that post-operative use of H2RA might have a negative

impact on healing and osseointegration. Accordingly, this study aimed to explore the effect of an H2RA, ranitidine, on bone healing and implant osseointegration in rats.

Materials and methods

Approval was obtained from the McGill Ethics Board Committee to perform this study (2012-7269). We used a total of 22 female rats (Sprague-Dawley) that were 12 weeks old and had an average weight of 250 g. The rats were housed at the Genome Animal Facility of McGill University in a controlled environment with a 12-hour day/night cycles (2 rats per cage). Conventional diet and water were provided ad libitum.

Titanium implants

The titanium implants used in our study were based on a previous publication from our laboratory (21). The custom-made Ti implants were fabricated by cutting a titanium rod (1.5 mm⌀, McMaster-CARR; Aurora, OH, USA) into 1.5 mm ⌀ x 2 mm long cylindrical implants. All implants were measured and sterilized prior to surgical insertion. No surface treatments were done.

Surgical procedure

Surgical experiments were conducted two weeks after the habituation period. Each rat was anesthetized using isoflurane (4% for induction and 2.5% for maintenance). After anesthesia, slow-release Buprenorphine (1 mg/kg) was administered. Then, both legs were shaved and disinfected using chlorohexidine before covering them with a sterile drape. A longitudinal incision (less than

10 mm) was made on the medial aspect of the proximal tibia using a 15 blade, and a periosteal elevator was used to deflect the muscle and expose the proximal medial diaphysis. A unicortical round defect (1.5 mm ⌀) was created with a cylindrical bur on the right tibia. Irrigated with saline to avoid damage by heat during the drilling process, and the defect was then filled with sterile custom-made titanium (Ti) implant. The same procedure was repeated on the contralateral tibia, and a 1.8 mm ⌀ unicortical defect was made but left empty.

After applying a single drop of lidocaine-mepivacaine local anesthesia at the surgical site, the incisions were closed using Vicryl 4.0, metal clips, and glue. All surgeries were done by a single surgeon blind to group allocation, using the same instruments, materials, and technique.

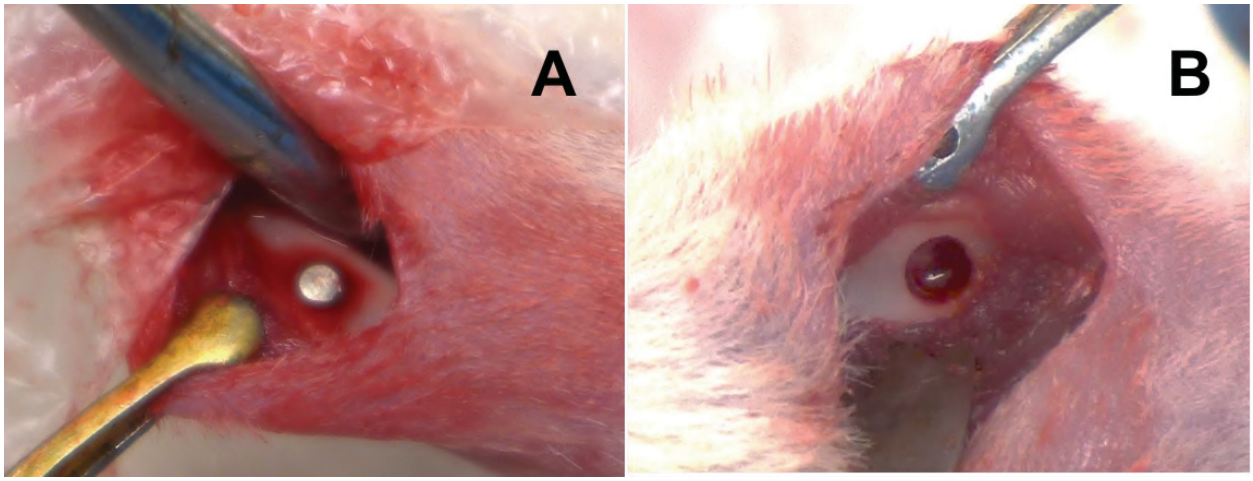

Figure 1: Surgery photographs. **A)** Right tibia unicortical defect with a custom-made titanium implant in place (1.5 mm ⌀ x 2.0 mm depth) to evaluate osseointegration. **B)** Left tibia unicortical defect (1.8 mm ⌀) was left without implant in site to evaluate bone healing.

Post-surgery

Subcutaneous injections of Carprofen (5 mg/kg) were given for analgesia on the day of surgery and daily until post-operative day 3. After surgery, a coin was flipped to decide which rat was going to be assigned randomly to either the experimental or control group. Post-operatively, the first group received ranitidine 30mg/kg via oral gavage, and the second group received saline as control, daily for 14 days. The dose of ranitidine was calculated based on previous studies done in rats to reflect the therapeutic dose given to humans (22). After two weeks period, all rats were euthanized using CO_2 asphyxiation, and both tibiae were harvested and stored in 70% alcohol. The samples were coded and labeled and the allocation to each group was blinded to the single person who did the analysis.

Micro-CT analysis

Micro Computed Tomography (μ-CT) (SkyScan1172; SkyScan; Kontich, Belgium) was used for 3D radiographic analysis. Two different sets of parameters were used to analyze the left (empty defect) and the right (titanium implant) tibiae.

The left tibiae defects were scanned and analyzed using the following setup: A resolution of 12.7 μm, 50 kV voltage, a rotation steps of 0.5° degrees, random movements of 10, and a 0.5 mm Aluminum filter. In order to measure the cortical thickness surrounding the defect, on a coronal section (figure 2. A), the bone volume of the cortical bone was calculated within a region of interest surrounding the defect, and the length of the region of interest span between the external and internal surfaces of the cortical bone, we used the equation: cortical thickness= cortical BV x L (Cortical BV: the bone volume of the cortical bone, L: the length of the region of interest). In figure 2. B, the defect volume was calculated within a region of interest that included the entire

defect site, using the equation: BV-TV (BV: bone volume, TV: total volume), bone architectures (trabecular thickness, trabecular number, and trabecular separation) were also calculated at the same time. Finally, the percentage of bone regeneration was calculated using the results of the cortical thickness and defect volume in the equation: Bone regeneration= (Total volume of original defect–defect volume) ÷ total volume of original defect, where the total volume of original defect= $(0.9)^2 \pi$ x cortical thickness

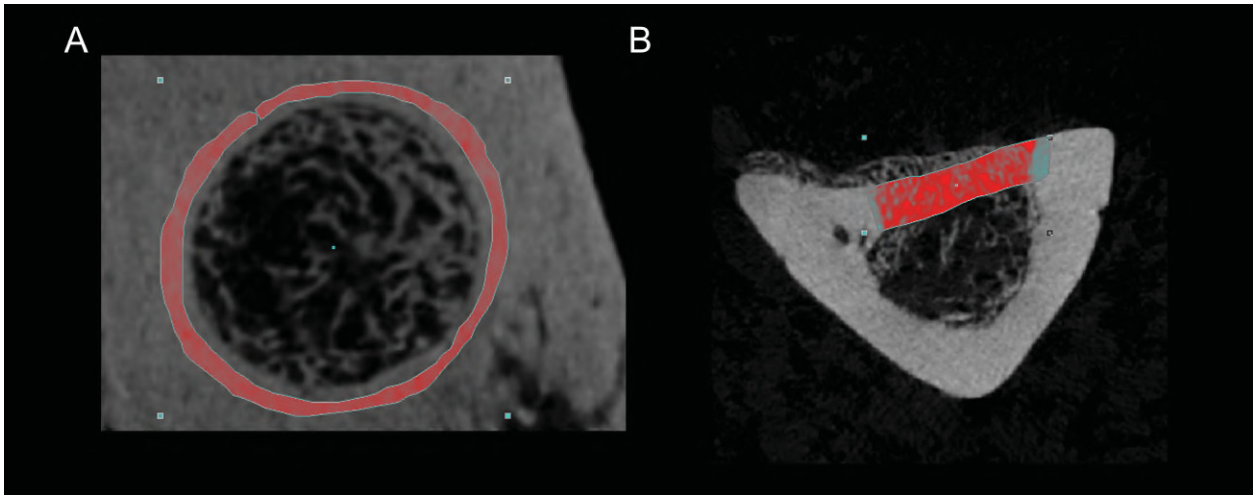

Figure 2: Bone defect analysis. **A)** Coronal image of a bone defect. Showing in red the region of interest used to measure the cortical thickness. Sagittal image of a bone defect. Showing in red the selected region of interest used for the analysis of defect volume, BV/TV, trabecular numbers, trabecular thickness, trabecular separation. **B)**. Analyses of the bone defects were done using CT-Analyser software (Skyscan1172, Bruker; Kontich, Belgium).

The right tibiae implants were scanned using specific criteria to ensure differentiation between highly radiopaque Titanium implants and the surrounding bone: Scans were done using a resolution of 4.5 µm, with a voltage of 100kV, a rotation step of 0.4° degrees, and an aluminum 0.5 mm/copper 0.04 mm filter, to minimize the beam hardening artifacts. After scanning and reconstruction, the reconstructed images were analyzed with two different parameters in order to select the correct area while avoiding dimensional errors and artifacts. To delimit the Ti implants, the minimum grayscale threshold was set at a value of 130, then a region of interest 50-70 µm away from the surface of implants was created for peri-implant bone analysis. Next, a gray scale ranging from a value of 6 to 255 was used to identify the bone surrounding the implant, and the region of interest created from the first analysis was imported to select the region 50-70 µm away from Ti implant in order to avoid the x-ray scatters from the implant surface, all analyses were done using CT-Analyser software (Skyscan1172, Bruker, Kontich, Belgium).

This technique for implant analysis was previously conducted and published by our laboratory (21, 23).

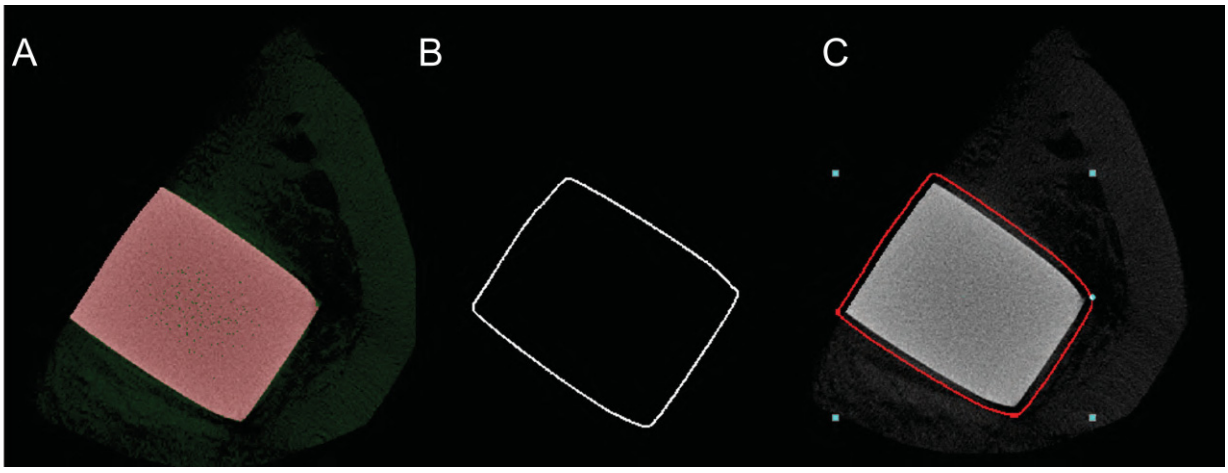

Figure 3: Implant osseointegration analysis with Micro-CT. **A)** Sagittal image of a Ti implant. Region indicated in red delimits the implant defined as having a radiopacity of more than 130 on

the gray scale. **B)** Sagittal image of the peri-implant region of interest 50-70 μm away from the Ti implant surface. **C)** Sagittal image of implant and its surrounding bone defined as having a radiopacity of 6-255 on the gray scale. The selected region of interest in red was used to calculate implant osseointegration percentage.

Histological analysis

All samples were dehydrated using an ascending concentration of ethanol, starting at 70% up to 100% before infiltration with polymethyl methacrylate histological resin. The resin-embedded samples were then sectioned using a diamond saw (SP1600, Leica Microsystems GmbH, Wetzlar, Germany). Sections were then prepared and stained using tartrate-resistant acid phosphatase (TRAP), acidified toluidine blue stains, and basic fuchsine/methylene blue to assess mineralization, osteoclastic activities, mast cells allocation, and peri-implant bone formation respectively. Images of the whole surgical site were captured at 40-power magnification using optical microscope ZEN-2012-SP2(Carl Zeiss Microscopy, Jena Germany). An oval region of interest comprising the bone regeneration area was delineated using ImageJ software (Wayne Rasband; NIH, Bethesda, MD, USA) as well as the peri-implant bone formation (bone volume % and bone-implant contact %), the number of osteoclasts (mm^{-2}), and the mast cell (mm^{-2}) and were quantified.

Statistical Analysis

The sample size for this experiment was calculated using a 0.8 power and a 0.5 α error to reject the null hypothesis, which states no difference in bone healing and osseointegration between ranitidine-treated and control groups. A difference of 10% was considered clinically significant,

and a 12% standard deviation was assumed based on a previous study.(24) We determined a sample size of 11 rats to be sufficient.

Statistical analysis was done using GraphPad Prism 7(GraphPad Software, San Diego, USA). Descriptive statistics were obtained, and normal distribution was tested using the Kolmogorov-Smirnov test. Student t-test was used to analyze statistical differences between experimental and control groups. Statistical significance was set at a P value of <0.05.

Results

The normality of data distribution was confirmed for both groups in all experiments. Bone defect analysis using μ-CT revealed a larger bone defect volume ($p=0.034$) in the ranitidine treated group (0.82 ± 0.13 mm^3) in comparison to the saline group (0.66 ± 0.16 mm^3). The cortical thickness was significantly ($p=0.026$) lower in the ranitidine group (0.54 ± 0.07 mm) compared to the saline group (0.63 ± 0.11 mm). Also, new bone formation within the defect site was lower ($p=0.003$) in the ranitidine group ($40 \pm 12\%$) compared to the saline group ($57 \pm 11\%$). No statistically significant differences between groups were found for trabecular numbers, trabecular separations, trabecular thickness, and trabecular pattern factor. (figure 5)

Histological analysis of osteoclastic activity (TRAP stain) at the bone defect site showed a decrease in the number of osteoclasts ($p=0.026$) in the ranitidine-treated group (4.8 ± 2.4 mm^{-2}) compared to the saline group (9.1 ± 2.1 mm^{-2}). Analysis of mast cell numbers (acidified toluidine blue stain) showed no statistically significant differences between the two groups ($p=0.61$). (Figure 6)

Implant site μ-CT analysis showed a reduction in osseointegration ($p=0.014$) in the ranitidine-treated group ($34.1 \pm 2.7\%$) compared to the saline-treated group ($43.5 \pm 2.1\%$). Histological

analysis of implants showed less total bone-implant contact (p=0.003) (11.2 ± 1.5% vs 24.7 ± 2.7), less medullary bone-implant contacts (p=0.021) (9.2 ±1.2% vs 14.9 ± 1.8%) and cortical bone-implant contacts (p=0.001) (18.3 ± 3.3% vs 40.8 ± 5.1%), and less peri-implant BV/TV (p=0.002) (31.5 ± 2.5% vs 48.4 ±2.6) in the ranitidine group in comparison to the control group. (Figure 7)

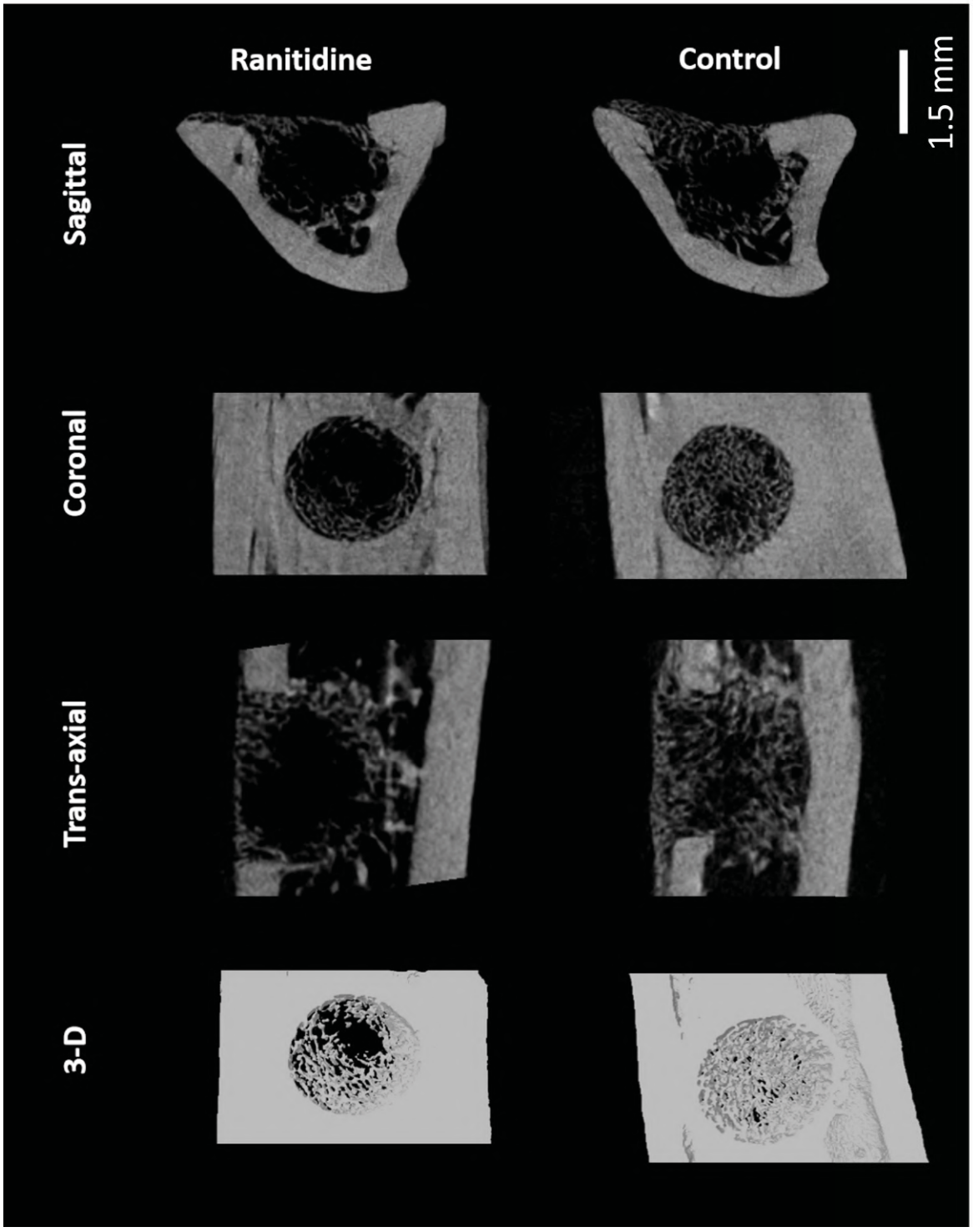

Figure 4: μ-CT images: Sagittal, coronal, trans-axial, and 3-D μ-CT images of bone defects showing compromised healing in ranitidine-treated animals in comparison to control group.

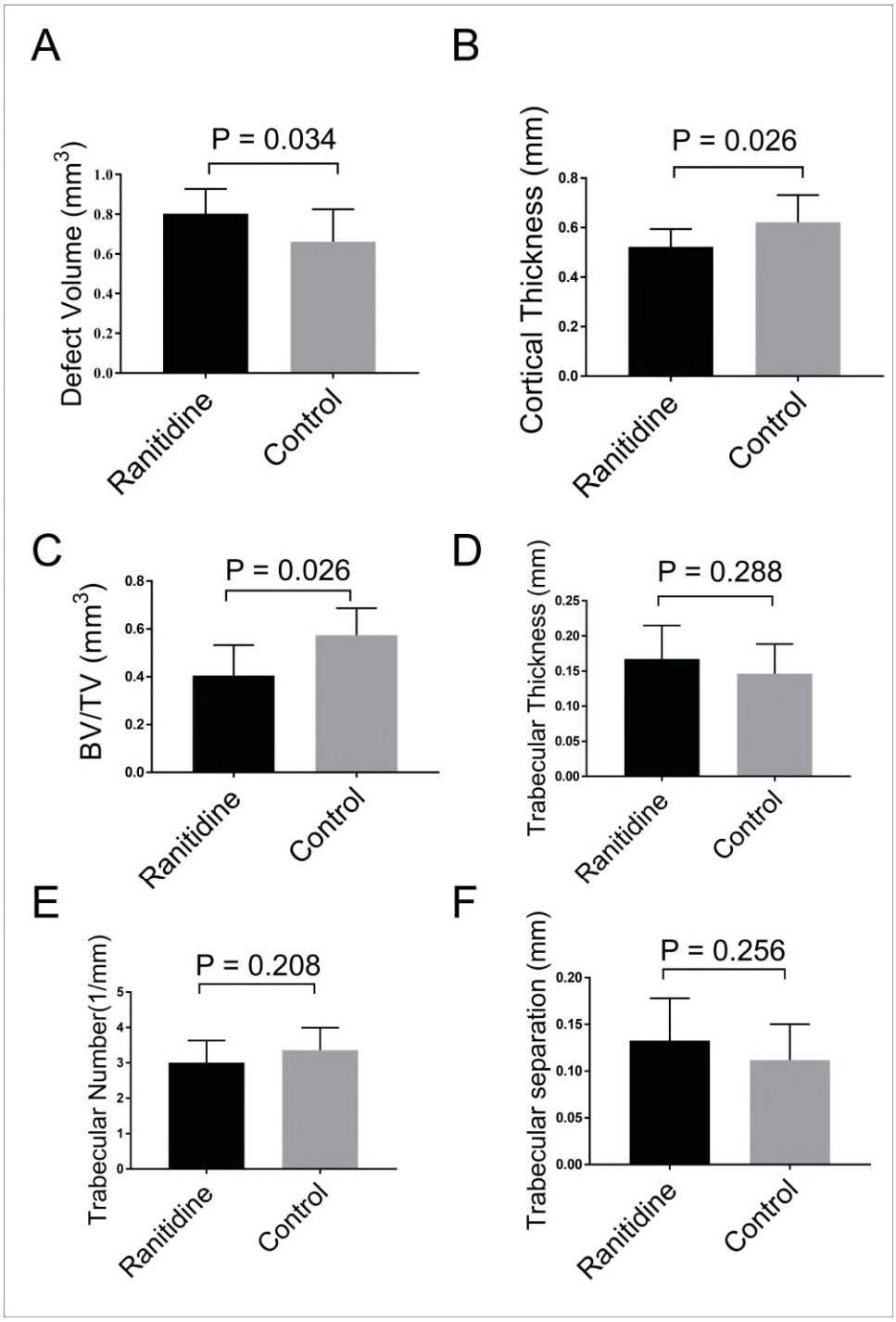

Figure 5: Bone defect healing: μ-CT analysis of bone defects comparing ranitidine-treated rats to controls showing differences in **A)** defect volume, **B)** cortical thickness, C. BV/TV, **D)** trabecular thickness, **E)** trabecular number, and **F**) trabecular separation. Statistical analysis was done using student's t-test.

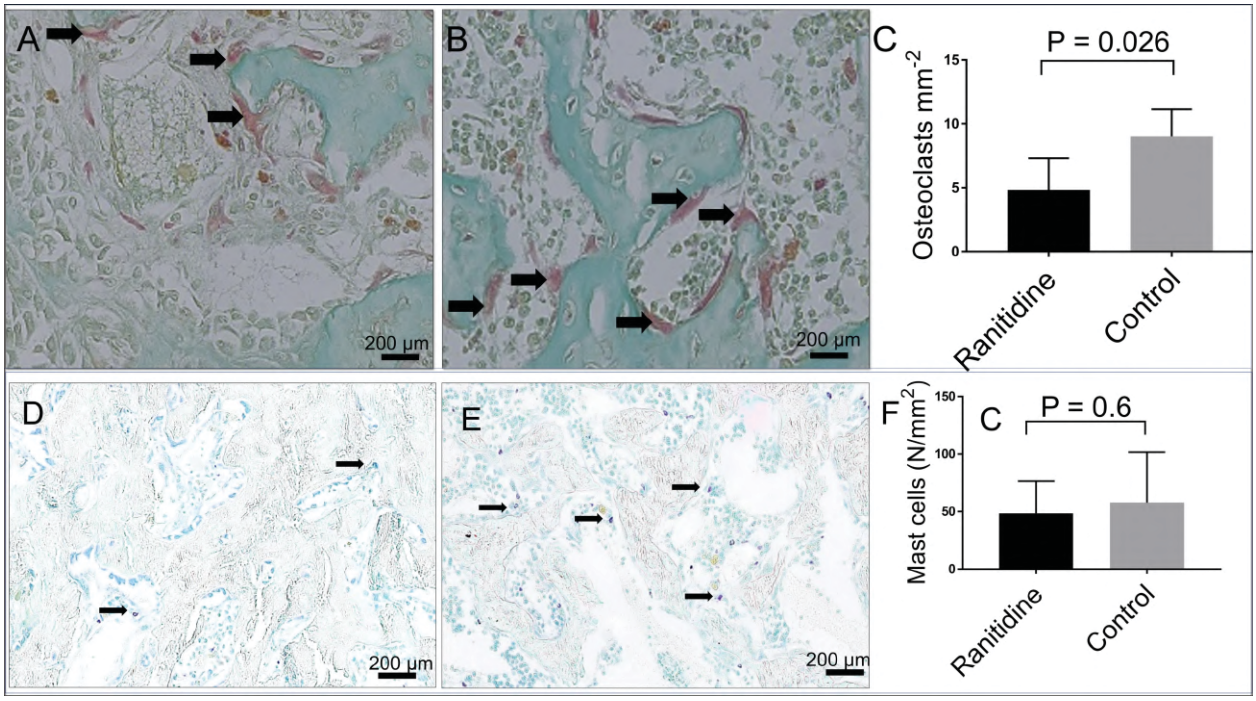

Figure 6: Analysis of TRAP-stained histological sections of bone defect samples comparing number of osteoclasts/mm^3 in ranitidine-treated rats to controls and Histological analysis of mast cells counts using acidified toluidine blue stain. **A.** Coronal histological section of bone defect in the tibia of a ranitidine-treated rat. **B.** Coronal histological section of bone defect in tibia of a control rat. **C)** Histomorphometric analysis of osteoclasts number. **D)** Coronal histological section of the bone defect in the tibia of a ranitidine-treated rat. **E)** Coronal histological section of bone defect in tibia of control group. **F)** Results of statistical analysis comparing mast cells number (mm^{-2}) in ranitidine group versus control group. Statistical analysis was done using student's t-test. Scale bar = 200 µm.

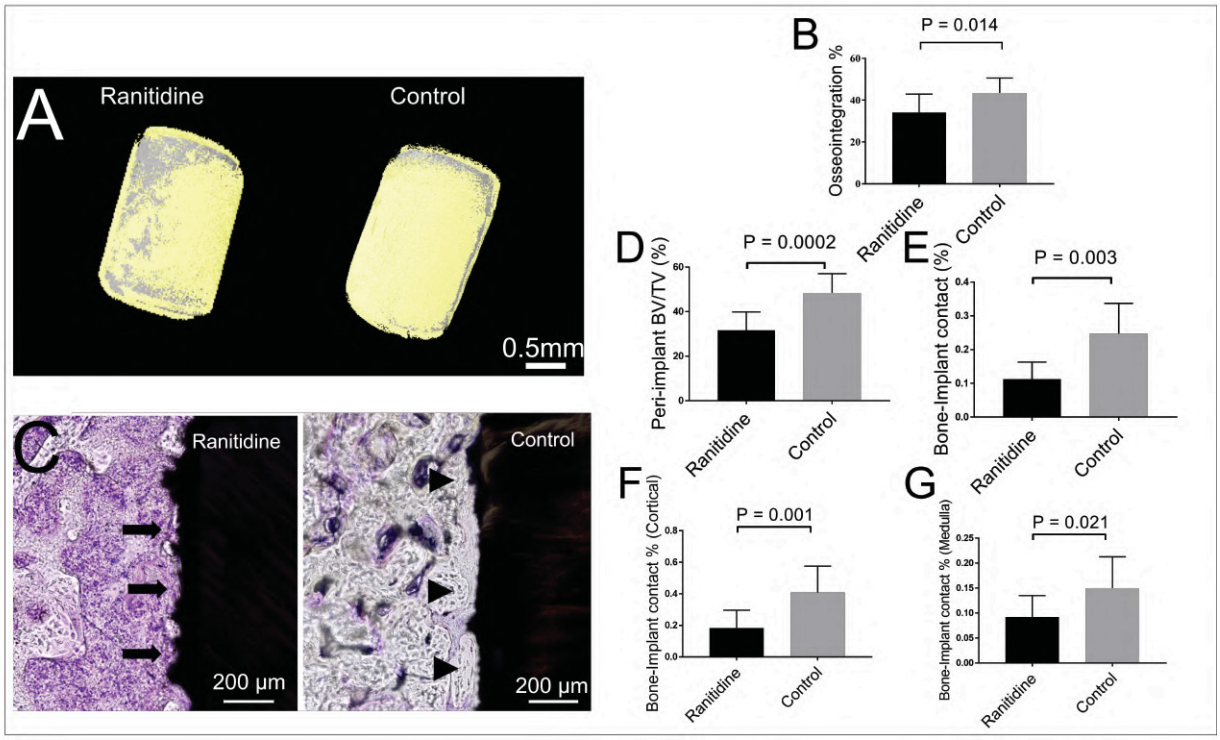

Figure 7: Implants osseointegration: **A)** 3D reconstruction of µ-CT images of implant (Grey) and bone (yellow) in ranitidine-treated group and control group. **B)** µ-CT analysis of implant osseointegration comparing ranitidine-treated rats to controls. **C)** Trans-axial histological sections of titanium implants in tibiae comparing ranitidine to control groups. Black arrows show soft tissues, black triangles show newly formed bones. Scale bar=100 µm. **D)** Histomorphometric analysis of osseointegration percentage (Peri-implant BV/TV). **E, F, G)** Total, Cortical, and Medullary bone implant contact respectively. Statistical analysis was done using student's t-test.

Discussion

To our knowledge, this is the first study that explores the effect of an H_2-receptor antagonist (Ranitidine) on bone healing and implant osseointegration. In the present study, we investigated the effect post-operative use of ranitidine on bone healing and implant osseointegration in a rat tibia model and found that post-operative use of ranitidine had a negative result on both.

Ranitidine decreases osteoclasts numbers in healing bone defects without affecting mast cells

Histological analysis of the bone defects showed a decrease in the numbers of osteoclasts in the ranitidine-treated group in comparison to the control group. This could be explained by the fact that ranitidine has the potential to hinder osteoclasts differentiation by impairing the expression RANKL in osteoblasts, as mature osteoblasts express H_2-receptors (13). RANKL, a cytokine from the tumor necrosis factor (TNF) superfamily, is expressed by osteoblasts in normal conditions and binds to RANK on osteoclast precursors playing a pivotal role in osteoclast differentiation and survival. Nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which has an integral role in osteoclasts differentiation, is also regulated by RANKL (25). Indeed, since osteoclasts precursors also express H_2-receptors, the use of H2RA (such as ranitidine, cimetidine, famotidine) have also been found to directly impair osteoclast precursors recruitment and differentiation (5).

Histamine can also affect osteoclastogenesis via signaling (RANKL) receptors indirectly by stimulating the production of macrophage colony-stimulating factor (M-CSF), interleukin-1 (IL-1) and IL-6, through H_1- and H_2-receptors on peripheral blood mononuclear cells, non-adherent mononuclear bone marrow cells, and macrophages (26, 27). These cytokines have a role in the formation of the extracellular matrix and differentiation from osteoclasts precursors to osteoclasts (28-30). M-CSF is produced by osteoblasts, fibroblasts, lymphocytes, and stromal cells, and it stimulates osteoclastogenesis through its effect on the c-Fms receptors, although, at high doses it can impair osteoclasts production (31). IL-1 and IL-6, produced by macrophages, B cells, monocytes, and cells of mesenchymal origin at the fracture site, are linked with the up-regulation of RANKL in osteoblasts and bone stromal cells which induces bone resorption (32). Also, IL-6 causes a decrease in RANKL-induced osteoclastogenesis by suppressing differentiation (33).

Histamine released from mast cells increases calvarial bone loss by promoting osteoclastic differentiation via H_2-receptors (34, 35), where H2RA (cimetidine) has been shown to increase osteoclast apoptosis and decrease RANKL expression in rats' periodontium (13).

Along with basophils, mast cells are one of the main producers of histamine. However, in our experiment, we did not find a statistically significant difference between the ranitidine and control groups in terms of the number of mast cells at the defect site. This could be explained by the fact that ranitidine acts only on H_2-receptors and does not affect other histamine receptors, and mast cells allocation and migration at bone healing sites is controlled by H_4-receptors (36).

Ranitidine has a negative effect on bone healing

Our μ-CT analysis of bone defects showed that ranitidine negatively affected bone formation, cortical thickness, and defect volume. This is probably related to the negative effect of ranitidine

on osteoclasts we observed. Osteoclast numbers and osteoclastic resorption are essential for bone healing because they regulate osteoblastic proliferation (37). During active bone resorption, multiple bone-resorption driven cytokines and growth factors incorporated within the bone matrix such as TGF-β, FGF, BMPs, PDGF and IGFs are released, leading to an increase in the osteoblast precursors proliferation proportional to the amount of resorption (38, 39). Also, *in vitro* and *in vivo* studies found that osteoclast-derived ephrinB2 promotes osteoblastic differentiation and bone formation through its direct contact-dependent action on EphB4 (40). Our results of impaired bone healing might be due to the decreased number of osteoclasts, which could negatively affect the osteoclast-osteoblast coupling mechanism.

Although our results showed a decrease in bone formation, we found no significant differences between the ranitidine and control groups in terms of trabecular number, trabecular thickness, and trabecular separation. These results correlate with the results of another study that investigated the effect of H2RA on bone accrual (41), and the lack of significant difference between the study groups might indicate that ranitidine does not affect the function of osteoblasts or osteoclasts but rather impacted cells availability. Further studies and analyses of these observations should be conducted to prove this hypothesis.

Table 2: Review of articles on the effect of histamine H_2-receptor antagonist (H2RA) on bone accrual (5, 13, 14, 16, 17, 41)

Author, Year	Study groups	Outcome
Matuszewska et al, 2018	Rats treated with ranitidine (n=10), and controls (n=10)	Long term use of ranitidine increased bone resorption and decreased BMD
Longhini et al, 2013	Rats treated with cimetidine (n=6), and controls (n=6)	Cimetidine decreased osteoclast numbers, increased osteoclast apoptosis, and reduced RANKL immunoexpression
Biosse-Duplan et al 2009	Rats treated with Histamine(n=6), mast cells degranulating agent c48/80(n=6), and controls (n=8)	Histamine promoted osteoclastogenesis and the expression of RANKL. The use of H2RA decreased osteoclast recruitment and bone resorption
Lesclous et al, 2006	Rats treated with cimetidine (n=8), controls (n=8), Ovariectomized rats treated with cimetidine (n=8), and ovariectomized controls (n=8)	Non-OVX rats showed no statistical differences between the two groups. In OVX rats, H2RA decreased the number histamine-releasing cells (mast cells and others), osteoclasts and increased bone resorption.
Lesclous et al, 2002	Rats treated with famotidine (n=10), controls(n=10), Ovariectomized rats treated with famotidine (n=10), and ovariectomized controls (n=10)	Non-OVX rats showed no statistical differences between the two groups. OVX rats treated with famotidine showed decreased osteoclast differentiation and bone resorption.
Kinjo et al, 2008	Human patient treated with H1RA (n=199), Treated with H2RA (n=297), and controls (n=4162)	H2RA users showed less femoral neck BMD compared to H1RA users and non-users

BMD: Bone mineral density, H1RA: H_1-Receptor antagonist, H2RA: H_2-Receptor antagonist

Ranitidine has a negative effect on implant osseointegration

μ-CT analysis of implants showed a significant decrease in osseointegration in ranitidine-treated rats compared to saline-treated rats. Histological analysis confirmed the results by showing a decrease in bone-implant contact percentage and peri-implant BV/TV in the ranitidine group in comparison to the control group. Our osseointegration results in the control group (43%) were similar to previous studies published using the same materials and methodology (21). However, the ranitidine group showed a significant decrease in osseointegration, which is known to affect the mechanical fixation of Ti implants negatively (20, 42).

A key for successful osseointegration is implant stability. This can be achieved through primary stability, which is a result of a good initial mechanical locking between the living bone and implant threads, and later on through secondary stability, which results from bone remodeling at the peri-implant area, following the same principles of bone remodeling during fracture healing (43). We expect that the negative impact of ranitidine on implant osseointegration was likely due to the impairment of the bone healing process we observed and discussed above.

Limitations and future direction

In our study, we tested the effect of ranitidine on bone healing and implant osseointegration at a single post-operative time point 2-weeks after surgery. This time point was selected as it roughly marks the period of which the cartilaginous callus is completely formed, and bone cells start to replace it, this period allows an excellent assessment of any effect drugs could have on the early stages of bone healing in terms of bone cells quantity and function (44). However, further studies

will be needed to study the pre-operative, immediate post-operative (day one), and long term use of ranitidine on bone healing during the maturation phase and on implant osseointegration.

Furthermore, this study was done in rats' tibiae with a surgical model that offers ease of accessibility, and reliable reproducibility of results. Nevertheless, further studies to investigate the effect on other bones (e.g. craniofacial bones vs long bone in tibia) and in humans would be recommended to further establish the result.

Clinical significance

Our findings indicate that ranitidine could have a negative impact on short-term bone healing, while the literature also reported that it does not influence long-term bone remodeling (45). On the other hand, proton pump inhibitors (e.g. omeprazole) have been proven to have a negative impact on bone healing and osseointegration on short and long-term remodeling (21, 46, 47). Further studies to investigate the effect of both drugs on bone healing and osseointegration on the short-term and long-term would be recommended to establish a comparison between both H2RA (ranitidine) and PPI (omeprazole).

Furthermore, ranitidine might pose a potential risk in bone and implant surgeries as it could delay healing and osseointegration of implants.

Conclusion

Our results indicate that the use of ranitidine post-operatively reduces bone formation in rats' tibia bone defects and implant osseointegration. The results showed significantly less number of osteoclasts.

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