The Effect of Zoledronate Pretreatment on BMP Induced Bone Formation in Mice

by

Marina Prichert

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Oral & Maxillofacial Surgery
Faculty of Dentistry
University of Toronto

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Abstract

Recombinant human bone morphogenetic proteins (rhBMPs) are increasingly used for reconstructing bony defects, fracture non-unions, and augmenting existing bone volumes. For the numerous patients taking bisphosphonates, the impact of prior bisphosphonate treatment on rhBMP bone induction is not well understood. **Objective:** to evaluate the effect of the prior treatment with zoledronate on rhBMP induced bone formation in mice. **Methods:** 42 mice were pre-treated with 0, 2, and 20 µg of zoledronate/mouse. The osteoinductive activity of a bioimplant, containing rhBMP-2, was assessed using the mouse muscle pouch assay, and analyzed with micro CT and histology. **Results:** micro CT demonstrated that BMP bioimplants placed in mice pretreated with 20 µg of zoledronate, formed bony ossicles of greater volume but reduced bone density compared to controls. Histologically, the heterotopic ossicles from the 20 µg group consisted of more immature bone than those from the other groups. **Conclusion:** bone induced by rhBMP-2 in mice pre-treated with a high concentration of zoledronate was immature as evidenced by radiographic and histologic appearance.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACS</td>
<td>Absorbable collagen sponge</td>
</tr>
<tr>
<td>ActR</td>
<td>Activin receptor</td>
</tr>
<tr>
<td>ADU</td>
<td>Arbitrary density unit</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>µA</td>
<td>Microamperes</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone morphogenetic protein receptor</td>
</tr>
<tr>
<td>rhBMP</td>
<td>Recombinant human bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Basic multicellular unit</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>Core-binding factor A1</td>
</tr>
<tr>
<td>CDMP</td>
<td>Cartilage-derived morphogenetic protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DBM</td>
<td>Demineralized bone matrix</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl diphosphate synthase</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kVp</td>
<td>Kilovolt potential</td>
</tr>
<tr>
<td>mCT</td>
<td>Micro CT</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDAF</td>
<td>Macrophage derived angiogenesis factor</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>msec</td>
<td>Milliseconds</td>
</tr>
<tr>
<td>N-BPs</td>
<td>Nitrogen-containing bisphosphonates</td>
</tr>
<tr>
<td>OP-1</td>
<td>Osteogenic protein 1</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly-methyl-methacrylate</td>
</tr>
<tr>
<td>PO</td>
<td>Per os (by mouth)</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear kappa-B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear kappa-B ligand</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SBPs</td>
<td>Simple (non-nitrogen-containing) bisphosphonates</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartarate-resistant acid phosphatase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Chapter 1
Introduction

1.1 Motivation and Study Objectives

BMPs are multifunctional cytokines that belong to the transforming growth factor beta (TGF-β) superfamily. They are able to induce bone formation when implanted in ectopic sites by the transformation of undifferentiated mesenchymal cells into osteoprogenitors [1]. The capability of bone morphogenetic proteins (BMPs) to induce bone formation has, since their discovery by Urist in 1965, attracted numerous investigations which explored the potential of exogenous application of BMPs in the management of skeletal injuries and diseases [2].

The osteoinductive capability of BMPs has been utilized to improve bone healing in fracture non-unions, long bone open fractures, and spinal fusion [3-5]. Approximately 25% of spinal fusion surgeries in the US employ BMPs to augment bony healing [6]. BMPs have also been employed in the reconstruction of mandibular defects [7] as well as maxillary sinus augmentation [8].

Bisphosphonates are synthetic analogs of the naturally occurring pyrophosphate with a high affinity for hydroxyapatite [9]. Since bisphosphonates are potent osteoclast inhibitors, they are used in the treatment of diseases exhibiting pathologic bone catabolism, namely osteoporosis [10], Paget’s disease, osteogenesis imperfecta of childhood, hypercalcemia associated with malignancy, and osteolytic lesions arising from any solid tumor such as breast, lung, or prostate cancer, as well as multiple myeloma [11]. The therapeutic goal, when treating with bisphosphonates, is to arrest bone loss, increase bone density, and decrease the risk of pathologic fractures.

Bisphosphonates administered via the oral route, are commonly prescribed to stabilize bone loss caused by osteoposis in post-menopausal women. Intravenous bisphosphonates are used to limit the proliferation of metastatic cancers in bone and
have been shown to improve the quality of life and even extend life in cancer patients [11].

There is a growing body of evidence suggesting that bisphosphonates affect cell types other than osteoclasts, including osteoblasts, and endothelial cells. However, these effects are not normally seen due to the low concentration of bisphosphonates in tissues other than bone.

The effect of bisphosphonates on cells associated with bone formation and repair raises a question as to the influence of bisphosphonates on the use of BMPs clinically. While studies have demonstrated that following BMP treatment with bisphosphonates results in delayed remodeling of BMP induced bone [12], there are no reports about the effect of prior bisphosphonate treatment on the ability of BMPs to induce bone formation. Thus, it is uncertain if the effect of BMPs in bisphosphonate users may be compromised, and a higher dose of BMPs may be required for these patients to achieve a result similar to patients not receiving bisphosphonates. The objective of this study was to evaluate the effect of pretreatment with the bisphosphonate zoledronate on BMP-induced bone formation in mice.
1.2 Bone Biology

Bone is a unique biological tissue composed of a rigid framework which integrates within it active cells. Bone serves a variety of functions, including structural support, leverage for muscles to permit locomotion, protection of internal organs, reservoir of calcium and phosphate ions to maintain serum homeostasis, and hematopoiesis within the marrow spaces [13]. A variety of cellular, hormonal, biomechanical, and pathological mechanisms influence the healing potential of bone in fracture repair and graft incorporation. The ability of bone to heal is facilitated by the continuous state of resorption, deposition, and remodeling [14].

1.2.1 Bone Biochemistry

Bone is composed of a combination of organic and inorganic elements, with 20% of its weight comprised of water. The remaining 80% of the weight is composed of inorganic calcium phosphate (65-70%) and organic matrix of collagen and protein (30-35%). Osteoblasts secrete a nonmineralized organic matrix called osteoid. Osteoid is composed of type I collagen (90%) and ground substance (10%). The ground substance includes proteoglycans, peptides, glycoproteins, noncollagenous proteins, carbohydrates, and lipids [14]. The collagen fibrils are laid down in a lamellar pattern with alternating orientation in every layer. The lamellae are either concentric or parallel, the former, when deposited around a channel or a blood vessel, and the latter, when deposited along a flat surface. The alternating orientations of collagen fibrils confer significant strength. In woven bone, the collagen fibrils are laid down in a disorganized manner as a result of rapid bone formation, making woven bone weaker than lamellar bone [13].

Whereas the organic matrix provides the elasticity and flexibility of bone, the mineralization of osteoid by inorganic salts provides the mechanical rigidity and the load-bearing strength of bone. Bone mineral content consists primarily of calcium carbonate and calcium phosphate with small quantities of fluoride, magnesium, and sodium. The mineral crystals precipitate as hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ producing
an orderly arrangement around the collagen fibrils [13]. As the bone matures, hydroxyapatite crystals enlarge by crystal growth and by aggregation. Osteoid begins to calcify a few days after secretion, and the calcification takes several months to complete [14].

1.2.2 Bone Anatomy

Osteoprogenitors, osteoblasts, osteocytes, osteoclasts, and hematopoietic bone marrow cells comprise the cellular components of bone. Osteoprogenitors are present in the deep layer of the periosteum and the endosteum, with the former surrounding the outer surface of bone and the latter lining the internal medullary surface. The periosteum is divided into two layers: a tough fibrous outer layer composed of dense connective tissue, and a thin inner cambium layer containing osteogenic cells. The endosteum is composed of only a thin osteocompetent layer [14].

Osteoblasts are specialized bone forming cells with several important functions, including the production of bone matrix proteins, mineralization of bone, and expression of osteoclastogenic factors. Osteoblasts develop from pluripotential mesenchymal cells that are also able to differentiate into chondrocytes, myocytes, and adipocytes in the presence of the appropriate regulatory transcription factors [15]. Osteoblast differentiation is regulated by the transcription factor Runx2 (runt-related transcription factor 2) that is also known as Cbfa1 (core-binding factor A1). Some osteoblasts are converted to osteocytes as they become entrapped within the osteoid, or the osteoblasts remain on the periosteal or endosteal bone surfaces [16].

Osteocytes are mature osteoblasts that are trapped within the matrix of bone in osteocytic lacunae. Each osteocyte maintains a network of cytoplasmic processes that extend through cylindrical canaliculi to blood vessels and other osteocytes. Osteocytes function via intercellular interactions in response to local stimuli by helping to regulate the extracellular concentrations of calcium and phosphate, and they also play a role in mechanosensation [13].

Osteoclasts are cells uniquely adapted to resorb bone. Osteoclasts are derived from mononuclear precursor cells of the monocyte-macrophage lineage. Osteoclast
formation is modulated by two key cytokines: RANKL (receptor activator of nuclear kappa-B ligand) and M-CSF (macrophage colony-stimulating factor) [15]. Osteoblasts and marrow stromal cells control the development of osteoclasts by synthesizing the osteoclastogenic factors RANKL and M-CSF. M-CSF is required for the proliferation and differentiation of osteoclast precursors, as well as the cystoskeletal arrangement and survival of mature osteoclasts [16]. RANKL is a member of the tumor necrosis factor (TNF) cytokine family and is found on the surface of osteoblasts and stromal cells. It interacts with RANK, a transmembrane protein receptor expressed on the surface of osteoclast precursors. The binding of RANKL to RANK, results in RANK activation and intracellular signal transduction, initiating osteoclast differentiation and activation. OPG (osteoprotegerin), a member of the TNF receptor superfamily that is secreted by osteoblasts, acts as a soluble receptor antagonist for RANK. Its binding to RANK inhibits the interaction with RANKL, and therefore, prevents osteoclast differentiation. The relative ratio of RANKL to OPG determines the number of osteoclasts formed [17].

Osteoclasts modulate bone resorption by secreting hydrogen ions and collagenolytic enzymes, including cathepsin K, tartrate-resistant acid phosphatase (TRAP), and matrix metalloproteinase 9 (MMP-9). Hydrogen ions acidify the resorption compartment and dissolve the mineral component of bone matrix, whereas the proteases digest the proteinaceous type I collagen matrix. Osteoclasts have integrin receptors in their cell membrane that bind to bone matrix peptides. This binding results in osteoclast membrane polarization producing a ruffled membrane border on the bone resorbing side. The ruffled border results in increased surface area and is produced by the fusion of cytoplasmic vesicles containing cathepsin K and the other proteases. Hydrogen ions are secreted from the ruffled border via a hydrogen ion-ATPase and chloride channels, producing an acidic space termed the Howship’s resorption lacuna. The actin cytoskeleton of the osteoclast reorganizes upon osteoclast contact with bone matrix, which results in the formation of a sealing zone around the osteoclast. This allows isolation of the resorption compartment from the surrounding bone [18].

Immature bone is referred to as woven bone, whereas mature bone is one of two types: cortical or cancellous bone. Woven bone is present during embryonic development, in
the fracture callus, as well as in certain pathologic states such as Paget’s disease of bone and hyperparathyroidism. Woven bone is composed of irregularly shaped vascular spaces lined by osteoblasts and randomly oriented collagen fibers. It has a lower mineral content and a higher proportion of osteocytes than mature bone. The osteocytes are positioned more closely together and distributed more randomly. This disorganized bone is functionally weaker and must be remodeled to mechanically withstand load. Upon remodeling, woven bone is normally replaced by cortical or cancellous bone [14].

Cortical bone, that is also known as lamellar or compact bone, is remodeled from woven bone by the invasion of vascular channels from its periosteal and endosteal surfaces. The osteon, also known as a haversian system, is the primary structural unit of cortical bone. An osteon consists of a longitudinally oriented vascular channel, called a haversian canal that is surrounded by cylindrically shaped lamellar bone. Adjacent osteons are connected by horizontally oriented canals, called Volkmann canals. The tight packing of osteons determines the mechanical strength of cortical bone [13].

Cancellous bone, that is also known as trabecular bone, is sandwiched between the cortices of bone and contains bone trabeculae with honeycombed interstitial spaces of hematopoietic elements. In order to provide structural support, the orientation of the trabeculae is mostly perpendicular to external forces. The endosteal surfaces are sites of continual remodeling of cancellous bone [14].

1.2.3 Bone Remodeling

Bone is a dynamic tissue that undergoes continual adaptation via modeling and remodeling throughout life to maintain the size, shape, and structural integrity of the skeleton, as well as mineral homeostasis. Bone modeling allows bones to change their overall shape in response to encountered mechanical forces, by coordinating bone formation and resorption to occur at anatomically distinct locations. Bone remodeling is the process by which damaged bone is renewed to maintain strength and ensure mineral homeostasis [13]. In remodeling, the processes of bone resorption and sequential placement of new bone matrix, which subsequently becomes mineralized,
are tightly coupled in the same anatomical location forming a structure known as a “basic multicellular unit” (BMU). A BMU has osteoclasts at the forefront resorbing bone followed by osteoblasts that secrete unmineralized bone matrix. Bone remodeling consists of four sequential phases: activation, resorption, reversal, and formation [16].

The activation phase involves the recruitment and activation of mononuclear monocyte-macrophage osteoclast precursors and their fusion to form multinucleated preosteoclasts. During each remodeling cycle, the resorption phase lasts approximately two to four weeks, at which time the osteoclasts create a “cutting cone” as they resorb bone [13]. The processes of formation, activation, and resorption of osteoclasts are mediated by various cytokines, including RANKL, OPG, interleukin-1 (IL-1) and -6 (IL-6), colony-stimulating factor, parathyroid hormone, vitamin D, and calcitonin [19]. The reversal phase transitions bone resorption to formation. The factors signaling the end of resorption and beginning of bone formation are not yet fully understood, but TGF-β1, insulin-like growth factors 1 and 2, and bone morphogenetic proteins (BMPs), released from bone matrix during resorption, were proposed as possible candidates, amongst others [16]. It takes approximately four to six months for the cycle to be complete, as osteoblasts synthesize the new collagenous organic matrix and regulate its mineralization. Each remodeling cycle produces a new osteon and the remodeling process follows the same biologic principles in cortical and cancellous bone [13].

1.2.4 Fracture Healing

Bone is unique in that it retains the ability to regenerate in adult life, unlike other tissues that heal by formation of a connective tissue scar. Fracture healing is a complex well organized process that integrates numerous factors at the molecular level together with physiological and biomechanical principles. Fracture healing is known to mimic embryological tissue formation with respect to the regulatory molecular mechanisms. Histologically, fracture healing has been divided into primary, gap, and secondary healing [20].

Primary healing occurs rarely in clinical situations as it requires complete anatomic reduction and stability of the fragments. The cortex attempts to reestablish new
haversian systems, but the interfragmentary gap is minimal and requires widening to establish continuity. Osteoclasts resorb bone by forming “cutting cones” across the fracture site that allow osteoblastic proliferation and vascular ingrowth. Osteoblasts, differentiated from vascular endothelial cells and perivascular mesenchymal cells, subsequently lay down new bone that later remolds, resulting in lamellar bone architecture bridging the fracture site. The presence of rigid fixation eliminates the need for the formation of a callus [21].

In fractures treated by rigid internal fixation, the presence of a gap between the fragments results in gap healing. Osteoclastic activity is not necessary and blood vessels from the periosteum, the endosteum, or pre-existing channels can grow into the gap and serve as a source of mesenchymal osteoblastic precursors. Osteoblasts deposit new bone directly on the fractured segments. Gaps under 1 mm between the fragments allow the formation of lamellar bone directly, whereas gaps exceeding 1 mm form woven bone that later remolds. Lamellar bone forms along a longitudinal orientation with respect to the fracture over the first six weeks, but it later remolds and reorients along the long axis of the fractured bones [22].

Secondary healing occurs in fractures treated without rigid fixation. It involves intramembranous and endochondral ossification, with subsequent callus formation. Intramembranous ossification involves bone formation without preceding cartilage development, resulting in the formation of a hard callus. The sources of osteoblasts are committed osteoprogenitors and undifferentiated mesenchymal stem cells residing in the periosteum [20].

Endochondral ossification is characterized by the recruitment, proliferation, and differentiation of mesenchymal stem cells into chondrocytes that lay down cartilage which calcifies and is eventually replaced by bone. The stages of endochondral ossification include haematoma formation and inflammation, angiogenesis and cartilage formation, cartilage ossification, cartilage removal, bone formation, and bone remodeling [22]. The soft callus formed in endochondral ossification stabilizes the fracture site via a fibro-cartilaginous union to avoid disruption of the newly formed blood vessels. The soft callus is weak in terms of resistance to motion, but by six weeks the
fracture segments are united by a bridge of woven bone. The callus may not ossify if proper immobilization is not used, resulting in an unstable fibrous union [21].

Mesenchymal stem cells (MSCs) are essential in fracture healing as they serve as a source of osteoblasts and chondrocytes. They are undifferentiated cells with the potential to give rise to multiple cell lineages producing bone, cartilage, muscle, tendon, ligament, marrow stroma, and fat. In fracture healing, numerous potential sources of mesenchymal stem cells exist, including bone marrow, the deep layer of the periosteum, the endosteum, granulation tissue, and the surrounding soft tissue [20].

Numerous signaling molecules interact with various cells types to affect the cascade of events in fracture healing (see Table 1.1). The signaling molecules are divided into three categories: (1) pro-inflammatory cytokines, (2) angiogenic factors, and (3) growth factors. The pro-inflammatory cytokines, including interleukin-1 (IL-1) and -6 (IL-6), as well as tumor necrosis factor-alpha (TNF-α) are secreted by macrophages and mesenchymal cells in the periosteum [22]. They function by exerting a chemotactic effect on other inflammatory cells, stimulating angiogenesis, and recruiting fibrogenic cells to the site of injury. Cytokines also regulate endochondral bone formation and remodeling. TNF-α promotes mesenchymal cell recruitment, stimulates osteoclasts, and induces apoptosis of hypertrophic chondrocytes during endochondral ossification. The main angiogenic factor involved in fracture healing is VEGF. VEGF is produced by endothelial cells, fibroblasts, and osteoblasts. It is an essential mediator of neo-angiogenesis and of mitogens specific for endothelial cells [20].

The growth factors involved in fracture healing include TGF-β (transforming growth factor-beta), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), insulin-like growth factors (IGFs), and BMPs (bone morphogenetic proteins). TGF-β is released by platelets, osteoblasts, and chondrocytes, and is stored in the bone matrix. It may be involved in the initiation of callus formation and is a potent chemotactic stimulator of MSCs. It enhances the proliferation of MSCs, chondrocytes, and osteoblasts [21]. PDGF is synthesized by platelets, macrophages, endothelial cells, and osteoblasts. It is responsible for macrophage chemotaxis and is a proliferative stimulus for MSCs and osteoblasts. FGF is synthesized by macrophages, MSCs, osteoblasts, and chondrocytes. It promotes the growth and differentiation of fibroblasts, osteoblasts,
and chondrocytes. IGFs are released from bone matrix, osteoblasts, and chondrocytes.
They promote bone matrix formation by osteoblasts, as well as cellular proliferation of
osteoprogenitors [22].

BMPs, the main source of which is the extracellular matrix, are synthesized by MSCs,
osteoprogenitors, osteoblasts, and chondrocytes. They induce a cascade of events for
chondrogenesis and osteogenesis, including chemotaxis, proliferation and
differentiation of MSCs and osteoprogenitors into osteoblasts and chondrocytes [20].

**Table 1.1**: The source, function, and temporal expression of signaling factors during
fracture healing (adapted from [20]).

<table>
<thead>
<tr>
<th>Signaling Factor</th>
<th>Source</th>
<th>Function</th>
<th>Temporal Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (IL-1, IL-6, TNF-α)</td>
<td>Macrophages, MSCs</td>
<td>Chemotactic for other inflammatory cells, stimulate angiogenesis, recruit fibrogenic cells, regulate endochondral bone formation and remodeling</td>
<td>Increased levels days 1-3, and during bone remodeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Endothelial cells, fibroblasts, osteoblasts</td>
<td>Mediator of neo-angiogenesis</td>
<td>Early stages of fracture healing</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Platelets, osteoblasts, chondrocytes</td>
<td>Initiation of callus formation, chemotactic for MSCs, mitogenic for MSCs, chondrocytes, and osteoblasts</td>
<td>Throughout fracture healing</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelets, macrophages, endothelial cells, osteoblasts</td>
<td>Chemotactic for macrophages, mitogenic for MSCs and osteoblasts</td>
<td>Early stages of fracture healing</td>
</tr>
<tr>
<td>FGF</td>
<td>Macrophages, MSCs, osteoblasts, chondrocytes</td>
<td>Mitogenic for fibroblasts, chondrocytes, and osteoblasts</td>
<td>Early stages of fracture healing until osteoblasts form</td>
</tr>
<tr>
<td>IGFs</td>
<td>Bone matrix, chondrocytes, osteoblasts</td>
<td>Mitogenic for osteoprogenitors, promote bone matrix formation by osteoblasts</td>
<td>Throughout fracture healing</td>
</tr>
<tr>
<td>BMPs</td>
<td>MSCs, osteoprogenitors, osteoblasts, chondrocytes</td>
<td>Chemotaxis, proliferation, and differentiation of MSCs and osteoprogenitors into osteoblasts and chondrocytes</td>
<td>Throughout fracture healing</td>
</tr>
</tbody>
</table>
1.3 Bone Morphogenetic Proteins (BMPs)

1.3.1 BMP Classification

The largest subfamily of the transforming growth factor β (TGF-β) superfamily of growth factors are bone morphogenetic proteins (BMPs). BMPs, like other members of the TGF-β superfamily, possess the characteristic cysteine-knot molecular arrangement (see section 1.3.2 BMP Structure for more detail). The BMP amino acid sequence is similar in multiple species, primarily in the carboxy terminus, and is thus considered to be conserved throughout evolution for approximately 600 million years [23].

Members of the BMP subfamily are further classified into groups based on genetic sequence homology and protein structure similarity of the mature carboxy terminus domain. Greater than 50% of sequence homology is present within the following groups: BMP-2 and 4, BMP-5 through 8, BMP-9 and 10, BMP-12 through 14, BMP-3 and growth and differentiation factor 10 (GDF-10), also known as BMP-3b. BMP-11 and 15 have less sequence homology and are thus more distantly related to the other BMPs (see Figure 1.1). Several reviews have discussed the classification of BMPs [24], [25], [26], [27].

There are currently 18 proteins that are labeled as BMPs. However, only BMP-2 to BMP-9 are considered to be osteoinductive [28]. BMPs-12, -13, and -14 are considered to be chondrogenic, and are thus labeled as cartilage-derived morphogenetic proteins (CDMP) [29]. BMP-8b, -10, -15, -16, -17, and -18 have other physiological roles (see Table 1.2). Other proteins are placed in the BMP subfamily based on sequence homology, but are labeled as growth and differentiation factors (GDFs), with some proteins receiving both a BMP and a GDF classification [30]. BMP-1 is not a member of the TGF-β superfamily as it does not contain the conserved amino acid sequence. It was mislabeled as a BMP, but it is actually a procollagen proteinase implicated in embryonic patterning, functioning to process procollagen to collagen [31].
**Figure 1.1**: Phylogenetic tree demonstrating a portion of TGF-β superfamily. Greater than 50% of sequence homology of the mature C-terminal domain is present within the following groups: BMP-2 and 4, BMP-5 through 8, BMP-9 and 10, BMP-12 through 14, and BMP-3 and BMP-3b. BMP-11 and 15 have less sequence homology and are thus more distantly related to the other BMPs (adapted from [26]).
### Table 1.2: Members of the BMP family and their main physiological roles (adapted from [28]).

<table>
<thead>
<tr>
<th>BMP</th>
<th>Alternative Name</th>
<th>Main Physiological Role</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone Morphogenetic Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-2</td>
<td>BMP-2a</td>
<td>Cartilage, bone, and heart formation in embryogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osteogenesis via differentiation of MSCs into osteoblasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apoptosis signaling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed in lung, spleen, and colon</td>
</tr>
<tr>
<td>BMP-3</td>
<td>Osteogenin</td>
<td>Negative regulation of osteogenesis</td>
</tr>
<tr>
<td>BMP-3b</td>
<td>GDF-10</td>
<td>Induce head-tail orientation</td>
</tr>
<tr>
<td>BMP-4</td>
<td>BMP-2b</td>
<td>Formation of teeth, limbs, and bone from mesoderm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung, eye, and kidney development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Formation of embryonic hematopoietic tissue</td>
</tr>
<tr>
<td>BMP-5</td>
<td>-</td>
<td>Bone and cartilage development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Early developmental skeletal patterning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed in lungs and liver</td>
</tr>
<tr>
<td>BMP-6</td>
<td>Vrg1, Dvr6</td>
<td>Bone and cartilage development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osteogenesis via differentiation of MSCs into osteoblasts</td>
</tr>
<tr>
<td>BMP-7</td>
<td>OP-1</td>
<td>Bone and cartilage development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eye and kidney development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bone and calcium homeostasis</td>
</tr>
<tr>
<td>BMP-8</td>
<td>OP-2</td>
<td>Bone and cartilage development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spermatogenesis</td>
</tr>
<tr>
<td>BMP-9</td>
<td>GDF-2</td>
<td>Osteogenesis via differentiation of MSCs into osteoblasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hepatogenesis, development of cholinergic neurons</td>
</tr>
<tr>
<td>BMP-11</td>
<td>GDF-11</td>
<td>Axial skeletal patterning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Development of eye, pancreas, kidney</td>
</tr>
<tr>
<td><strong>Cartilage-Derived Morphogenetic Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-12</td>
<td>CDMP-3, GDF-7</td>
<td>Chondrogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ligament and tendon formation and repair</td>
</tr>
<tr>
<td>BMP-13</td>
<td>CDMP-2, GDF-6</td>
<td>Chondrogenesis and hypertrophy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ligament and tendon formation and repair</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed in long bones during embryogenesis</td>
</tr>
<tr>
<td>BMP-14</td>
<td>CDMP-1, GDF-5</td>
<td>Chondrogenesis and angiogenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expressed in long bones during embryogenesis</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-8b</td>
<td>OP-3</td>
<td>Spermatogenesis</td>
</tr>
<tr>
<td>BMP-10</td>
<td>-</td>
<td>Cardiac development</td>
</tr>
<tr>
<td>BMP-15</td>
<td>GDF-9b</td>
<td>Ovary physiology</td>
</tr>
<tr>
<td>BMP-16</td>
<td>Nodal</td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td>BMP-17</td>
<td>Lefty</td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td>BMP-18</td>
<td>Lefty</td>
<td>Embryonic patterning</td>
</tr>
</tbody>
</table>
1.3.2 BMP Structure

All members of the TGF-β superfamily, including BMP molecules, are linked by disulfide bonds as either homodimers or heterodimers [32]. They are synthesized as large precursor molecules that are subsequently cleaved to produce the mature protein [28]. Before secretion from the cell, BMPs contain a signal peptide, a pro-domain, and the mature peptide. The signal peptide is cleaved, followed by glycosylation and dimerization [1]. The pro-domain is cleaved from the dimer prior to secretion from the cell to liberate the active carboxy terminal domain, and the active BMP molecule is released as a homodimer of the mature region (see Figure 1.2) [33].

BMPs, like other members of the TGF-β superfamily, have a conserved motif of seven cysteine residues in the mature domain. Six of the cysteine residues form three intrachain disulfide bonds that result in the three-dimensional cysteine-knot molecular arrangement. The remaining cysteine residue is responsible for interchain dimer formation [32].
Figure 1.2: Schematic structure of the BMP molecule processing. BMP is synthesized as a precursor molecule with a signal peptide and a pro-domain, in addition to the mature peptide. The signal peptide is cleaved before the precursor is dimerized. As the dimer is secreted from the cell, the pro-domain is cleaved, releasing the mature peptide dimer in its active form (adapted from [1]).

1.3.3 BMP Receptors

After BMPs are secreted from the cells, the active molecule interacts with surface membrane receptors that transmit the signal intracellularly by activating SMAD proteins. BMP family members bind two types of serine-threonine kinase receptors, type I and type II, both of which are required for signal transduction [1]. Once a BMP molecule is bound to both type I and type II receptors, type II receptors use their serine-threonine domains to phosphorylate domains on type I receptors. Type I receptors are thus
activated and transmit the signal intracellularly. Hence, the type I receptor determines the specificity of intracellular signals [34].

BMPs bind four different type I receptors of the seven type I receptors currently identified. The type I receptors that BMPs bind are called activin receptor-like kinases (ALKs) and include ALK-2 (ActR-IA), ALK-3 (BMPR-IA), and ALK-6 (BMPR-IB) [35]. All type I receptors have a conserved cysteine-rich region in the extracellular portion, however, ALK-3 and ALK-6 are more structurally similar to each other than ALK-2. ALK-6 receptors are found only in chondrocytes and osteoclasts, while most cells possess the ALK-3 receptor, including osteoblasts. It has been found that BMP-2 preferentially binds ALK-3 and ALK-6 receptors (see Table 1.3) [34].

Type II receptors also have a conserved cysteine residue that is different from type I receptors. Type II receptors are larger due to a long carboxy terminal tail present on the intracellular portion after the serine-threonine domain. There are three type II receptors currently identified, including BMPR-II, ActR-II, and ActR-IIB. BMP-2 binds all three type II receptors [35].

Table 1.3: Serine-threonine receptors and ligands (adapted from [24])

<table>
<thead>
<tr>
<th>Serine-Threonine Receptor Type</th>
<th>Receptor Subtype</th>
<th>Alternative Name</th>
<th>BMP Binding Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>BMPR-IA</td>
<td>ALK-3</td>
<td>BMP-2, -4, -7, -13</td>
</tr>
<tr>
<td></td>
<td>BMPR-IB</td>
<td>ALK-6</td>
<td>BMP-2, -4, -7, -14, -15</td>
</tr>
<tr>
<td></td>
<td>ActR-IA</td>
<td>ALK-2</td>
<td>BMP-2, -7, -14</td>
</tr>
<tr>
<td></td>
<td>ActR-IB</td>
<td>ALK-4</td>
<td>BMP-3, -11</td>
</tr>
<tr>
<td>Type II</td>
<td>BMPR-II</td>
<td>T-ALK</td>
<td>BMP-2, -4, -6, -7, -14</td>
</tr>
<tr>
<td></td>
<td>ActR-II</td>
<td></td>
<td>BMP-2, -6, -7, -14</td>
</tr>
<tr>
<td></td>
<td>ActR-IIB</td>
<td></td>
<td>BMP-2, -6, -7, -14</td>
</tr>
</tbody>
</table>
1.3.4 Intracellular Signaling

The binding of the BMP ligand to the transmembrane complex formed by type I (BMPR-I) and type II (BMPR-2) receptors allows BMPR-II to phosphorylate BMPR-I. The activated BMPR-I recognizes and phosphorylates pathway-specific receptor-regulated Smads (R-Smads). Smad proteins are the major signal transducers for these serine-threonine kinase receptors [34]. The specific Smad activated is determined by the member of the TGF-β superfamily binding and the type of BMPR-I. Signal transduction is mediated by R-Smad 1, 5, and 8 for the BMP subfamily. Activated Smads are released from the receptor and form a complex with phosphorylated Smad 4. Smad 4 is used by all members of the TGF-β superfamily in the signaling pathways, hence it is a common mediator Smad or co-Smad. The R-Smad/Co-Smad complex then translocates into the nucleus where it regulates the expression of specific genes by interacting with transcription factors (see Figure 1.3) [28]. Transcription factors are master regulators of gene expression. They bind DNA and help initiate increased or decreased transcription of specific genes. A significant transcription factor that is induced by the R-Smad/Co-Smad complex is PEB2αA/Cbfa1. Lack of PEB2αA/Cbfa1 in embryogenesis results in the complete absence of skeletal ossification in homozygous mice [36]. The transcription factor Cbfa1 is crucial in osteogenesis as it regulates the expression of key proteins, including osteocalcin. Osteocalcin is produced by BMPs and it is an accepted marker of mesenchymal cell commitment to the osteoblastic lineage [37].
Figure 1.3: Schematic diagram of the BMP receptor signaling cascade through Smad proteins (adapted from [34]). BMP ligand binding to the complex of type I and type II transmembrane receptors allows BMPR-II to phosphorylate BMPR-I. Phosphorylated BMPR-I activates intracellular Smad proteins. The specific Smad activated is determined by the member of the TGF-β superfamily binding and the type of BMPR-I. Signal transduction is mediated by Smad 1, 5, and 8 for the BMP subfamily. The activated Smad complexes with phosphorylated Smad 4 and translocates into the nucleus where it interacts with transcription factors to regulate expression of specific genes.
1.3.5 Physiologic Roles of BMPs

The essential role of BMPs in a wide variety of developmental processes, including skeletal development and embryonic patterning, as well as adult skeletal healing and remodeling (see Table 1.2), was uncovered by studying the phenotypes of mice with mutations in genes encoding for various BMPs and their receptors [38].

BMPs have been shown to play a role in the dorso-ventral specification of the mesoderm layer. Specifically, BMP-4 was shown to have a critical role in the establishment of the embryonic body plan [39], whereas BMP-3 and BMP-3b were shown to induce the head-tail orientation [40]. BMPs appear to be involved in the development of numerous organs and tissues, including the lung, heart, kidney, skin, nervous system, teeth, and gonads [41], [42], [43].

Certain BMPs have specific functions in particular organs. Mice with a mutation in the gene encoding BMP-5 displayed compromised perichondral and periosteal growth with multiple mesenchymal defects including short ears, long bones that are thin, and altered skull morphology [23]. Study models with a BMP-7 knockout demonstrated poor nephrogenesis, as well as compromised ocular and skeletal development [44]. Defects in BMP-8 resulted in compromised spermatogenesis [45]. Disruption of BMP-2 resulted in nonviable mice with severe cardiac defects [46].

BMPs have been shown to affect various stages of bone formation and affect fracture healing. Immunohistochemical analysis of a rat fracture healing model demonstrated BMP-2 and BMP-4 increased in the cambium layer of the periosteum near the fracture ends and in the mesenchymal cells that migrated into the fracture gap. BMP-2 and BMP-4 were also upregulated in chondroid precursor cells as well as in osteoblasts in the newly formed trabecular bone [47]. In another study of rat fracture healing, the expression of BMP-7 was enhanced in the intramembranous and endochondral ossification sites. BMP-2, -4, and -7 were expressed in newly formed trabecular bone and in osteoclast-like cells, demonstrating that BMPs play a role of in bone remodeling [48].
1.4 Bone Graft Healing

1.4.1 Autografts

Currently, the process of autogenous bone graft healing is regarded as incorporation [49], defined as viable new bone growing to replace resorbed necrotic tissue [50]. Bone graft incorporation proceeds through five overlapping stages, namely, inflammation, revascularization, osteoinduction, osteoconduction, and remodeling [49].

Surgery results in injury and stimulates an inflammatory response with a twenty-four hour peak and a duration of approximately one week [20]. Hemostasis begins immediately and involves vasoconstriction, platelet aggregation, and formation of a fibrin clot. Degranulation of platelets releases essential signaling molecules, specifically, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), epithelial growth factor, and vascular endothelial growth factor (VEGF) [22]. These molecules trigger chemotaxis and signal transduction leading to mitosis.

Placing a graft into a host bed with surgically induced vascular compromise, results in a hypoxic recipient site environment and an acidic pH [51]. The oxygen gradient established at the surgical site, in addition to other chemoattractants, recruits tissue monocytes, which differentiate into macrophages [52], [53]. Macrophages play a key role in wound healing. They phagocytose microorganisms, foreign particles, and degenerating cells, release proteolytic enzymes that aid in debridement, and stimulate angiogenesis by secreting macrophage derived angiogenesis factor (MDAF) [52], [54]. Thus, host capillary angiogenesis and mitogenesis of the grafted osteocompetent cells is initiated by the wound environment as well as various factors released by platelets and macrophages [55].

Graft revascularization significantly overlaps with the inflammatory phase. It is generally regarded that the graft is invaded by host capillaries by the third day, but is not fully revascularized until two to three weeks after placement [56], [57], [58]. At the end of the first week, platelet supply of growth factors diminishes, but macrophages take over and secrete similar growth factors until the end of revascularization. Completion of vascularization results in oxygen and nutrients reaching the grafted osteocompetent cells, and stimulating the production of osteoid [56].
Autogenous bone grafts are considered osteogenic as they contain living cells that are able to or have already differentiated into osteoblasts. In the osteogenic phase, viable cells that are transferred within the graft play a key role. These include periosteal and endosteal osteoblasts, as well as cancellous marrow MSCs. Most osteocompetent cells die during the transplantation due to surgically induced ischemia, resulting in an area of necrosis. However, mesenchymal stem cells are more resistant to ischemia, and their survival may contribute to increased efficacy of autogenous grafts compared to allografts, which lack cells [59]. Osteoid production by the surviving osteocompetent cells increases as vascularization progresses and lasts approximately for the period of two to eight weeks post-operatively [56].

Osteoinduction refers to the provision of a biologic stimulus that incites progenitor cells to differentiate into mature osteoblasts [59]. Osteoinduction in graft incorporation is stimulated in part by osteoclasts, delivered from systemic circulation by increased vascularity, that resorb necrotic areas of the graft. This resorption liberates bone morphogenetic proteins (BMPs) contained in the necrotic host trabeculae. BMPs are also produced by macrophages [60]. The released BMPs then further stimulate mesenchymal progenitor chemotaxis, division, and commitment to the osteogenic lineage [22].

An osteoconductive substance functions as a scaffold for the formation of bone as it allows the migration of osteoprogenitor cells into the wound site and bone formation along a scaffold [59]. The mechanical framework of the graft and the fibrin scaffold in the organized clot serve as the matrix upon which new bone is formed. The scaffold allows even distribution of the newly formed bone and helps to coalesce the osteoid islands and unite them to the recipient bone. This ensures adequate mechanical strength to support function [61]. The production of osteoid by osteoblasts in intratrabecular spaces and around the necrotic trabeculae, occurs approximately two to four weeks post-operatively. The process of replacement of old necrotic bone by new immature bone, that is later remodeled, is referred to as “creeping substitution” [50].

Remodeling occurs concurrently with the other stages of bone graft incorporation, beginning with the revascularization phase delivering osteoclasts to the site. Immature woven bone and old necrotic bone are resorbed, and replaced by less cellular, more
mineralized, and more structurally organized bone. The remodeled bone forms in response to function and remodeling continues throughout life as part of normal bone turnover. Four months after graft placement, periosteum and endosteum develop [55]. Six months post-operatively, graft incorporation is considered functional, and at one year, it is considered complete [49].

1.4.2 Allografts & Xenografts

Tissue harvested from one individual and implanted into another individual of the same species is termed an allograft [59]. Allografts must undergo rigorous processing to reduce the risk of disease transmission, remove antigenic components, and decrease the host's immune response. The challenge lies in achieving this without significantly affecting the biologic properties of the graft since the processing of allografts results in the death of all viable cells [62]. Classically, allografts can be prepared fresh, frozen, freeze-dried, mineralized, or demineralized. Although fresh and frozen allografts are the most osteoinductive and osteoconductive, they are not commonly utilized due to increased risk of disease transmission and significant host immune response. Grafts that are freeze-dried and vacuum packed have a long shelf life and can be stored at room temperature. They have reduced immunogenicity, but the mechanical strength is compromised due to microfractures as a result of dehydration [63]. Demineralized bone matrix (DBM) is produced by decalcifying cortical bone. Demineralization allows BMPs within the extracellular matrix to become locally accessible and stimulate host mesenchymal cells to differentiate into bone-forming cells. Thus, DBM has osteoconductive and osteoinductive properties [63], [64]. Conversely, mineralized bone allografts, support bone formation through osteoconduction only, as BMPs entrenched within the mineralized matrix, exert no action. Mineralized allografts, however, have superior mechanical properties compared to DBM [64].

Tissue derived from a different species is termed a xenograft [59]. To reduce a severe immune response, xenografts must be deproteinized and defatted to have all of the organic material removed. Thus, xenografts support bone formation through osteoconduction only [62].
Allograft and xenograft incorporation resembles the five stages of autograft healing. The initial inflammatory response is more intense than with autografts and is responsible for impaired revascularization. Host vessels may grow rapidly, but they are surrounded by a dense inflammatory infiltrate that results in occlusion and degeneration of vessels, leading to graft necrosis. The immune response of the host also delays osteoinduction and osteoconduction. Bone formation via osteogenesis is not present in allografts and xenografts. Osteoconduction results in very slow bone formation, accompanied by slow remodeling via resorption of the necrotic graft [49].

1.4.3 Alloplasts/Synthetic Grafts

The surgical morbidity associated with autografts and the risk of disease transmission and immunologic reaction associated with allografts, has stimulated the development of alloplasts or synthetic grafts. Alloplasts are passive scaffolds that mimic some of the chemical and/or some of the physical properties of bone. However, unlike bone, synthetic grafts possess only the capability of osteoconduction [65]. Similarly to allografts, alloplasts become osteointegrated via resorption and remodeling. Capillaries invade the porous spaces and host derived osteoprogenitor cells migrate into the scaffold. Osteoid is formed along the porous structure and later remodeled into mature bone [66].

1.4.4 Growth Factor Based Grafts

Growth factors are a group of molecules responsible for the proliferation and differentiation of cells throughout the life cycle of an organism. Growth factors can behave as mitogens and morphogens, with the former enhancing cellular proliferation, and the latter inducing differentiation of certain cells. Growth factors exert their effect on target cells by interacting with transmembrane receptors. This interaction generates an intracellular signaling cascade leading to the migration of various factors into the nucleus. These factors modulate transcription of specific genes that alter the activity or the phenotype of cells [67].
To facilitate skeletal reconstruction, growth factors involved in bone induction or regeneration can be exploited in an attempt to modify the complex interaction of inflammation, angiogenesis, and cellular differentiation in favour of osteogenesis. Platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), and bone morphogenetic proteins (BMPs) have been investigated for their abilities to improve bone healing. VEGF was shown to be involved in the mineralization of the fracture callus and to influence osteoblast differentiation, in addition to the stimulation and regulation of angiogenesis. Hence, applying exogenous VEGF to a fracture site can improve angiogenesis, providing more nutrients to the wound, and increase differentiation of mesenchymal stem cells into osteoblasts [68]. PDGF was shown to increase the differentiation and chemotaxis of osteoblasts, as well as to increase osteopontin production [67].

BMPs have been found to induce osteogenic and chondrogenic differentiation of mesenchymal stem cells, thus, resulting in greater bone formation at the graft site [69]. BMPs may also aid in angiogenesis as they have been shown to stimulate chemotaxis of endothelial progenitor cells that may later differentiate into endothelial cells [70]. BMPs have also been shown to stimulate the expression of VEGF locally [71]. VEGF was shown to play a significant part in the differentiation, activity, and recruitment of entothelial cells, osteoclasts, osteoblasts [72], [73]. Thus, growth factor based grafts promote osteogenesis by attempting to augment the normal wound physiology.

1.4.4.1 Clinical Applications of BMPs

Basic science research has elucidated the physiologic roles of BMPs and fueled ideas on the wide range of potential human applications. A study that found that systemic administration of rhBMP-2 increased mesenchymal stem cell activity and reversed ovariectomy-induced bone loss, has led researchers to suggest systemic administration of rhBMP-2 for the treatment of osteoporosis [74]. In the field of endodontics, studies demonstrated that BMP exposure induced the formation of reparative dentin, resulting in a possible role for BMP in maintaining tooth vitality via pulp capping [75], [76]. BMPs
were also investigated in periodontal regeneration and enhancement of osseointegration of dental implants [77].

Of all the members of the BMP family, only BMP-2 and BMP-7 received FDA approval for specific indications in human applications. They have also been utilized for off label uses. In 2002, rhBMP-2 (Infuse® Bone Graft by Medtronic) was approved to replace autogenous graft for anterior lumbar interbody spinal fusion procedures used in combination with the LT-Cage (lumbar tapered cage) device [78]. In 2004, Infuse® Bone Graft was approved for the treatment of open tibial fractures [79]. In 2007, Infuse® Bone Graft was approved as an alternative to autogenous grafting for maxillary sinus augmentation and post-extraction socket augmentation of alveolar ridges [80]. In 2008, Infuse® Mastergraft Posterolateral Revision Device received a humanitarian device exemption (HDE) for the repair of symptomatic posterolateral lumbar spine pseudoarthrosis for patients in whom autologous bone graft harvest is not feasible or is not expected to promote fusion [81]. Whereas a full FDA approval requires scientifically valid clinical investigations to demonstrate that the device is safe and effective for its intended purpose, an HDE requires only evidence that the product does not pose unreasonable health risks for its intended use. HDE is granted to products intended to treat medical conditions that annually affect fewer than 4000 individuals [82].

In 2001, OP-1® Implant (Stryker, Biotech) received a humanitarian device exemption (HDE) for use in long bone fracture nonunions [83]. In 2004, OP-1® Putty was granted another HDE to be used in posterolateral lumbar spinal fusion revision surgery in situations where autogenous grafting was not possible or was not expected to provide a stable outcome [84].

Studies in maxillary sinus floor augmentation [85] and extraction socket augmentation [86] demonstrated that rhBMP-2 induced bone formation suitable for the placement of implants. This bone was found to be histologically similar to native bone, capable of implant osseointegration, and able to support dental prostheses in functional loading.

Several case reports described off-label use of rhBMP-2 to reconstruct mandibular continuity defects [87], [88], [89]. A collagen sponge with rhBMP-2 was used to successfully reconstruct defects ranging from 4 to 8 cm in length. However, in spite of the favourable short-term results, a note of caution is required for the use of BMPs to
reconstruct segmental mandibular defects, as various problems are becoming apparent in the orthopaedic literature. The adverse effects described include excessive resorption, ectopic bone formation, severe swelling, as well as seroma and hematoma formation [90], [91]. Theoretically, BMPs may also act as carcinogens and teratogens [92]. Thus, additional long-term clinical trials are necessary to establish safety and efficacy.

1.5 Bisphosphonates

1.5.1 Structure & Classification of Bisphosphonates

Bisphosphonates are stable synthetic analogues of the naturally occurring pyrophosphate [93]. In a pyrophosphate molecule, the two phosphate moieties are bound by an oxygen atom (P-O-P), whereas bisphosphonates have a carbon atom binding the two phosphate groups (P-C-P). This substitution confers biological stability and resistance to chemical and enzymatic degradation [94]. Studies on the relationship between structure and function of bisphosphonates suggest that the biological activity of the molecule is dependent on two properties: affinity to bone mineral and potency [93].

With respect to bone mineral affinity, the central carbon atom can form two additional covalent bonds and these groups are referred to as the R₁ and R₂ side chains (see Figure 1.4). This P-C-P structure allows bidentate binding to calcium ions. The presence of a hydroxyl group (OH) at the R₁ position improves binding affinity to mineral as it allows the formation of a tridentate bond [95]. Therefore, the two phosphate groups and a hydroxyl group at the R₁ position, together act as a “bone hook” and account for the tissue specific targeting of bisphosphonates to bone mineral.

The potency of the molecule is determined by the chemical composition of the R₂ side chain and its spatial relationship to the two phosphate groups [96]. A primary amino-nitrogen group (i.e. NH₂) at the R₂ position increases potency 10 to 100-fold. The most powerful antiresorptive properties are conferred by an R₂ side chain with a nitrogen atom within a heterocyclic ring, as in zoledronate (see Figure 1.5), which is 10000-fold more potent than bisphosphonates without nitrogen [97].
Bisphosphonates are classified into two groups with different modes of action: non-nitrogen-containing bisphosphonates or simple bisphosphonates (SBPs), and nitrogen-containing bisphosphonates (N-BPs). Clodronate, etidronate, and tiludronate are part of the SBP group, while alendronate, risedronate, ibandronate, pamidronate, and zoledronate are N-BPs [97]. See Table 1.4 for the classification, relative potency, and route of administration of the currently available bisphosphonates.

\[
\begin{align*}
\text{OH} & \quad \text{R}_1 \quad \text{OH} \\
\text{O} \equiv \text{P} \quad \text{C} \quad \text{P} \equiv \text{O} \\
\text{OH} & \quad \text{R}_2 \quad \text{OH}
\end{align*}
\]

**Figure 1.4**: Chemical structure of a bisphosphonate molecule. The P-C-P backbone confers biological stability, the R\(_1\) group determines mineral affinity, and the R\(_2\) group establishes the antiresorptive potency (adapted from [93]).

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{O} \equiv \text{P} \quad \text{C} \quad \text{P} \equiv \text{O} \\
\text{OH} & \quad \text{CH}_2 \quad \text{OH} \\
& \quad \text{N} \quad \text{N}
\end{align*}
\]

**Figure 1.5**: Chemical structure of zoledronate, with an OH group at the R\(_1\) position, contributing to increased mineral-binding affinity, and a nitrogen-containing heterocyclic
ring at the R₂ position, conferring significantly increased antiresorptive potency (adapted from [95]).

**Table 1.4**: Classification, sidechains, relative potency, and route of administration of bisphosphonates (adapted from [98]).

<table>
<thead>
<tr>
<th>Bisphosphonates</th>
<th>R₁</th>
<th>R₂</th>
<th>Relative Potency</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Etidronate</td>
<td>OH</td>
<td>CH₃</td>
<td>1</td>
<td>Oral or Intravenous</td>
</tr>
<tr>
<td>• Clodronate</td>
<td>Cl</td>
<td>Cl</td>
<td>10</td>
<td>Oral</td>
</tr>
<tr>
<td>• Tiludronate</td>
<td>CH₂</td>
<td>-NHCH₃(CH₂)₅</td>
<td>100</td>
<td>Oral</td>
</tr>
<tr>
<td>Nitrogen-Containing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkyl-Amino</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Pamidronate</td>
<td>OH</td>
<td>(CH₂)₂-NH₂</td>
<td>100</td>
<td>Intravenous</td>
</tr>
<tr>
<td>• Alendronate</td>
<td>OH</td>
<td>(CH₂)₃-NH₂</td>
<td>1000-1000</td>
<td>Oral</td>
</tr>
<tr>
<td>• Ibandronate</td>
<td>OH</td>
<td>(CH₂)₂-NHCH₃(CH₂)₅</td>
<td>1000-10000</td>
<td>Oral</td>
</tr>
<tr>
<td>Cyclic Nitrogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Risedronate</td>
<td>OH</td>
<td></td>
<td>1000-10000</td>
<td>Oral</td>
</tr>
<tr>
<td>• Zoledronate</td>
<td>OH</td>
<td></td>
<td>≥ 10000</td>
<td>Intravenous</td>
</tr>
</tbody>
</table>
1.5.2 Mechanisms of Action of Bisphosphonates

Numerous studies have demonstrated that bisphosphonates affect osteoclast mediated bone resorption through intracellular effects [99], [95]. Specific targeting of bisphosphonates for bone mineral, allows close association of the molecule with osteoclasts. Bisphosphonates are taken up by osteoclasts via endocytosis, and subsequently released into the cytosol where they exert their biological action [100]. Various biochemical mechanisms have been proposed to explain the inhibition of bone resorption. Currently, however, there are two accepted mechanisms of action, upon which the classification system of bisphosphonates is based [100].

Non-nitrogen-containing or simple bisphosphonates (SBPs) are converted intracellularly into nonhydrolyzable analogues of adenosine triphosphate (ATP). These ATP analogues accumulate inside cells and induce apoptosis [99], [95], [100]. A proposed mechanism resulting in apoptosis is inhibition by ATP analogues of an enzyme located in the mitochondrial membrane. This alters the membrane permeability of mitochondria and commits the cell to apoptotic death. SBPs are less potent than nitrogen-containing bisphosphonates (N-BPs) [99].

Nitrogen-containing bisphosphonates (N-BPs) interfere with the mevalonate pathway by inhibiting a key enzyme, farnesyl diphosphate synthase (FPPS) (see Figure 1.6). FPPS utilizes diphosphates as substrates, and N-BPs can act as inactive analogues of these molecules [93]. Blocking the mevalonate pathway interrupts the formation of isoprenoid lipids, that participate in post-translational protein modification, that is also known as prenylation. This post-translational modification includes the prenylation of small GTPases that belong to the subfamilies of Ras, Rho, and Rab [101]. Prenylation produces farnesylated and geranylgeranylated proteins by transferring a farnesyl or a geranylgeranyl lipid group onto a cysteine residue. The lipid groups render the proteins functional as they anchor the proteins to cell membranes and participate in protein interactions [94]. These small prenylated GTPases play a significant role in the regulation of osteoclast activity by involvement in multiple intracellular signaling pathways, including gene transcription, cell growth, control of cell morphology, differentiation, and apoptosis. In other cell types, GTPases are known to regulate the actin cytoskeleton and their functions are crucial for the control of cell shape and
motility.[101]. Thus, the deficiency in prenylation results in the loss of osteoclast function and leads to apoptosis.

Several studies provide data that indicate that bisphosphonates may have indirect effects on antiresorptive activity via their action on osteoblasts [102], [103]. The most plausible explanation is the influence of osteoblasts on osteoclast formation via the RANK-RANKL/OPG interaction of osteoblasts with osteoclast precursors [104].

A study has demonstrated that pamidronate and zoledronate increase the expression of OPG mRNA and protein in human osteoblasts [103], thus leading to decreased osteoclast differentiation. Pamidronate was also shown to decrease the expression of RANKL mRNA in a rat osteoblast cell line [102], resulting in reduced bone resorption. Another study demonstrated that zoledronate indirectly inhibited osteoclast maturation by increasing OPG protein secretion and reducing RANKL protein expression in human osteoblast-like cells [104].

There are reports suggesting that N-BPs can interfere with angiogenesis. Pamidronate and zoledronate were shown to reduce the circulating levels of vascular endothelial growth factor (VEGF), a potent mediator of angiogenesis, in metastatic breast cancer patients [105], [106]. A rat study demonstrated that bisphosphonates inhibit angiogenesis in the prostate gland [107]. Zoledronate was shown to inhibit the differentiation of endothelial progenitor cells in vitro [108]. Interestingly, zoledronate was shown to inhibit angiogenesis in an FPPS-independent manner in vivo in rats [109]. Bisphosphonates have also been shown to elevate acute phase inflammatory markers [110], [111].
Figure 1.6: Schematic diagram of the mevalonate pathway. Nitrogen-containing bisphosphonates (N-BPs) inhibit farnesyl diphasphate (FPP) synthase, thus inhibiting prenylation of proteins (adapted from [94], [97], and [100]).

1.5.3 Pharmacology

The uptake and retention of bisphosphonates was measured by using radiolabeled bisphosphonates and following their distribution within the body. Bisphosphonates are recycled back into the circulation unchanged as they are not metabolized by the body. Measuring the amount of bisphosphonates excreted in urine within the first 24 hours, indicated the amount retained in bone, as bisphosphonates have minimal protein binding. Oral bisphosphonates have poor absorption (less than 1%), whereas intravenous bisphosphonates exhibit skeletal incorporation of approximately one to two thirds of the total dose administered, with most of the rest being excreted in urine unchanged within the first few hours [112]. The amount of bisphosphonate retention was greater in patients with higher bone turnover and impaired renal function. As well, bisphosphonates with higher affinity for hydroxyapatite had higher retention in bone [99].
The distribution of radiolabeled zoledronate was studied in rats [113]. It was found that following intravenous administration, approximately 40% of zoledronate was excreted unchanged in urine, with the remainder bound to bone. The concentration of zoledronate in bone, soft tissue, and blood was measured 1 day, 15 days, 2 months, 4 months, and 8 months after the injection. The concentration in bone remained approximately the same over the 8 months (~10^5 picomoles/g), whereas the concentration in blood decreased from 10^2 picomoles/g on day 1 to 10 picomoles/g at 8 months. An interesting finding was that trace amounts of zoledronate were found in soft tissue with the concentration of the drug decreasing from 10^3 picomoles/g on day 1 to 10^2 picomoles/g at 8 months [113].

Dosing regimens of bisphosphonates are based on the fact that the total dose administered was a major determinant of effects, regardless of the dosing frequency [114]. This lead to transitioning from lower more frequent to higher less frequent dosing, minimizing the side effects of oral administration (i.e. esophagitis) and decreasing the inconvenience of intravenous treatment. However, higher bone turnover states as in patients with bone metastases, necessitate more frequent administration to effectively reduce osteolysis [115].

Due to the high affinity of bisphosphonates for bone mineral, bisphosphonates localize to regions of osteoclast resorption and osteoblast mineral deposition. Thus, bisphosphonates are targeted to areas of high bone turnover and may be incorporated into the skeleton for prolonged periods of time. In accelerated bone turnover states, bisphosphonates are eliminated from the skeleton more rapidly and can re-enter the systemic circulation. Bisphosphonates are released from the skeleton over a period of weeks to months after stopping treatment, as trace amounts of bisphosphonates can be detected in the urine [10]. This implies that bisphosphonates can recirculate in the circulation and be available for re-uptake into bone long after the initial elimination. This concept may explain the prolonged duration of action of bisphosphonates. Sustained reduction in bone turnover for at least 12 months was reported after a single dose of zoledronate 1 mg IV [116].

Bisphosphonates can be released from the skeleton via chemical desorption and osteoclastic resorption. Desorption is characterized by debonding of the
bisphosphonate from hydroxyapatite and is more likely to occur when the drug is closer to the surface of bone and the extracellular concentration of the bisphosphonate is low [117].

As bisphosphonates are eliminated from the body by the kidney, they have the potential to cause adverse renal effects when present at sufficiently high plasma concentrations. Renal excretion occurs by passive glomerular filtration and active transport in the renal proximal tubular cells [118]. Several early studies demonstrated that rapid intravenous infusion of bisphosphonates was associated with acute renal failure, likely due to nephrotoxic effects on tubular cells of high systemic bisphosphonate concentrations [119], [120]. Since rapid intravenous infusion (< 5min) of zoledronate for the treatment of metastases demonstrated acute increases in serum creatinine, while a slower infusion rate did not, the FDA label recommends dose adjustment based on baseline creatinine clearance and a slower infusion rate of zoledronate over 15 min [121].

1.5.4 Indications for Bisphosphonates

As noted above, as a result of their inhibition of bone resorption, bisphosphonates are used in diseases with pathologic bone catabolism, namely osteoporosis [10], Paget’s disease, osteogenesis imperfecta of childhood, hypercalcemia associated with malignancy, and osteolytic lesions arising from any solid tumor such as breast, lung, or prostate cancer, as well as multiple myeloma [11]. The therapeutic goal is to arrest bone loss, increase bone density and decrease the risk of pathologic fractures. Table 1.5 represents a summary of the accepted bisphosphonate regimens for various conditions.

Bisphosphonates administered via the oral route, are commonly prescribed to stabilize bone loss caused by osteoposis in post-menopausal women, making osteoporosis the most common clinical condition treated with bisphosphonates. Osteoporosis is an imbalance between bone resorption and deposition resulting in compromised bone strength and increased risk of fracture. By suppressing osteoclast activity, bisphosphonates retard bone resorption and increase bone density [122]. Since oral bisphosphonates have been shown to significantly reduce the incidence of pain,
fracture, and disability in women with osteoporosis, there were 22.4 million prescriptions of alendronate (Fosamax®) written in the US in 2005 [123]. Alendronate and risedronate demonstrated reduction in vertebral and hip fractures, and in progression of vertebral deformities, as well as in height loss. The oral regimens for alendronate and risedronate are 70 mg and 35 mg, respectively, administered orally once per week. Since bisphosphonates are poorly absorbed orally, they are administered on an empty stomach with water and followed by 30-60 minutes of nothing by mouth to improve absorption. As well, patients are required to stand or sit upright for 30-60 minutes after administration to minimize esophagitis. As a result, oral bisphosphonates are often associated with poor patient compliance. Thus, new regimens of intravenous bisphosphonates evolved as an option for patients unable to tolerate the oral form. A single administration of zoledronate 5 mg intravenously administered over 15 min once yearly, demonstrated significant fracture reduction and bone mineral density increase [124].

Paget’s disease manifests as disordered bone remodeling characterized by abnormal resorption and deposition of bone. The most commonly affected bones are the lumbar vertebrae, pelvis, skull, and femur. The bones become thickened, enlarged, and weakened. Clinically, the disease may present as bowing of weight-bearing joints, increase in head circumference, bone pain, headaches, hearing loss, and ill-fitting dentures. Bisphosphonates are used in the management of Paget’s disease to suppress increased bone resorption in order to decrease bone pain and prevent progression of disease. The oral regimens involve alendronate and risedronate at 40 mg and 30 mg, respectively, administered orally for 6 and 2 months, respectively [125]. In cases where intravenous administration is preferred, pamidronate 30 mg IV over 2 hours for 3 consecutive days or zoledronate 5 mg IV over 15 min administered once yearly can be given [126].

Osteogenesis imperfecta is a heritable disorder characterized by impaired collagen maturation leading to diminished bone mass and severe fragility. The treatment with pamidronate IV in 3-day cycles every 2-4 months to an annual dose of 9 mg/kg, has been shown to improve the functional status and increase bone mineral density [127].
Numerous cancers are osteoporotic and to metastasize to skeleton (e.g., breast, prostate, lung, and kidney) or grow within bone marrow (multiple myeloma). This results in hypercalcemia, severe bone pain, skeletal destruction, and pathologic fractures. Thus, intravenous bisphosphonates are used to limit the proliferation of metastatic cancers in bone and have been shown to improve the quality of life and even extend life in cancer patients [11]. Breast cancer metastatic to bone treated with pamidronate 90 mg IV over 2 hours or zoledronate 4 mg IV over 15 min, administered every 3-4 weeks, was shown to relieve skeletal pain and reduce skeletal complications [128].

Multiple myeloma is the most common malignancy to involve bone. It manifests as clonal proliferation of malignant plasma cells in bone marrow, resulting in osteolysis and skeletal destruction [129]. Multiple myeloma is a disease with significant patient morbidity as greater that 85% of patients have bone involvement, approximately 40% have a pathologic fracture within the first year of diagnosis, more than 60% experience a fracture during the course of the disease, and about 15% develop hypercalcemia [130]. Hence, therapies to reduce bone destruction can significantly improve the quality of life in multiple myeloma patients. The pathophysiology of multiple myeloma involves the production of cytokines by the malignant cells that induce increased levels of RANKL and reduced levels of OPG, resulting in increased osteoclast differentiation and activity [129]. Bisphosphonates (specifically, pamidronate and zoledronate) were shown to decrease the frequency of skeletal events and delay their development, significantly improving the quality of life in multiple myeloma patients. The American Society of Clinical Oncology released practice guidelines on administration of bisphosphonates in multiple myeloma. Patients with lytic destruction of bone or compression fractures of the spine due to osteopenia should receive pamidronate 90 mg IV over 2 hours every 3-4 weeks or zoledronate 4 mg IV over 15 min every 3-4 weeks. The duration of therapy is 2 years, at which time bisphosphonates are stopped in patients with responsive or stable disease. Bisphosphonates are, however, resumed upon relapse with new skeletal events [131].

Bisphosphonates demonstrate evidence-based reduction in skeletal-related events and improve the quality of life in patients when prescribed for the management of
osteoporosis, Paget’s disease, osteogenesis imperfecta, and various malignancies. Thus, it is likely that the number of patients taking bisphosphonates will increase in the coming years.

Table 1.5: Accepted bisphosphonate regimens for various conditions (adapted from [124], [125], [126], [127], [128], [131]).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Zoledronate</th>
<th>Pamidronate</th>
<th>Alendronate</th>
<th>Risedronate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoporosis</td>
<td>5 mg IV* over 15 min once yearly</td>
<td></td>
<td>70 mg PO** once weekly</td>
<td>35 mg PO once weekly</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td>4 mg IV over 15 min every 3-4 weeks for 2 years</td>
<td>90 mg IV over 2 hours every 3-4 weeks for 2 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastases to Bone</td>
<td>4 mg IV over 15 min every 3-4 weeks for 2 years</td>
<td>90 mg IV over 2 hours every 3-4 weeks for 2 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paget’s Disease</td>
<td>5 mg IV over 15 min once yearly</td>
<td>30 mg IV over 2 hours for 3 consecutive days</td>
<td>40 mg PO once daily for 6 months</td>
<td>30 mg PO once daily for 2 months</td>
</tr>
<tr>
<td>Osteogenesis Imperfecta</td>
<td>9 mg/kg/year IV divided into 3 day cycles every 2-4 months</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*IV - intravenous  
**PO - oral
1.5.5 Bisphosphonates & Osteonecrosis of the Jaw

Improved survival of cancer patients revealed complications associated with prolonged administration of potent bisphosphonates, namely bisphosphonate related osteonecrosis of the jaws (BRONJ). BRONJ was first reported by Marx in 2003. It is defined as “exposed bone in the maxilla or mandible that fails to heal within eight weeks in a patient receiving or who has received a systemic bisphosphonate and who has not received local radiation therapy to the jaws” [132]. Clinically, early findings of BRONJ include tooth mobility, spontaneous or trauma induced areas of exposed non-vital bone with erythematous and edematous mucosal tissue margins that are sensitive to palpation and are often secondarily infected, and are usually refractory to debridement. Late clinical findings include paraesthesia as a result of nerve compression, cutaneous or mucosal fistula, and possibly pathological fractures of the maxilla or mandible [133].

BRONJ is a debilitating chronic condition that is usually resistant to therapeutic intervention. Conservative treatment strategies for BRONJ include an antibacterial mouth rinse and antibiotic therapy. More severe cases of BRONJ require surgical intervention such as minor debridement under local anaesthetic, major surgical sequestrectomies, as well as marginal and segmental mandibular resections, and partial or complete maxillectomies in the case of pathologic fractures [134].

Accurate assessment of the incidence of BRONJ is difficult since there is no record of all of the patients receiving bisphosphonates and not all of the cases of BRONJ are reported. However, the incidence of BRONJ appears to be dependent on dose and duration of therapy, as well as the specific bisphosphonate used, and estimates range from 1% - 12% for intravenous bisphosphonates at 36 months of exposure [135]. Zoledronate is associated with higher risk than pamidronate and the risk of BRONJ increases with time of exposure. Moreover, the incidence of BRONJ is likely to rise with increased recognition of the condition, duration of exposure to bisphosphonates, and follow-up.

Patients on prolonged intravenous administration of amino bisphosphonates that concomitantly undergo dentoalveolar procedures such as extractions, dental implant
placement, periapical surgery, or periodontal surgery involving osseous injury, have the greatest risk of developing BRONJ [132].

The exact physiological mechanisms by which osteonecrosis manifests in bisphosphonate users are unknown. Inhibition of osteoclastic activity leads to severe inhibition of physiologic bone remodeling. One hypothesis for the pathogenesis of BRONJ is that, as there is a high demand for remodeling in the oral cavity due to physiologic microdamage and microfractures that occur daily from masticatory forces, infections such as periodontal disease, as well as local trauma from denture irritation or an extraction, the inhibition of remodeling may be responsible for BRONJ [136]. Bisphosphonate-induced suppression of bone turnover was demonstrated by a study that subjected mature beagle dogs to three years of daily treatment with oral alendronate at doses equivalent to the clinical dose for osteoporosis, and histologically assessed the level of bone remodeling [137]. The control animals demonstrated that remodeling rates were ten-fold greater in the alveolar region of the mandible than in long bones, supporting the theory of a high demand for remodeling in the oral cavity. The animals receiving alendronate had significantly suppressed bone turnover, especially in the alveolar region compared to the controls. This study, however, did not establish a direct cause-and-effect relationship between inhibition of remodeling and BRONJ.

In support of the theory of oversuppression of bone remodeling, recent case reports have emerged reporting subtrochanteric or femoral shaft fractures in patients on long term (usually greater than four years) oral bisphosphonate therapy. These fractures displayed characteristics that are atypical for osteoporotic fractures, namely bilateral fractures and association with thick rather than thin bone cortices. These reports raised concerns that long-term suppression of bone remodeling by bisphosphonates inhibited the ability to repair the accumulated microdamage, resulting in stress fractures [138], [139], [140], [141], [142]. However, further studies are required to elucidate the cause.

The role of vasculature in the pathophysiology of BRONJ was implicated by studies that demonstrated antiangiogenic properties of bisphosphonates, namely, in the use of bisphosphonates to suppress angiogenesis associated with tumor growth [143]. A
reduction in angiogenesis may adversely affect wound healing and explain why surgical procedures in the oral cavity are a major precipitating event for BRONJ [136].

1.5.6 Bisphosphonates and BMPs

Bone healing and graft incorporation involve the formation of a new bone matrix and resorption of damaged or necrotic bone, as well as remodeling of immature bone. A balance of the anabolic osteoblast-mediated formation and catabolic osteoclast-mediated resorption is required for normal bone metabolism. The provision of BMPs stimulates the cells of the osteoblastic lineage as well as a rapid production of osteoclasts [144]. Since the formation of osteoclasts occurs before osteoblast formation, large doses of BMPs may lead to an initial wave of resorption [145]. The knowledge of the inhibitory effect of bisphosphonates on osteoclastic bone resorption generated the idea of combining the anticatabolic effects of bisphosphonates with BMPs. The rationale was to maximize the BMP-induced bone formation by reducing bone resorption [146].

The addition of zoledronate to OP-1 in a rat critical defect model increased the bone volume and mineral content of the callus compared to OP-1 alone [147]. The zoledronate was administered systemically at the time of surgery in one group and two weeks post-operatively in another group, with the latter group showing a greater increase. The explanation provided by the authors was that the delay in zoledronate administration allowed the anabolic response to start, and the initial bone formation increased the availability of target mineral sites, thereby increasing the effective dose of zoledronate.

Another study with a rat bone chamber model added OP-1 to cancellous grafts at the time of implantation, with systemic administration of zoledronate two weeks post-operatively [148]. The group receiving both the OP-1 and the zoledronate demonstrated a 4-fold increase in bone volume compared to the controls and 2.5-fold increase in bone volume compared to OP-1 alone.

The existing studies primarily focused on using bisphosphonates to increase the amount of bone formed by BMP, and thus, bisphosphonates were administered at the
time of surgery or post-operatively. The general consensus was that bisphosphonates and BMPs work synergistically, but further studies are required to elucidate the appropriate bisphosphonate dose and the optimal timing of administration. There are currently no reports that evaluate BMP activity in patients with a longstanding history of bisphosphonate use, and thus, the present study explored this topic.

1.6 Study Aim & Hypothesis

The aim of this study was to ascertain the effect of pretreatment with the bisphosphonate zoledronate on the osteoinductive activity of a bioimplant containing rhBMP. This was addressed via an in vivo study, which compared the quality and the quantity of bone induced by a collagen sponge containing rhBMP-2 (Infuse®), in mice pretreated with zoledronate. The null hypothesis was that zoledronate will have no effect on the osteoinductive capability of rhBMP-2.
Chapter 2
Materials & Methods

2.1 Experimental Design

This experiment was designed based on earlier studies where a BMP-containing bioimplant was inserted into a rat or mouse muscle pouch [149, 150]. The analysis methods developed in these prior studies, were utilized in this experiment.

In the current study, 42 CD-1 male mice that weighed 30.9 ± 2.2 grams were divided into 3 groups of 14 mice per group. Groups 2 and 3 received an intraperitoneal injection with 2 µg of zoledronate and 20 µg of zoledronate in 0.2 mL of phosphate-buffered saline (PBS), respectively. Group 1 served as control and received an intraperitoneal injection of PBS alone (see Table 2.1 for cohort breakdown). Twenty eight days later, all of the animals received the same injection according to their group, and a bioimplant was placed into their biceps femoris muscle containing rhBMP-2 (Infuse®). Twenty eight days subsequent to bioimplant placement, the mice were euthanized. Their hind limbs were dissected out and placed in fixative for histologic and radiographic analyses.

Table 2.1 : Cohort breakdown

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Specimen #</th>
<th>Zoledronate (µg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>1 - 8, 10, 11, 13 - 16</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>17 – 24, 26, 27, 29 - 32</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>33 – 40, 43 - 48</td>
<td>20</td>
</tr>
</tbody>
</table>
2.2 Pre-Treatment with Zoledronate

The mice were injected with zoledronate from Sequoia Research Products (Pangbourne, UK). The low dose of 2 µg/mouse was selected as being equivalent to the dose received in multiple myeloma treatment, that is, 4 mg IV over 15 min every 3-4 weeks [131]. The calculation was as follows: assuming that an average person weighing 60 kg (60,000 g) received 4 mg, then the average mouse weighing 30 g should receive 30 x 4 ÷ 60,000 = 0.002 mg = 2 µg. The high zoledronate dose of 20 µg/mouse was selected as ten times the equivalent dose to simulate extended prior treatment.

2.3 Fabrication of Bioimplant

The Infuse® bone graft (Medtronic) consisted of two main components: the rhBMP-2 as its active ingredient, and a carrier delivery system in the form of an absorbable collagen sponge (ACS) [151]. The ACS primarily served to retain the protein at the implanted site for a required period of time, and it was able to act as scaffold for the formation of new bone [152]. The rhBMP-2 was supplied as a freeze-dried powder form that was reconstituted with sterile water. The ACS was prepared from bovine Achilles tendon type I collagen. It was manufactured by processing the purified collagen material into aqueous solution, followed by lyophilazation (a.k.a. freeze-drying) to attain biocompatibility. Cross-linking by chemical methods prevented premature degradation in vivo, and the final step in the ACS preparation process was sterilization [152].

The Infuse® Medtronic kit contained 4.2 mg of rhBMP-2 powder in a vial, 5 mL of sterile water in a vial, and two ACSs with dimensions 25 mm x 50 mm. To allow the ACS to fit into the #5 gelatin capsule, the ACS was cut into 4 mm x 8 mm sections. The #5 gelatin capsules with the ACS were placed in sterilization packages and sealed. Sterilization was performed by 4 hour exposure to chloroform gas. The chloroform vapours were then removed by placing the packages into a running biological safety hood overnight. Prior to surgical implantation, 4.2 mg of rhBMP-2 powder was reconstituted with the sterile water provided according to package instructions. According to the Medtronic package insert, the resulting solution of 2.8 mL, contained 1.5 mg/mL of rhBMP-2.
During the surgery, the dry Infuse® gelatin capsule with an ACS was inserted into the mouse muscle pouch. Using a 200 µL micropipette, 35 µL was drawn from the reconstituted solution (this contained 52.5 µg of rhBMP-2). This amount was then added to the inside of the open gelatin capsule.

2.4 Surgical Phase

2.4.1 Implant Placement

The location of the surgery was at the University of Toronto, Faculty of Dentistry in the animal surgical suite. All animal work was approved by the University of Toronto Animal Care Committee. CD1 mice were used from Charles River. They were present at the experimental facility for 2 weeks prior to the initial injection and were labeled with ear tags. The mice were anaesthetized with isoflurane inhalational anaesthetic delivered in nitrous oxide and oxygen via a nasal cone. Induction of anaesthesia was achieved with 4% isoflurane delivered in 1 L of oxygen. Anaesthetic was maintained with 1.5% isoflurane in 600 mL of nitrous oxide and 300 mL of oxygen. The mice were weighed and electric clippers were used to shave an area of approximately 2 cm x 2 cm over the dorsal aspect of the lumbar spine, as well as medial and lateral aspects of one hind limb, alternating right and left sides. The shaven skin was cleansed with iodine and the surgical site was draped with sterile disposable paper drapes.

A 3 cm long incision was made with a #10 scalpel blade along the dorsal aspect of the midline parallel to the lumbar spine. The biceps femoris muscle was exposed via a blunt dissection. A muscle pouch was formed by making a small incision into the muscle and bluntly dissecting the fibers apart. The dry Infuse® gelatin capsule with a collagen sponge was inserted into the pouch. A micropipette was used to add 35 µL (52.5 µg) of the aqueous rhBMP-2 (1.5mg/mL) to the absorbable collagen sponge (ACS) through the opening in the capsule. None of the liquid leaked out into the muscle sheath as all of it was absorbed by the ACS. Adding the liquid to the capsule made it very easy to disintegrate, and thus, the gelatin capsule was not sealed after the addition of the rhBMP-2. The skin wound was closed using three Michel surgical clips; these were removed on post-operative day 4 (see Figure 2.1).
Each mouse received a subcutaneous injection of buprenorphine (Temgesic®) 0.1 mg/kg for post-operative pain control, as well as an intraperitoneal injection of 0.2 mL of PBS containing either 0, 2, or 20 µg of zoledronate per mouse. Each subject was then allowed to recover in a private cage before returning to its initial cage population of 4 mice per cage. After the procedure, the cages were returned to a mammal dormitory in the animal care facility.

All subjects tolerated the surgery well and regained normal mobility of their hind limbs. No would dehiscences or complications were evident on post-operative day 1 or any time subsequent to that, and their progress was regularly monitored during the following 28 day period.

2.4.2 Sample Harvest

On post-operative day 28, the animals were euthanized using a sealed carbon dioxide chamber. This was followed by cervical dislocation. Skin was dissected off the hind quarters with surgical scissors (see Figure 2.2) and the spinal column was severed with bone cutting forceps cephalad to the pelvis. The feet were removed at the level of the ankles. The kidneys were also dissected out to examine for signs of renal toxicity. Sealable 50 mL polypropylene test tubes were labeled and filled with 10% buffered formalin solution. The dissected samples were then placed into these test tubes for tissue fixation.
Figure 2.2: Skin dissection off the hind quarter of the mouse in preparation for sample harvest

2.5 Radiographic Evaluation

2.5.1 CT Protocol, Calibration, & Reconstruction

The femurs with heterotopic ossicles were imaged with a microcomputed tomography system (MicroCT40, Scanco Medical, Basserdorf, Switzerland). The scanner had numerous poly-methyl-methacrylate (PMMA) tube holders of different diameters to contain the sample. The size of the mouse hind limbs dictated the use of the larger tube holder (diameter 36.9 mm). For scanning, each specimen was placed into the large tube holder with the pelvis oriented inferiorly.

The system was calibrated on a weekly basis with a PMMA block containing five hydroxyapatite (HA) samples of different densities. The scanner accurately reproduced the density values provided by the manufacturer during the period of time that our samples were analyzed, indicating that the machine was well-calibrated.

The specimens were scanned at 70 kVp and 114 μA in a high resolution mode with an X, Y, Z resolution of 36 μm. Acquisition files were obtained at 1000 projections with 2048 samples each (per 180° of rotation), 0° angle increment, with three-frame
averaging and 300 ms integration time (see Table 2.2 for explanation of micro CT scanning parameters). The scanning time for each specimen was approximately $5^{1/2}$ hours.

The final images were composed of approximately 800 slices, each one 36 µm thick. Reconstruction of the images was performed using the software provided by the manufacturer (see Figure 2.3).

**Table 2.2**: Explanation of micro CT scanning parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kVp)</td>
<td>Potential energy difference that exists between source and target determines the number of electrons generated by the source</td>
</tr>
<tr>
<td>Current (µA)</td>
<td>Acceleration of electrons from source to target</td>
</tr>
<tr>
<td>1000 Projections with 2048 samples each (per 180º of rotation)</td>
<td>As the sample rotates, for the duration of every $\frac{1}{2}$ turn (or 180º), the sample stops 1000 times, and for every stop, 2048 images are generated</td>
</tr>
<tr>
<td>Angle Increment (º)</td>
<td>Degree of holder tilting (0 means that the holder was not tilted) option allows to tilt the holder if sample inside is not well positioned</td>
</tr>
<tr>
<td>3-Frame Averaging</td>
<td>Scan is processed 3 times and the final images are averages of 3 processes increases resolution</td>
</tr>
<tr>
<td>Integration Time (msec)</td>
<td>The time required to produce each slice</td>
</tr>
</tbody>
</table>
Figure 2.3: 3-D reconstruction of the hind limb with BMP-induced ossicle A) control group, B) low zoledronate group, and C) high zoledronate group

2.5.2 Selection of Region of Interest

A region of interest (ROI) had to be selected for each reconstructed image to ensure that image analysis applied only to the BMP induced ossicle, and excluded the femur and pelvic girdle. This ensured that only the appropriate area was analyzed for bone content. Outlining of the ROI is termed splining.

Splining involved outlining the periphery of the BMP induced ossicle on individual CT slices (see Figure 2.4). This was manually done approximately every 10 slices, and the software interpolated the contours in the intermediate slices (a.k.a. autosplining). This procedure was performed by one individual who also analyzed the autosplined slices to ensure outlining of the appropriate areas as the shape of the newly formed bone varied throughout the depth of the sample (see Figure 2.5). This rendered a three-dimensional ROI containing the BMP induced ossicle.
Figure 2.4: Acquisition file showing the vertebral spine above, with the arrow pointing to the outlined region of interest (ROI) located to the left of the femur
Figure 2.5: Acquisition files demonstrating variation in the region of interest (ROI) along the depth of the sample.
2.5.3 Threshold Value

Computed tomography (CT) is an imaging technique that generates cross-sectional images in the axial plane resulting from the interaction of x-rays with the object of study. The density of tissues determines the amount of radiation that is blocked (or attenuated), and thus, does not reach the detector. Objects that are more dense (e.g. bone, metal) appear more radiopaque than less dense tissues (e.g. air), which appear more radiolucent. Data (projection) is acquired via detectors by summing the total attenuation of the beam by the tissues. Two-dimensional CT images are then derived by mathematical analysis of projection data and are comprised of pixels (picture elements). The pixels are assigned an arbitrary density unit (ADU) depending on the amount of radiation attenuated. The ADU is proportional to the density of the material, in this case, to the density of calcium. Rendering of a three-dimensional image requires segmenting the data to select the tissue of interest. Addition of another dimension to a pixel, converts it to a voxel (volumetric pixel) [153].

The outlined ROI contained a variety of tissues, including bone, soft tissue, and fluids (see Figure 2.6). Quantitative and qualitative analysis of bone in the ROI required determining if each individual voxel represented bone or soft tissue. This was done by selecting a threshold ADU value, below which the voxels were grouped into the non-bone phase. Hence, all voxels with values equal to or greater than the threshold value were considered to represent bone.

The threshold value was selected by choosing a number and visually analyzing the samples to maximize the volume of bone without including other tissues. Pixels below this value were not represented in the image. The threshold value of 130 ADU was selected as it was deemed to accurately represent the volume of bone without over- or underestimation (see Figure 2.7, Figure 2.8, and Figure 2.9). The same threshold value was used for all of the samples.
**Figure 2.6** : Outlined region of interest (ROI) containing bone and soft tissues

**Figure 2.7** : Outlined region of interest (ROI) with a threshold value underestimating the volume of bone
Figure 2.8 : Outlined region of interest (ROI) with a threshold value overestimating the volume of bone

Figure 2.9 : Outlined region of interest (ROI) with a threshold value accurately representing the volume of bone
2.5.4 Image Analysis

The ROI was analyzed using the “Full Morphometry” protocol. The parameters included the total volume (TV), bone volume (BV), bone volume fraction (BV/TV), total volume bone mineral density (TV-BMD), and bone volume bone mineral density (BV-BMD). TV is the entire volume in the ROI, including bone, soft tissue, and fluids. BV is the volume of bone in the ROI, including voxels that are greater than the threshold value. BV/TV is the fraction of bone greater than the threshold value compared to the total volume in the ROI. TV-BMD is the density of the mineral in the ROI. BV-BMD is the mineral density in the ROI, including only voxels greater than the threshold value. More detailed descriptions of these parameters can be found in Table 2.3.

Table 2.3: Explanation of micro CT bone analysis parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acronym</th>
<th>Definition</th>
<th>Threshold Dependence</th>
<th>Evaluates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume (mm$^3$)</td>
<td>TV</td>
<td>Total volume in the ROI, including bone, soft tissue, and fluids</td>
<td>No</td>
<td>Quantity</td>
</tr>
<tr>
<td>Bone Volume (mm$^3$)</td>
<td>BV</td>
<td>Volume of bone in the ROI, including only voxels greater than the threshold value</td>
<td>Yes</td>
<td>Quantity</td>
</tr>
<tr>
<td>Bone Volume Fraction</td>
<td>BV/TV</td>
<td>Fraction of bone greater than the threshold value compared to the total volume in the ROI (Bone volume ÷ Total volume)</td>
<td>Yes</td>
<td>Quality</td>
</tr>
<tr>
<td>Total Volume Bone Mineral Density (mg/cc)</td>
<td>TV-BMD</td>
<td>Mineral density in the ROI</td>
<td>No</td>
<td>Quality</td>
</tr>
<tr>
<td>Bone Volume Bone Mineral Density (mg/cc)</td>
<td>BV-BMD</td>
<td>Mineral density in the ROI, including only voxels greater than the threshold value</td>
<td>Yes</td>
<td>Quality</td>
</tr>
</tbody>
</table>
2.6 Histologic Evaluation

After the microCT analysis was completed, the samples were prepared for histologic evaluation. The previously fixed specimens were decalcified for six weeks in a solution containing 45% formic acid in 20% sodium citrate. After completion of decalcification, the samples were embedded in paraffin wax and sectioned perpendicular to the long axis of the femur through the newly formed ossicle. The sections were then stained with hematoxylin and eosin (H & E) and examined using a light microscope to assess the quality of the induced bone. The kidneys were embedded in paraffin wax without decalcification and stained with H & E to examine for signs of renal toxicity.

2.7 Statistics

The weight and weight changes of the mice, as well as the micro CT bone analysis parameters were analyzed with Sigma Stat version 3.5 statistical software. The purpose of the analysis was to elucidate any statistically significant differences among the control, low zoledronate, and high zoledronate groups. The data was evaluated for normality and equal variance. Normally distributed data was analyzed using one-way ANOVA, and the post hoc analysis was done using the Student-Newman-Keuls method. Non-parametric data and/or data with abnormal variance was analyzed with Kruskal-Wallis one-way ANOVA. Statistical significance was defined by a p-value < 0.05.
Chapter 3

Results

3.1 Necroscopy

When the samples were harvested, button shaped plaques of hard tissue were seen under the muscle sheath. There were no grossly observable differences in the ossicles formed amongst the three groups. There were also no grossly evident differences in the kidneys amongst the three groups.

3.2 Investigation of Systemic Effects

Injection of a high bisphosphonate dose warranted investigation of any detrimental systemic effects, including organ dysfunction and hormonal imbalances. These may affect bone formation or remodeling, and thus, confound the results otherwise attributed to the direct action of bisphosphonates.

3.2.1 Weight

Normal growth and weight gain were assumed to reflect minimal systemic effects of a high bisphosphonate dose. The mice were weighed on Day 0 (administration of 1st zoledronate dose), Day 28 (administration of 2nd zoledronate dose and BMP bioimplant placement), as well as Day 56 (necropsy). There were no statistically significant differences in the weights amongst the groups at any time during the study period. In the overall weight change for the duration of the study period, there were no statistically significant differences amongst the groups (see Table 3.1, Table 3.2, and Figure 3.1).
Table 3.1: Mice Weights and Weight Changes

<table>
<thead>
<tr>
<th>Zoledronate (µg/mouse)</th>
<th>n</th>
<th>Weight (g) MEAN ± SD</th>
<th>Weight Change Day 0-56</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>0</td>
<td>14</td>
<td>31.1 ± 1.9</td>
<td>39.1 ± 3.8</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>31.3 ± 2.4</td>
<td>39.7 ± 4.0</td>
</tr>
<tr>
<td>20</td>
<td>14</td>
<td>30.3 ± 2.3</td>
<td>36.8 ± 2.6</td>
</tr>
<tr>
<td>P(ANOVA)</td>
<td></td>
<td>0.280</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Table 3.2: Statistical summary of weights and weight changes

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Group*</th>
<th>Normal Dist.</th>
<th>Test</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Median</th>
<th>25% Value</th>
<th>75% Value</th>
<th>P Value</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0</td>
<td>No</td>
<td>[Kruskal-Wallis One Way ANOVA – median]</td>
<td>30</td>
<td>31</td>
<td>30</td>
<td>29</td>
<td>32</td>
<td>0.280</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>One Way ANOVA</td>
<td>39.1</td>
<td>39.7</td>
<td>3.8</td>
<td>4.0</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>One Way ANOVA</td>
<td>36.8</td>
<td>36.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0</td>
<td>Yes</td>
<td>One Way ANOVA</td>
<td>39.1</td>
<td>39.7</td>
<td>3.8</td>
<td>4.0</td>
<td>2.6</td>
<td>0.062</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>One Way ANOVA</td>
<td>36.8</td>
<td>36.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>One Way ANOVA</td>
<td>43.6</td>
<td>43.6</td>
<td>5.1</td>
<td>5.3</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 56</td>
<td>0</td>
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<td>One Way ANOVA</td>
<td>43.6</td>
<td>43.6</td>
<td></td>
<td></td>
<td></td>
<td>0.165</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>One Way ANOVA</td>
<td>40.5</td>
<td>40.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>One Way ANOVA</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
<td>10</td>
<td>15</td>
<td>0.125</td>
<td>No</td>
</tr>
<tr>
<td>Weight Change Day 0 - 56</td>
<td>0</td>
<td>No</td>
<td>[Kruskal-Wallis One Way ANOVA – median]</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
<td>10</td>
<td>15</td>
<td>13</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>One Way ANOVA</td>
<td>9</td>
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<tr>
<td></td>
<td>20</td>
<td></td>
<td>One Way ANOVA</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Group: 0 – Control; 2 – 2 µg/mouse of zoledronate; 20 – 20 µg/mouse of zoledronate
Figure 3.1: Weight gain from Day 0 to Day 56
According to the Kruskal-Wallis One Way ANOVA, the difference in the weight change during the study period amongst the three groups was not statistically significant (p = 0.125). The box represents 25\textsuperscript{th}-75\textsuperscript{th} percentiles, the line within the box is the median, the whiskers represent the minimum and the maximum values, and the points connected by a line represent the mean.
3.2.2 Kidneys

The kidneys did not demonstrate any histological differences amongst the three groups. Sections of the renal cortex in the control, the high zoledronate, and the low zoledronate groups demonstrated glomeruli, tubules, and interstitium that appeared similar in the three samples. See Figure 3.2, Figure 3.3, and Figure 3.4 for a more detailed description.

**Figure 3.2**: Photomicrograph of H & E stained renal cortex from the control group demonstrating a glomerulus (G) surrounded by Bowman’s space (BS) and squamous cells (S) lining the Bowman’s capsule. The numerous nuclei in the glomerulus represent endothelial cells, mesangial cells, and podocytes. The tubules (T) are lined by cuboidal or columnar epithelium. (40x magnification)
Figure 3.3: Photomicrograph of H & E stained renal cortex from the low zoledronate group displaying glomeruli (G) and tubules (T). The tubules are lined by cuboidal and columnar epithelial cells. There are no differences in the glomeruli, the tubules, or the interstitium between this group and the control.

(40x magnification)
Figure 3.4: Photomicrograph of H & E stained renal cortex from the high zoledronate group demonstrating a glomerulus (G) and tubules (T). The tubules are lined by cuboidal and columnar epithelial cells. There are no differences in the glomeruli, the tubules, or the interstitium between this group and the low zoledronate group or the control.

(40x magnification)
3.3 Micro CT Analysis

The micro CT results were analyzed using numerous parameters, specifically, the total volume (TV), bone volume (BV), bone volume fraction (BV/TV), total volume bone mineral density (TV-BMD), and bone volume bone mineral density (BV-BMD). See Table 3.3 and Table 3.4 for a statistical summary of the micro CT results.

Table 3.3: Statistical summary of microCT results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group*</th>
<th>Normal Dist.</th>
<th>Test</th>
<th>Mean</th>
<th>Std Dev</th>
<th>Median</th>
<th>25% Value</th>
<th>75% Value</th>
<th>P Value</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV (mm³)</td>
<td>0 2 20</td>
<td>Yes</td>
<td>One Way ANOVA</td>
<td>341.5</td>
<td>302.7</td>
<td>427.2</td>
<td>154.0</td>
<td>107.7</td>
<td>178.7</td>
<td>0.092 No</td>
</tr>
<tr>
<td>BV (mm³)</td>
<td>0 2 20</td>
<td>No</td>
<td>Kruskal-Wallis</td>
<td>25.1</td>
<td>21.1</td>
<td>55.9</td>
<td>19.2</td>
<td>15.3</td>
<td>36.9</td>
<td>0.002 Yes</td>
</tr>
<tr>
<td>TV-BMD (mg/cc)</td>
<td>0 2 20</td>
<td>Yes</td>
<td>One Way ANOVA</td>
<td>35.4</td>
<td>34.8</td>
<td>63.6</td>
<td>28.4</td>
<td>25.3</td>
<td>22.7</td>
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</tr>
<tr>
<td>BV-BMD (mg/cc)</td>
<td>0 2 20</td>
<td>Yes</td>
<td>One Way ANOVA</td>
<td>408.0</td>
<td>382.1</td>
<td>363.4</td>
<td>30.1</td>
<td>20.1</td>
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<tr>
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<td>One Way ANOVA</td>
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<td>0.1</td>
<td>0.2</td>
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<td>0.05</td>
<td>0.06</td>
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*Group: 0 – Control; 2 – 2 µg/mouse of zoledronate; 20 – 20 µg/mouse of zoledronate
Table 3.4: Post hoc analysis using the Student-Newman-Keuls Method

<table>
<thead>
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<th>Parameter</th>
<th>Groups* Compared</th>
<th>P Value</th>
<th>Significant</th>
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<tr>
<td>BV (mm³)</td>
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</tr>
<tr>
<td></td>
<td>20 vs. 0</td>
<td>0.006</td>
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</tr>
<tr>
<td></td>
<td>20 vs. 2</td>
<td>0.013</td>
<td>Yes</td>
</tr>
<tr>
<td>TV-BMD (mg/cc)</td>
<td>0 vs. 2</td>
<td>0.038</td>
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<td>20 vs. 0</td>
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<tr>
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<td>20 vs. 2</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>BV-BMD (mg/cc)</td>
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<td>20 vs. 0</td>
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<td></td>
<td>20 vs. 2</td>
<td>0.004</td>
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</tr>
<tr>
<td>BV/TV</td>
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<td>0.006</td>
<td>Yes</td>
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<tr>
<td></td>
<td>20 vs. 0</td>
<td>0.013</td>
<td>Yes</td>
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<tr>
<td></td>
<td>20 vs. 2</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Group: 0 – Control; 2 – 2 µg/mouse of zoledronate; 20 – 20 µg/mouse of zoledronate
3.3.1 Total Volume (TV)

This measurement represented the total volume of the ROI, which included bone and soft tissue (marrow) present within the ossicle (see Figure 3.5). The data demonstrated a normal distribution. The means for the control, low zoledronate, and high zoledronate groups were $341.5 \pm 154.0 \text{ mm}^3$, $302.7 \pm 107.7 \text{ mm}^3$, and $427.2 \pm 178.7 \text{ mm}^3$, respectively. One Way ANOVA did not demonstrate any significant differences amongst the 3 groups ($p = 0.092$).

![Figure 3.5: Total Volume](image)

One way ANOVA demonstrated no statistically significant difference in the total volume amongst the three groups ($p = 0.092$).
3.3.2 Bone Volume (BV)

This measurement represented the number of voxels in the ROI with a grayscale value higher than the threshold value (see Figure 3.6). The data was not normally distributed. The medians for the control, low zoledronate, and high zoledronate groups were 25.1 mm$^3$, 21.1 mm$^3$, and 55.9 mm$^3$, respectively. Using the Kruskal-Wallis One Way ANOVA, there was a statistically significant increase in the volume of bone in the high zoledronate group relative to the other two groups ($p = 0.002$). There was no statistically significant difference between the volume of bone in the low zoledronate group and the control.

Figure 3.6: Total Bone Volume

The Kruskal-Wallis one way ANOVA demonstrated a statistically significant increase in the total bone volume in the high zoledronate group compared to the other two groups ($p = 0.002$). There was no statistically significant difference between the low zoledronate group and the control. The box represents 25$^{th}$-75$^{th}$ percentiles, the line within the box is the median, the whiskers represent the minimum and the maximum values, and the points connected by a line represent the mean.
3.3.3 Bone Volume Fraction (BV/TV)

This value was calculated by dividing the volume of bone (i.e. voxels in the ROI with a grayscale value higher than the threshold value) by the total volume of the ROI (see Figure 3.7). The data demonstrated a normal distribution. The means for the control, low zoledronate, and high zoledronate groups were 0.1 ± 0.05, 0.1 ± 0.05, and 0.2 ± 0.06, respectively. According to the One Way ANOVA, there was a statistically significant increase in the bone volume fraction in the high zoledronate group relative to the other two groups (p = 0.002). There was no statistically significant difference between the bone volume fraction in the low zoledronate group and the control.

![Bone Volume Fraction Chart](image)

**Figure 3.7: Bone Volume Fraction**

One way ANOVA demonstrated a statistically significant increase in the fraction of bone within the total volume in the high zoledronate group compared to the other two groups (p = 0.002). There was no statistically significant difference between the low zoledronate group and the control.
3.3.4 Total Volume Bone Mineral Density (TV-BMD)

This measurement represented the mineral density in the entire ROI (see Figure 3.8). The data demonstrated a normal distribution. The means for the control, low zoledronate, and high zoledronate groups were 35.4 ± 28.4 mg/cc, 34.8 ± 25.3 mg/cc, and 63.6 ± 22.7 mg/cc, respectively. One Way ANOVA demonstrated a statistically significant increase in the total volume bone mineral density in the high zoledronate group relative to the other two groups (p = 0.006). There was no statistically significant difference between the mineral density in the low zoledronate group and the control.

![Figure 3.8: Total Volume Bone Mineral Density](image)

*Figure 3.8: Total Volume Bone Mineral Density*

One Way ANOVA demonstrated a statistically significant increase in the mineral density within the entire ROI in the high zoledronate group relative to the other two groups (p = 0.006). There was no statistically significant difference between the low zoledronate group and the control.
3.3.5 Bone Volume Bone Mineral Density (BV-BMD)

This measurement represented the mineral density of bone volume within the ROI (see Figure 3.9). The data demonstrated a normal distribution. The means for the control, low zoledronate, and high zoledronate groups were 408.0 ± 30.1 mg/cc, 382.1 ± 20.1 mg/cc, and 363.4 ± 16.8 mg/cc, respectively. According to the One Way ANOVA, there was a statistically significant decrease in the bone volume bone mineral density in the high zoledronate group relative to the other two groups (p < 0.001). There was also a statistically significant decrease in the bone volume bone mineral density in the low zoledronate group relative to the control (p = 0.005).

![Figure 3.9: Bone Volume Bone Mineral Density](image)

Figure 3.9: Bone Volume Bone Mineral Density

One Way ANOVA demonstrated a statistically significant decrease in the mineral density of the bone volume in the high zoledronate group relative to the other two groups (p < 0.001). There was also a statistically significant decrease in the bone volume bone mineral density in the low zoledronate group relative to the control (p = 0.005).
3.4 Histological Analysis

Histological specimens of the BMP induced ossicles in the high zoledronate group exhibited qualities more consistent with immature bone as compared to the control and to the low zoledronate group. The ossicle from the control group displayed an outer thin cortical shell of bone with cancellous bone on the inside and yellow marrow. The cortical matrix was uniform with a high mineral content and few osteocytes. The high zoledronate ossicle demonstrated a less uniform matrix with red marrow on the inside. There was a lower mineral content and a higher proportion of osteocytes. The osteocytes were positioned more closely together and distributed more randomly than in the other two groups. The low zoledronate ossicle displayed features intermediate between the other two groups (see Figure 3.10, Figure 3.11, and Figure 3.12).

Figure 3.10: Photomicrograph of H & E stained induced ossicle from the control group displayed an outer thin cortical shell of bone with cancellous bone on the inside and yellow marrow. The cortical matrix appeared fairly uniform with a high mineral content and few osteocytes. (10x magnification)
Figure 3.11: Photomicrograph of H & E stained induced ossicle from the low zoledronate group demonstrated a moderately uniform cortical matrix with a slightly lower mineral proportion and more osteocytes than the control.

(10x magnification)
Figure 3.12: Photomicrograph of H & E stained induced ossicle from the high zoledronate group demonstrated a less uniform matrix within the trabecula with red marrow on the inside. There was a lower mineral content and a higher proportion of osteocytes than in the control or the low zoledronate group. The osteocytes were positioned more closely together and distributed more randomly than in the other two groups. These characteristics were consistent with immature bone.

(10x magnification)
Chapter 4
Discussion

Autogenous bone grafts have been the gold standard for reconstructing bone defects, fracture non-unions, and augmenting existing bone volumes for a long period of time. Hence millions of surgical procedures are performed every year to harvest autogenous bone. However, autogenous bone graft harvest can result in significant donor site morbidity as it requires a second surgical site, potentially prolongs hospital stay, and increases operating time. It carries the risk of general complications, including pain, infection, and neurovascular disturbance, as well as site-specific complications, including iliac fracture, peritoneal perforation, gait disturbance and pneumothorax. Various studies have described a 10-30% rate of donor site complications [154], [155], [156]. Hence, surgeons have sought alternatives to autogenous grafting, however, these alternatives have been used with mixed results. Currently, the success and relative predictability of autogenous grafting has not been replicated by any alternative methods.

Growth factors, most commonly bone morphogenetic proteins (BMPs), have recently received significant attention as initial studies demonstrated great potential for induction of bone. Several concerns have been raised over the use of BMPs instead of autogenous grafts, namely, safety, predictability, and cost. However, recent intensive research was undertaken to demonstrate the reliability and safety of rhBMP use in humans. It was found that rhBMP has to be combined with a carrier or a delivery system to form a bioimplant in order to be effective in clinical applications.

Currently rhBMP-2 with an absorbable collagen sponge (Infuse®) was one of the two BMPs approved for human applications by the FDA and Health Canada. Approximately 126,000 procedures utilized BMPs in 2008 in the U.S., with rhBMP-2 employed in more than 94% of cases [157].
Numerous patients are taking oral bisphosphonates to stabilize bone loss associated with osteoporosis or the more potent intravenous bisphosphonates, namely zoledronate, to treat skeletal complications associated with bone metastases or multiple myeloma. The effect of bisphosphonates on bone metabolism introduces the possibility of an effect on BMP induced bone formation. Thus, it would be prudent to explore the quantity and the quality of bone induced by BMPs in the presence of bisphosphonates in the system.

To date, all of the studies that explored the effect of bisphosphonates on BMPs administered the bisphosphonate either at the time of surgery or at a later time. Thus, it was the aim of this study to elucidate the effect of pre-treatment with bisphosphonate on the bioactive ability of BMP.

4.1 Study Design

The Urist mouse muscle pouch assay is the standard for evaluating the osteoinductive activity of BMP bioimplants in an in vivo model [149]. The muscle pouch holds great potential for bone induction due to its significant vascularity and vast source of precursor cells. It is ideal for studying the effect of the implanted BMP and isolating it from endogenous BMPs that would be present at skeletal sites. A similar model was utilized in our lab to compare the osteoinductivity of rhBMP-2 and rhBMP-7 [158].

In this investigation, the low dose of zoledronate that was equivalent to the dose used in multiple myeloma, was comparable to doses used in other studies. Previous studies that injected mice or rats with zoledronate to augment bone formation induced by BMPs, used a total zoledronate dose of 0.1 mg/kg [147], [148], [159]. Our study utilized 2 µg/mouse which was equivalent to 0.07 mg/kg. The high zoledronate dose of 20 µg/mouse was selected to simulate extended prior treatment. The present study was different from previous studies in that it was designed to evaluate the activity of BMPs introduced into an environment of bisphosphonates, and thus, the mice were pretreated...
with zoledronate. The above-mentioned studies, however, were designed to attempt to reduce resorption and maximize the amount of bone formed by BMPs. Hence, zoledronate was injected either at the time of surgery, three days later, or two weeks later.

4.2 Validation of Experimental Model

In any investigation that involves animal manipulation by injection of medication, one might expect behavioural or systemic physiological changes. In this study, the mice tolerated the experiment well and no behavioural differences were noted. As well, the mice did not exhibit any clinically significant systemic complications throughout the study period. Upon histological examination, the kidneys did not demonstrate any abnormalities grossly or at the microscopic level. The use of a very high bisphosphonate dose in one experimental group warranted investigation of any detrimental systemic effects, including organ dysfunction and hormonal imbalances. These may have affected bone formation or remodeling, and thus, confounded the results otherwise attributed to the direct actions of bisphosphonates.

Examination of the kidneys was prompted by previous reports of acute renal toxicity in patients receiving high doses of intravenous bisphosphonates [160], [161]. The renal effects on rats of supraphysiologic doses of zoledronate deemed to be nephrotoxic were studied by Pfister et al. [162] to detect any subclinical renal damage. The animals received intermittent (every 3 weeks over a 25 week period) intravenous injections of zoledronate (1 and 3 mg/kg). Cumulative renal damage, assessed with creatinine clearance and histopathology, was evident with both the high and the low zoledronate doses. However, there was no histopathologic damage evident after a single injection of 1 mg/kg of zoledronate. In our study, the 2 µg/mouse and 20 µg/mouse doses of zoledronate, when converted to mg/kg (i.e. 0.07 mg/kg and 0.7 mg/kg, respectively), were significantly lower than the nephrotoxic doses used in the Pfister study. Thus, it was consistent that no histopathologic renal changes were evident in our subjects.
To ensure normal growth and weight gain, the mice were weighed at three instances during the study period. There were no statistically significant differences in mice weights or weight changes amongst the three groups.

These results served to validate the experimental model by demonstrating that a high bisphosphonate dose produced minimal systemic effects on the mice. Thus, any observed effect on bone metabolism can be attributed directly to the actions of bisphosphonates.

4.3 Explanation of Results

The microCT and histological results consistently demonstrated that the high zoledronate group had less mature ossicle formation than the control and the low zoledronate group displayed intermediate characteristics. Interpretation of these results necessitated an understanding of BMP induced formation of ectopic bone.

Recombinant human BMP-2 has previously been shown capable of inducing ectopic bone in animal models [163], [164], [165]. Bone formation at heterotopic sites was shown to occur via the intramembraneous route in areas close to blood vessels and via the endochondral route in less vascular areas [149].

The morphogenesis of ectopic bone was described in a study that examined the molecular progression of ectopic bone development with rhBMP-2 and a collagen type I carrier in the hind quarter muscles of mice [166]. The specimens were examined on days 3, 5, 6, 7, 8, 14, and 21 after BMP implantation. On day 3, the BMP sponge was still directly surrounded by muscle tissue with various cell types migrating towards the surface of the sponge. In previous studies, it was shown that these cells are polymorphonuclear lymphocytes and mesenchymal cells [2]. The mesenchymal cells demonstrated the morphology of fibroblasts and expressed type I collagen mRNA. This is consistent with the process of wound healing with macrophages and fibroblasts being recruited to the site of injury.
On day 5, the migrating cells formed a continuous tissue layer surrounding the collagen sponge. The cells in this layer consisted mainly of fibroblasts expressing type I collagen. As well, cells surrounding the BMP implant started to express high levels of alkaline phosphatase, which is an early marker of osteogenesis. Chondrocytes appeared for the first time on day 6 and clusters of cartilage formed with proliferating and hypertrophic chondrocytes on days 7-8. In another study, on day 9, osteoblasts appeared and calcification was detected near the hypertrophic chondrocytes and in the peripheral matrix, signifying the progress of chondrogenesis and osteogenesis. The developing ossicle was also being invaded by blood vessels at this point. Numerous multinucleated osteoclasts appeared on day 10 and cartilage disappeared by day 11.

From day 11 and onwards, the osteoclasts were breaking down the implant and remodeling the woven bone, that was slowly becoming more mineralized [167]. By day 14, cartilage was fully replaced by bone, as it was no longer detected at that point. The vascularization of mineralized hypertrophic cartilage and the replacement of cartilage by bone, led to bone marrow formation and hematopoiesis, characterized by irregular honeycomb trabeculae of woven bone that was being remodeled. On day 21, a thin ring of cortical bone surrounded an almost completely resorbed sponge, embedded by bone marrow [166].

This investigation explored whether rhBMP-2 was able to induce bone formation in all three groups, however, the heterotopic ossicle in the high zoledronate group was different from the other two groups. The microCT results demonstrated that the BMP induced ossicle in the high zoledronate group, compared to the low zoledronate group and to the control, was of a similar size (TV) but had a larger volume of bone (BV). This resulted in a higher fraction of the volume of bone to the total volume (BV/TV).

It was found that the mineral content of bone matrix was lower in the high zoledronate group as evidenced by decreased BV-BMD. This parameter provided a comparison of the amount of calcium per unit of bone. Bone maturity may be inferred from the degree of mineralization, with higher mineralization implying greater maturity. The mineral content of the total volume of tissue (TV-BMD) was less than the density of bone (BV-TMD), consistent with the assumption that the majority of the mineral content resided in
bone. The TV-BMD was greater in the high zoledronate group due to the higher volume of bone in that group. As well, the low zoledronate group demonstrated a decrease in the mineral content of bone matrix compared to the control.

In summary, these results suggested that the ossicle formed in the high zoledronate group, compared to the other two groups, had larger bone volume, but was less mineralized, consistent with being more immature. The low zoledronate group demonstrated a less mineralized ossicle compared to the control. These results suggested that bisphosphonates affected mineralization or remodeling.

The interpretation of the microCT results was confirmed histologically. The ossicle in the high zoledronate group exhibited characteristics more closely resembling immature bone, whereas the induced bone in the control group appeared more mature. The low zoledronate group displayed intermediate characteristics at the microscopic level.

These findings suggested that bisphosphonates affected bone remodeling, since at high doses, the BMP induced ossicle was immature compared to the low dose and the control. Initially, rhBMP-2 induced the formation of immature bone in all three experimental groups. However, only the control group and to some extent the low zoledronate group retained the ability to remodel that bone and replace it with a more mature ossicle. Nevertheless, this effect was evident in the high zoledronate group and to a lesser degree at the clinically relevant low zoledronate dose, indicating a dose-dependent relationship between bisphosphonates and BMPs. Since a similar amount of woven bone was formed in all three groups, the effect of bisphosphonates on osteogenesis was negligible, and it can be inferred that the effect was only on remodeling.

Considering the pharmacokinetic properties of zoledronate and the location of BMP implantation in our study, it is likely that the zoledronate released into systemic circulation from the bone reservoir interacted with the BMP induced ossicle. Following intravenous administration of zoledronate, a rapid peak in its concentration occurred at the end of the infusion period. According to previous studies, approximately 60% of the infused dose bound with high affinity to the hydroxyapatite of bone, and the rest was quickly excreted by the kidneys unchanged. This was evidenced by the fact that there
was less than 10% of the peak concentration present 4 hours post-infusion and less than 1% 24 hours post-infusion. The urinary excretion was complete at 24 hours after drug administration and amounted to approximately 40% of the initial dose, implying that 60% of the dose was retained in the skeleton [168].

Thus, when initially introduced into the system during its concentration peak, zoledronate did not have significant opportunity to affect bone formation and remodeling induced by rhBMP-2, since mineralization at the surgical site did not appear until day 9 and osteoclasts were not present until day 10 after BMP implantation [167]. At this time, the concentration of zoledronate in blood and muscles was below detectable levels [169]. Hence, the observed effect on remodeling was not a result of the peak zoledronate concentration after injection of the drug. A possible explanation is that during skeletal resorption and remodeling, the zoledronate that was retained in the skeleton, could have been slowly released into the systemic circulation, delivered to the site of the heterotopic ossicle, and taken up by the osteoclasts, resulting in impaired remodeling. The fact that the concentration of zoledronate in blood and muscle was very low at this time, signified that very small amounts of zoledronate are sufficient to inhibit bone resorption.

Several studies measured the concentration of bisphosphonates in different tissues at various time points after dose injection. According to one study, the recirculation of zoledronate occurred for a prolonged period after the initial injection as levels of zoledronate in soft tissue and blood, albeit low, were measured in rats eight months after intravenous injection [113].

A more recent study measured the elimination and distribution in bone, plasma, and noncalcified tissues (including muscle) of zoledronate in rats and dogs after single or multiple injections [169] and suggested that the presence of bisphosphonates in noncalcified tissues was a result of slow release from bone. The rats received either a single dose or 16 consecutive doses of 0.15 mg/kg of zoledronate administered as a bolus intravenously. The tissues were harvested at 5 min, 4 and 24 hours after the first dose, 24 hours after the eighth dose, and 1, 16, 64, 128, and 240 days after the sixteenth dose. The dogs received an intravenous injection of 0.15 mg/kg of
zoledronate and the tissues were harvested 4 days later. At 5 min after a single injection, the highest zoledronate concentration was in plasma, which declined very rapidly to 0.5% and 0.07% of the initial peak dose at 4 and 24 hours, respectively. At 4 hours, the highest zoledronate concentration was in bone, which demonstrated continuous uptake up to 24 hours. It is interesting that at 24 hours, the zoledronate concentration in bone was 7-fold higher after the eighth dose and 10- to 12-fold higher after the sixteenth dose. In both the rats and the dogs, the concentration of zoledronate showed a rapid decline in plasma and noncalcified tissues, but a very slow decline in bone to approximately 50% of the peak dose at 240 days. The terminal half-lives, however, were very similar in bone and noncalcified tissues, ranging from 50-200 days. This suggested that the distribution of zoledronate in noncalcified tissues was governed by the extensive uptake into bone and slow release from it rather than from prolonged retention in noncalcified tissues [169].

4.4 Clinical Applications

This findings of this investigation demonstrated that bone induction by BMPs occurs in the presence of bisphosphonates. The high zoledronate dose produced a less mature BMP induced ossicle than the other two groups, whereas the low zoledronate dose demonstrated a less pronounced, albeit existent, effect on remodeling.

The concept that pretreatment with zoledronate interferes with remodeling of the BMP induced bone has significant clinical implications. It suggests that any process requiring bone remodeling may be compromised in bisphosphonate users, and is consistent with the accepted pathophysiology of BRONJ. The premise of the remodeling hypothesis of the pathophysiology of BRONJ is that the jaw has a high remodeling rate, and bisphosphonates inhibit remodeling [136]. As well, to increase the success rate of non-urgent surgical procedures utilizing BMPs, a bisphosphonate drug holiday may be recommended as it has been suggested that a drug holiday can improve bone healing [170]. A drug holiday of sufficient length would decrease the concentration of
bisphosphonate in bone and thus, would allow a smaller amount of drug to redistribute to the rest of the organism. Given the results of this investigation, a drug holiday would potentially improve the healing of BMP bioimplants. In a previous study [169], the concentration of zoledronate in bone declined by 50% over 8 months in rats, however, it is not known whether this decline would produce a clinically significant improvement in remodeling. Since bisphosphonates are avidly bound to bone, a reservoir of drug accumulates after years of treatment and this reservoir is gradually released over months or years. Hence, the recommendation of a 3-month drug holiday by the American Association of Oral and Maxillofacial Surgeons updated position paper on BRONJ [170] and Marx's recommendation of a 4-6 month drug holiday [171] may not be sufficient. The duration of the drug holiday will depend on the risk of stopping the medication, the duration and frequency of bisphosphonate administration, and the bone binding affinity of the particular bisphosphonate. It is a variable that is yet to be determined by future studies.

In addition to the drug holiday, patients subjected to high doses of potent bisphosphonates may also require an increase in the amount of BMPs to achieve optimal results. Currently, there is a gap in the literature on this topic, as there are no published reports on the application of BMPs in patients on bisphosphonates. The concept that BMPs can induce bone formation in the presence of bisphosphonates, brings forth the idea of using BMPs in the prevention and treatment of BRONJ. Osteoclasts have a role in osteoinduction as resorption liberates BMPs from the resorbed trabeculae. The released BMPs further stimulate mesenchymal progenitor chemotaxis, division, and commitment to the osteogenic lineage [22], which ensures proper healing and remodeling. Therefore, there is a potential that the amount of BMPs is significantly decreased in patients on bisphosphonates due to impaired osteoclast function. Hence, exogenous supplementation of BMPs may help to overcome this phenomenon. Another theoretical basis for using BMPs to treat or prevent BRONJ is that BMPs may be able to overcome the impaired remodeling indirectly by stimulating the production of osteoblasts, which in turn, stimulate osteoclast differentiation via the RANK/RANKL interaction. Although, this idea requires further theoretical development and experimental testing, it offers a potentially simple solution to a complex problem if,
for instance, it can be demonstrated that placing BMP into an extraction socket of patients on bisphosphonates can prevent BRONJ.

4.5 Critical Appraisal

It is noteworthy that the behavior of BMPs in the presence of bisphosphonates in a rodent model may differ from behavior in humans. It is possible that the pluripotential cells of a mouse respond differently to human BMP than they would respond to mouse BMP. However, it was reported that the mature coding region of human and mouse BMP-2 is identical [172]. Animal model results may be difficult to reproduce in humans as there may be a species-specific dose response [173], a difference in the recruitment of osteoprogenitors [28], or a larger quantity of pluripotential cells in the bone and soft tissues of small animals [174].

Bisphosphonate concentration in the immediate vicinity of bone (i.e. a situation with more clinical relevance) would be expected to be greater than in a muscle pouch. Hence, BMP induced bone formation may be affected more significantly when BMP bioimplants are placed at skeletal sites.

It must be noted that interference with remodeling was seen primarily in the high zoledronate group. The dose of 20 µg/mouse corresponded with 10x the zoledronate dose used clinically. The intention of this dose was to simulate extended prior treatment. Since administration of multiple doses leads to higher accumulation of the drug in bone, a single high dose may be similar to multiple dosing with respect to the total amount of drug in the reservoir of bone. However, it is difficult to estimate the accuracy of this simulation. Thus, it is not recommended to extrapolate direct clinical applications from these results. Nonetheless, this investigation was aimed to establish the existence of an effect of bisphosphonate pretreatment on the ability of BMPs to induce bone, and study its characteristics. Hence, bisphosphonate doses thought to elicit this effect were employed in this study.

To elucidate whether both zoledronate injections contributed to the inhibition of remodeling, two other experimental groups could have been added. Specifically, the
suggested groups would be: (1) injection at the time of implantation only, and (2) injection one month prior to BMP implantation only. This would allow one to determine if pretreatment with bisphosphonate made a significant difference compared with bisphosphonate administration at the time of BMP implantation only. Extrapolation from a previous study that demonstrated a higher concentration in bone after repeated dosing [169], leads to the assumption that with repeated dosing and a higher concentration of bisphosphonate in bone, a greater amount would be available for redistribution to other tissues.

Studying the BMP implant at 3, 5, 7 and 14 days post implantation, in addition to the current study’s 28 days, may have helped to establish differences amongst the groups at various stages of the BMP induced ossicle formation. For instance, the finding of the sites appearing similar in the three groups at 14 days, but at 28 days demonstrating the differences noted in this study, would support the idea of impaired remodeling of the BMP induced ossicle due to the inhibition of osteoclasts by zoledronate.

4.6 Future Directions

It can be said with certainty that a void on this topic exists in the current literature and that more studies on the topic are required. Future studies should be directed to establish more accurately the clinical relevance of the effects of bisphosphonate pretreatment on the activity of BMPs. Suggestions include implanting BMPs in critical sized bony defects in subjects pretreated with bisphosphonates to determine if vicinity to bone results in a more pronounced impediment to remodeling. Also, since intravenous bisphosphonates are usually administered for many months, using clinically relevant doses with a longer pretreatment period will more closely replicate the clinical situation. Alternatively, varying the amounts of BMPs used may help to establish if increasing the dose of BMPs can overcome the effect of bisphosphonates. Finally, implanting BMPs after a drug holiday and varying the duration of the drug holiday will help to establish the clinical utility of stopping bisphoshonates prior to BMP related surgery.
Chapter 5
Conclusions

This study demonstrated that BMPs can function in the presence of bisphosphonates. Bone induced by rhBMP-2 in mice pre-treated with a high concentration of zoledronate (20 µg) was immature compared to the low zoledronate group (2 µg) and to the control, as evidenced by radiographic and histologic findings. Pretreatment with a clinically relevant dose of zoledronate (2 µg) demonstrated reduced effects on remodeling in the BMP induced ossicle. Thus, a potential exists for using BMPs in patients treated with bisphosphonates, but further studies are required to elucidate specific clinical applications.
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