



T.C.

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DEPARTMENT OF ORAL AND MAXILLOFACIAL SURGERY

**HISTOLOGICAL AND HISTOMORPHOMETRIC
EVALUATION OF THE EFFECTS OF BONE
MORPHOGENETIC PROTEIN 2 (BMP 2) AND
METFORMIN ON BONE FORMATION IN
CRITICAL SIZE DEFECT MODELS**

DOCTOR OF PHILOSOPHY THESIS

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 30.06.2021 and numbered

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Orkun UYGUN

30.06.2021



DEDICATION

I dedicate this thesis to my late grandparents for looking after me with affection and love and their dedicated partnership for success in my life.



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LIST OF SYMBOLS AND ABBREVIATIONS

AGE	Advanced glycosylated end products
Akt	Protein kinase B
ALK	Anaplastic lymphoma kinase
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
AMPK	Adenosin monophosphate kinase
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
CCHB	Corticocancellous human bone block
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene related peptide
COX	Cyclooxygenase
CSD	Critical size defect
D	Dimensional
DFDBA	Demineralized freeze dried bone allograft
DM	Diabetes mellitus
DPP	Dipeptidyl peptidase
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular receptor kinase
FDA	Food and Drug Administration
FDBA	Freeze dried bone allograft
FFB	Fresh frozen bone
FGF	Fibroblast growth factor
g	gram
GDF	Growth and differentiation factor
GLP	Glucagon like peptide
GLUT	Glucose transporter
GTP	Guanidine triphosphate
HbA1c	Hemoglobin A1c
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HMH-CoA	β -hydroxy β -methylglutaryl CoA

H ₂ CO ₃	Carbonic acid
IGF	Insulin like growth factor
IL	Interleukin
IRS	Insulin receptor substrate
kDA	Kilodalton
kg	kilogram
LDL	Low density lipide
LIF	Leukemia inhibitory factor
M	Molarity
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage stimulating colony factor
MC3T-E1	Murine ostoblastic cell line
mm	milimeter
mL	mililiter
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NFATc1	Nuclear factor of activated t cells
NO	Nitric oxide
NSAID	Non steroid antiinflammatory drug
NK	Natural killer
pc	piece
PDGF	Platelet derived growth factor
PGE-2	Prostoglandin E-2
PGF-2 α	Prostoglandin F-2 α
pH	Power of hydrogen
pkg	package
PPAR	Peroxisome proliferator aktivated receptor
PPI	Proton pump inhibitor
PTH	Parathormon
RANKL	Receptor aktivator of nuclear factor kapa B ligand
rhBMP	recombinant human bone morphogenetic protein
Ras	Reticular activating system
Ros	Reactive oxygen species
Rpm	Rotation per minute

Runx	Runt related transcription factor
OPG	Osteoprotegerin
OP-1	Osteogenic protein 1
OSF-1	Osteoblast specific factor 1
Osx	Osterix
SGLT	Sodium glucose linked transporter
SSRI	Serotonin reuptake inhibitors
tabs	tablets
TGF- β	Transforming growth factor beta
TNF- α	Tumor necrosis factor alpha
TRAP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factor
Wnt	Wingless related integration site
%	percent
μ	piko
<	less than
$\varnothing$	diameter
$^{\circ}\text{C}$	Celcius degree
®	registered trademark

ABSTRACT

Uygun O. (2021). Histological and Histomorphometric Evaluation of the Effects of Bone Morphogenetic Protein 2 (BMP 2) and Metformin on Bone Formation in Critical Size Defect Models. Yeditepe University, Institute of Health Sciences, Department of Oral and Maxillofacial Surgery, PhD Thesis. Istanbul.

The aim of this study is to evaluate the possibility of metformin administration to achieve increased bone healing in non diabetic patients. It was stated that metformin increases the release of bone morphogenetic protein 2 (BMP 2), a growth factor which is effective in osteoblast differentiation. Recombinant human BMP 2 (rhBMP 2) can be applied with absorbable collagen sponges. In this study osteogenic effects of rhBMP 2 implantation to that of metformin administration were compared. Gelatin sponges were used as rhBMP 2 carriers and scaffolds. Forty two Sprague Dawley rats were randomly divided into six groups and a 5 mm calvarium defect was created in the parietal bone of each rat. The defect site was left empty in Group 1. Gelatin sponge was implanted on the defect site in Group 2. Gelatin sponge containing 2.15 µg rhBMP 2 was implanted into the defect site in Group 3. Systemic metformin hydrochloride (HCl) (255 mg/kg) was administered after the operation once daily for 14 days and the defect was left empty in Group 4. Gelatin sponge was implanted into the defect and systemic metformin HCl was administered after the operation once daily for 14 days in Group 5. 2.15 µg of rhBMP 2 impregnated gelatin sponge was implanted into the defect and systemic metformin HCl (255mg/ kg) was administered after the operation once daily for 14 days in Group 6. All rats were sacrificed on day 28. Calvarial bones were evaluated via histological and histomorphometric methods. The average weight of the rats increased after 28 days in all groups. No foreign body reaction, necrosis or inflammation were observed. During healing period new bone formation gained advantage over fibrosis in all groups, however no pairwise comparisons were statistically significant in terms of mean new bone percentage. The results of this study showed that metformin does not promote osteogenesis as effective as stated in similar studies, yet a relationship between BMP2 and metformin could be possible as the combined therapy group (Group 6) portrayed the highest levels of new bone formation.

Keywords: Bone Morphogenetic Protein 2, Metformin, Gelatin sponge, Bone

ÖZET

Uygun O. (2021). Kritik Boyutlu Defekt Modellerinde Kemik Morfogenetik Protein 2 (BMP 2) ve Metforminin Kemik Oluşumuna Etkilerinin Histolojik ve Histomorfometrik Olarak Değerlendirilmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Ağız Diş ve Çene Cerrahisi Anabilim Dalı, Doktora Tezi. İstanbul.

Bu çalışmanın amacı diyabetik olmayan hastalarda artmış kemik iyileşmesi elde etmek için metformin uygulama olasılığını değerlendirmektir. Metforminin, osteoblast farklılaşmasında etkili bir büyüme faktörü olan kemik morfogenetik protein 2 (BMP 2) salınımını arttırdığı belirtilmektedir. İnsan rekombinant BMP 2 (rhBMP 2) emilebilir kolajen süngerlerle uygulanabilmektedir. Bu çalışmada rhBMP 2 implantasyonunun osteojenik etkileri, metformin uygulamasınıninkiyle karşılaştırıldı. rhBMP 2 taşıyıcı ve iskelet olarak jelatin süngerler kullanıldı. Kırk iki Sprague Dawley sıçanı rastgele altı gruba ayrıldı ve her bir sıçanın paryetal kemiğinde 5 mm lik bir kalvaryum defekti oluşturuldu. 1. Grupta defekt bölgesi boş bırakıldı. 2. Grupta defekt bölgesine jelatin sünger implante edildi. 3. Grupta defekt bölgesine 2.15 µg rhBMP 2 içeren jelatin sünger implante edildi. 4. Grupta operasyon sonrası 14 gün boyunca sistemik metformin hidroklorid (HCl) (255 mg/kg) uygulandı ve defekt boş bırakıldı. 5 Grupta defektin içerisine jelatin sünger implante edildi ve operasyon sonrası 14 gün boyunca sistemik metformin HCl uygulandı. 6. Grupta 2.15 µg rhBMP 2 emdirilmiş jelatin sünger defekt içerisine yerleştirildi ve operasyondan sonra 14 gün süreyle sistemik metformin HCl (255 mg/kg) uygulandı. Tüm sıçanlar 28. günde sakrifiye edildi. Kalvaryaya kemikleri histolojik ve histomorfometrik yöntemlerle değerlendirildi. Tüm gruplarda sıçanların ortalama ağırlığı 28 gün sonra arttı. Yabancı cisim reaksiyonu, nekroz veya enfeksiyon gözlenmedi. İyileşme periyodu sırasında yeni kemik oluşumu tüm gruplarda fibrozise göre avantaj sağlamıştır, ancak hiçbir ikili karşılaştırmada ortalama yeni kemik yüzdesi istatistiksel olarak anlamlı bulunmamıştır. Bu çalışmanın sonuçları, metforminin, osteogenezisi benzer çalışmalarda belirtildiği kadar etkili şekilde desteklemediğini, ancak kombine tedavi grubunda (6. Grup) en yüksek seviyelerde yeni kemik oluşumu gözlemlendiği için BMP 2 ile arasında bir ilişkinin mümkün olabileceğini göstermiştir.

Anahtar Kelimeler: Kemik Morfogenetik Protein 2, Metformin, Jelatin sünger, Kemik

1. INTRODUCTION AND PURPOSE

Recovery of the anatomical integrity and replacement of the lost tissue with adequate function are the main goals of dentoalveolar surgery. Tooth and bone loss may occur due to injuries such as trauma and lesions including cysts and tumors (1). Rehabilitation of the lost tissues can be maintained, through augmentation procedures. In this fashion sufficient bone gain can be obtained for dental implants. Aside from the gold standard of augmentation procedures, autogenous bone grafting, different approaches such as homogeneous allogenic or heterogenic bone implants, recombinant human bone morphogenetic protein (rhBMP-2), tissue regeneration and distraction histogenesis are available together with their advantages and disadvantages (2). The medical history and the medication regimen of the patient significantly affect bone healing (3). According to the studies, parathyroid hormone (PTH), peptides, simvastatin, prostaglandin EP4 receptor agonist, vitamin D and strontium ranelate have anabolic properties on bone formation (4). On the other hand, as stated in studies, thiazide diuretics, β - blockers, anti-inflammatory drugs, proton pump inhibitors or serotonin reuptake inhibitors (SSRIs) may have catabolic effects (5-10).

Metformin is one of the first biguanide derivatives. It is prescribed for the treatment of type 2 diabetes mellitus due to its hypoglycemic, insulin sensitizing and vascular protective effects (11,12). Studies have shown that metformin ameliorates conditions like polycystic ovary syndrome and improves prognosis of cancer patients. As the results imply, metformin is a candidate for multiple indications (13,14). The effect of metformin on bone formation generates questions that remain unanswered. As reported in the literature, it provides proliferation and differentiation of osteoblastic cells proosteogenically. Furthermore, it increases the release of bone morphogenetic protein 2 and osteoprotegerin as well as alkaline phosphatase activity and bone matrix synthesis while decreasing the release of receptor activator of nuclear factor kappa B ligand (RANKL) (15-23). As reported by research oral administration of metformin induces osteoblastic differentiation of bone marrow progenitor cells. It is stated that the latter leads to expression of osteoblast specific transcription factor (Runx2) and activation of AMPK in non-diabetic animals (24). On the other hand, there is also conflicting information regarding its effects. In another study, it was reported that no osteogenic effect of metformin was observed in ovariectomized mice (25).

Bone morphogenetic proteins are multifunctional growth factors belonging to the transforming growth factor beta (TGF- β) superfamily (26). According to the clinical studies, bone morphogenic protein 2 (BMP2) is used for its osteoinductive properties in bone defects, fracture healing and osteoporosis surgery (27). In a study it was reported that a block of deproteinized bovine bone and a cortico cancellous human bone block (CCHB) with rhBMP2 showed similar results on new bone formation. It was stated that CCHB did not seem to maintain and release rhBMP2 in practical terms (28). On the contrary, undesirable results such as post-operative inflammation, ectopic bone formation, osteoclast-related bone resorption were observed in high-dose applications (29,30). As stated the desired bone formation could be achieved with a suitable delivery material, allowing a slower release (31,32).

Gelatamp® is an absorbable gelatin sponge which reserves silver colloidal ions (33). It is suggested to act as a facilitating matrix for clot formation during coagulation (34). It shows bactericidal effect through silver ion release and resorbs in about 4 weeks (35). Gelatin is a denatured collagen, a biodegradable polymer (36). Various materials were tested for BMP 2 delivery. Eventually collagen sponges were approved as effective materials (37,38). According to the studies, rhBMP2 soaked in collagen sponge showed a sudden release pattern in initial hours. However it was succeeded by a gradual loss of rhBMP2 from the collagenous implants over a 14 day period. Less than 5% of the initially implanted dose remained in the implants after 14 days (31,39,40).

Some in vitro studies report that metformin may increase osteogenesis and promote BMP2 expression. Moreover some in vivo studies state that metformin may have positive effects on bone density and quality, while contradictory results exist. Ultimately, more studies are needed to prescribe metformin for its osteogenic properties and to gain insight to its effects. The present study is undertaken to investigate the possibility of metformin administration to achieve increased bone healing in non diabetic patients. In this regard, we wanted to support our thesis by comparing the osteogenic effects of BMP 2 implantation to that of metformin administration. We used gelatin sponges as BMP 2 carriers and scaffolds. We hypothesed that there could even be a synergy between BMP 2 and metformin administrations. Our experiment was conducted on rat calvarial defect models and the results were evaluated using histological and histomorphometric methods.

1. LITERATURE REVIEW

2.1. Bone

Bone is a dynamic tissue which provides the skeletal system of all vertebrates (41). It functions in support, preservation, mineral storage and hematopoiesis and provides articulation or movement with its specialized cartilage-covered ends (42). Histologically, bone is a special connective tissue that has a hard, mineralized, extracellular matrix including osteocytes (43).

The inorganic component of the bone is mostly composed of hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ (41). The difference that distinguishes bone from other connective tissues is its mineralized matrix containing calcium phosphate in the form of hydroxyapatite crystals (44). It drastically differs from cartilage with the content of only 25% water in its matrix. This rate is around 75% in cartilage tissue (43). The organic component of the matrix mostly contains type I collagen and a small amount of type V collagen, which accounts for about 90% of its weight (45). The remaining part consists of non-collagenous proteins that constitute the basic substance (44).

Non-collagenous proteins can be examined in four main groups. First group is comprised of proteoglycan macromolecules which contribute to the compressive strength of the bone by combining different glycosaminoglycan (hyaluronan, chondroitin sulfate, keratan sulfate) chains around the core proteins. Second group is of multiadhesive glycoproteins which are responsible for the adhesion of bone cells and collagen fibers to the mineralized base. These are osteonectin and osteopontin or sialoproteins such as sialoprotein I-II. Third group consists of bone-specific proteins dependent on vitamin K. Those proteins such as osteocalcin contribute to bone remodeling of osteoclasts by binding calcium in circulation. Protein S is involved in the elimination of cells that undergo apoptosis. Fourth are growth factors and cytokines. These include insulin-like growth factors (IGF), tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β), platelet-derived growth factor (PDGF), bone morphogenic proteins (BMP) and interleukins (IL- 1, IL-6). BMPs are unique because they stimulate differentiation of mesenchymal cells to bone producing cells, osteoblasts. Clinically, BMPs are used to provide bone growth in large defects (44).

2.1.1. Bone Cells

The basic cells that constitute the bone are osteoblasts, osteoclasts, osteocytes, bone lining cells along with precursor cells, bone marrow cells and immune system regulating cells (46).

2.1.1.1. Osteoblast

They derive from primitive mesenchymal stem cells in the bone marrow called osteoprogenitor cells (44,47). The evolution of mesenchymal stem cells into osteoprogenitor cells requires the release of certain specific genes, including the synthesis of members of bone morphogenetic proteins (BMP) and Wingless (Wnt) pathways (48). Also transforming growth factor (TGF- β), leukemia inhibitory factor (LIF), fibroblast growth factors (FGF), platelet derivative growth factors (PDGF) and runt related transcription factor 2 (Runx2), home box protein Msx-2 (Msx2) and transcription factors such as transcription factor Sp7 or osterix (Osx) play a role in the process (49).

Osteoprogenitor cells can be found in the outer and inner parts of the bone as well as in the areas where microcirculation is provided. Osteoprogenitor cells are located in the inner layers of the tight fibrous connective tissue surrounding the bone called the periosteum, or inside the connective tissue cells surrounding the bone cavities called the endosteum. In addition, it can be located in the vascular structure in the middle of the cylindrical units called osteon (Haversian system) and on the canals, reaching the osteonal canal through the endosteal and periosteal surfaces, called Volkman canals (44).

Osteoblasts are cuboid cells that make up approximately 4-6% of all bone cells and are located on the bone surface. These cells are involved in bone formation and have well-developed organelles that express protein, such as granular endoplasmic reticulum and Golgi body (50,51). Although osteoblasts are present for a life time, embryological skeleton formation and growth period are the most active. In adults, they are activated during bone defect regeneration or when the bone matrix is reduced (51).

Osteoblasts do not respond quickly as formation of newly synthesized matrix is a coordinated process which takes about 4 months (53). By synthesizing and secreting collagen, osteoblasts form the unmineralized bone matrix called osteoid and regulate calcium phosphate flow and take part in osteoid calcification (46). Osteoblasts are fully differentiated cells that synthesize membrane-related alkaline phosphatase (ALP) and also regulate the accumulation of matrix proteins such as type 1 collagen and some non-collagenous proteins (54). A non-collagenous protein synthesized by osteoblasts, osteocalcin is synthesized in the calcification phase of the bone and has the property of binding to apatite and calcium. Osteonectin, produced by osteoblasts, is a glycoprotein that binds to sodium, collagen and calcium. It plays a role in the calcification process (55).

Osteoblasts also produce growth factor, cytokines and enzymes (55,56). Moreover, they transform factors such as transforming growth factor beta (TGF- β), bone morphogenetic proteins (BMPs), platelet derivative growth factors (PDGF) and insulin-like growth factor. Matured osteoblasts have parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D receptors that play a role in bone metabolism and mineral homeostasis. In addition, they synthesize the receptor activator (RANK) ligand of the nuclear factor kappa B, which plays an important role in differentiation of osteoblasts into osteoclasts (41). Osteoblasts can affect osteoclast formation, differentiation and apoptosis by means such as OPG / RANKL / RANK. This communication between the two cells can occur through direct cell contact, cytokines and extracellular matrix relationships (57).

Some researchers advocate that bone cells affect other systems, such as the nervous system and involve in energy metabolism and in serum glucose balance. For example, it is demonstrated that osteocalcin affects pancreatic insulin release (58).

Mechanical stimuli have great effect on osteoblast differentiation. According to Wolff's law, bone grows towards functional forces under physiological conditions (59). In vivo bending studies showed that osteoblasts are distributed on the periosteal surface and the ALP signal is increased in response to the force application. Gravity, vibration, compression, shear forces affect bone homeostasis (60).

2.1.1.2. Osteocyte

Osteocytes are of mesenchymal cell origin and generated by differentiation of osteoblasts (61). Osteoblast are surrounded and compressed by the bone matrix they produce and eventually give rise to osteocytes (41). They constitute approximately 90% of all bone cells and are the most common and longest living bone cells (62). Osteocytes are located in lenticular shaped spaces called lacunae in the bone matrix and have protrusions extending into narrow spaces called canaliculi (42,43). Organelles such as granular endoplasmic reticulum and Golgi apparatus are diminished resulting in decreased protein synthesis and release. They are responsible for ensuring the sustainability of the bone matrix. They respond to the forces applied to the bone and play a role in mechanotransduction (44).

Osteocytes are the main signal sensors, integrators and transducers of the skeleton (63). They have the ability to stimulate osteoblasts and environmental matrix by synthesizing osteoblast specific factor-1 (OSF-1) (41). Major osteocytes synthesize a protein called sclerostin which inhibits bone formation under certain physiological conditions, thereby regulating bone density. Sclerostin release depends on mechanical factors, local cytokines and endocrine factors (64).

Experimental studies in type 2 diabetic rats, treated with sclerostin antibody, resulted in increased bone density and improved bone defect regeneration (65). Some studies suggest that bone defect regeneration is impaired in patients with type 2 diabetes due to early callus resorption prior to adequate ossification (66,67).

Implant failure is occasionally associated with resorption of bone around the implant and osteocyte death (68). According to the studies, the particles abraded from the implants cause alkaline phosphatase (ALP) inactivation and cell apoptosis and change the osteocyte function (69).

According to the studies, when sclerostin antibody was applied to the rat implant models, it was observed that the negative effect of the worn implant particles did not appear and the bone formation was increased (70,71).

2.1.1.3. Osteoclast

Osteoclasts from monocyte / macrophage lineage are multinuclear cells derived from hemapoetic progenitor cells (41,42). These cells are primarily responsible for resorption. They are located on bone tissue where resorption occurs. Owing to osteoclast activity, a resorption bay (Howship's lacuna) is noticed. Their cytoplasm is acidophilic and shows a high histochemical reaction to acid phosphatase due to the large number of lysosomes. When lysosomal enzymes including Cathepsin K and matrix metalloproteinases are released, matrix collagen and other proteins are degraded (41,44).

Digestion is initiated when the environmental pH decreases. Carbonic anhydrase II produces carbonic acid (H_2CO_3) which dissociates to bicarbonate and proton. ATP dependent proton pumps transfer protons to the part of the cell in contact with bone, rendering the environment suitable for resorption (44). Tartrate resistant acid phosphatase (TRAP) is used as a marker to assess osteoclast activity and differentiation (72). Osteoclast formation has a close relationship with stroma cells in the bone marrow as these cells synthesize the necessary cytokines which allow them to differentiate (44).

Osteoclasts have colony-stimulating factor 1, calcitonin and RANK receptors (42). After the precursor cells, which will be transformed into osteoclasts, secrete two different transcription factors, the RANK-RANKL mechanism activates the differentiation and growth of the cells. Alternatively, inflammatory processes can trigger osteoclast-related bone resorption (44). This pathway can be inhibited by osteoprotegerin (OPG), a member of the TNF receptor family and synthesized by osteoblasts (42,44). Interleukin 1, 6, 11 (IL-1,6,11), TGF- α , TNF affect the activity and development of osteoclasts (73,74). Under physiological conditions osteoclast formation requires intercellular contact where a number of factors such as RANKL play a role. In pathological conditions, such as rheumatoid arthritis, where bone resorption is triggered, macrophages or T cells synthesize inflammatory cytokines such as TNF- α and IL-1. In this case, intercellular contact is not observed (41).

Macrophage stimulating colony factor (M-CSF), induced by parathormone (PTH), initiates osteoclastic activity while calcitonin synthesized from parafollicular cells of the thyroid gland reduces osteoclastic activity (42,44).

On the other hand, when parathormone is secreted intermittently, it has been observed that it increases bone formation and causes the synthesis of growth factors that directly stimulate osteoblasts and stimulate IGF 1 or other osteogenic factors (44,75).

As stated in research, osteoclastic activity may differ according to bone types. Osteoclasts in the bone marrow in long bones form faster than those in the jaw (76).

Abnormal osteoclast formation and activity may cause diseases such as osteoporosis with a decrease in bone density and increase the fracture rate. In some cases, including bone metastases and inflammatory arthritis, abnormal osteoclast activity can cause erosions and painful osteolytic lesions around the joint. Chemical mediators such as IL-6 and RANKL produced by cells in periodontitis lesions due to bacteria trigger osteoclast migration resulting in bone resorption (77).

2.1.1.4. Bone Lining Cells

These cells are assumed to originate from osteoblasts. They are composed of flat cells with reduced cytoplasm in bone tissue where bone remodeling does not occur. Those on the outer surface of the bone are called periosteal cells, and those on the inner surface are called endosteal cells. These cells are adjacent to Haversian canals and are suggested to act as barriers between the bone surface and the cells and contribute to ion exchange. It is thought that these cells provide bone matrix regulation, as the ends of these cells extend to the canalicular channels of the surrounding bones and communicate with the osteocytic processes through gap junctions. In this regard, it can be said that they have similar features with osteocytes (44).

2.1.2. Bone Types

Bones can be classified according to their anatomical shape. Those are long bones, short bones, flat bones and irregular bones. According to various sources, sesamoid bone is a separate class (41,43). Microscopically it is classified as two types: primary (immature or woven) bone and secondary (mature or lamellar) bone, based on their collagen sequence. According to its macroscopic structure, bone is divided into two types: compact (dense / cortical) and cancellous (spongy / trabecular) bone (42,43).

Primary bone depicts a less mineralized, unorganized, scattered order and involves in bone formation in the developing fetus or during fracture healing where rapid bone formation occurs (43). It has a low mineral content (42). It consists of randomly placed collagen fibers and a high number of osteoblasts and osteoprogenitor cells. Although it is the major bone type in the developing fetus, it can be seen in the regions where remodeling occurs in adults. This bone type is noted in the extraction socket and tendon bone junction in adults. Apart from these examples, it is considered pathological in adults (41).

Secondary bone, on the other hand, shows a well-mineralized and regular sequence and provides protection and support function in adults (43,78). Secondary bone replaces primary bone in adulthood. Lamellae appear as parallel lines of accumulated bone in a polarized light microscope. It consists of well-organized collagen fibers. The lamellae are arranged in a circular and tubular form, forming structures called osteon. The middle part of this tube is the Havers channel, which contains nervous and vascular structures. Volkman channels are observed as passages connecting Haversian systems to each other and blood vessels in the periosteum (41).

Compact bone is uniform and does not contain trabeculae. It is in high density and has circular lamellar arrangement and is located in the outer (cortical) part of the bone. It provides protection and support (41).

Cancellous bone is irregular and consists of trabeculae around the bone marrow. It is low in density and has parallel lamellae. It is located in the inner (medullary) part of the bone and provides support and production of blood cells (41). It is always surrounded by a compact bone layer (42).

2.1.3. Ossification Types

The development of bones originates from three different lineages. The first are cranial neural crest cells. They give rise to flat bones of the skull, clavicle and majority of the cranial bones. The second are somites that form the axial bone. Finally, it is the lateral plate mesoderm that forms the long bones. Bone formation requires a scaffold for development (79).

Bone development is evaluated as endochondral ossification and as intramembranous ossification. Endochondral ossification requires a cartilage template while the latter undergoes a simpler process. Weight bearing areas such as extremities and vertebrae develop by endochondral ossification, while the flat bones of the skull, mandible and clavicle develop by intramembranous ossification (44).

In intramembranous ossification, osteoprogenitor cells accumulate in regions where the new bone will form and differentiate into osteoblasts that will synthesize the new bone matrix (41). Osteoblasts form an unmineralized collagen proteoglycan matrix called osteoid, to which calcium can bind. As the calcium binds to the osteoids, the matrix hardens and osteoblasts get trapped. This compression causes osteoblasts to turn into osteocytes (80). Centers of the bone which are developed through spicular fusions become trabeculae. Trabeculae surround blood vessels, and fusion of bony trabeculae forms spongy bone. Meanwhile the other undifferentiated mesenchymal cells form the bone marrow (42,80). The surfaces of trabeculae are populated with osteoblasts which interact with osteoclasts. It causes bone to be remodeled. Newly formed bone is called woven bone (42). Mesenchymal cells form the periosteum on the outer surface of the bone. Its inner surface differentiates into osteoblast and synthesizes osteoid parallel to the matrix and creates a compact (cortical) bone (80).

Endochondral ossification begins with the conversion of mesenchymal cells derived from mesoderm into chondrocytes owing to fibroblastic growth factors (FGF) and BMPs (44). Chondrocytes proliferate rapidly and produce extracellular matrix, forming a cartilage model for bone. Perichondrium is formed in the membrane around this structure, which will ossify in the future. The chondrocytes near the center undergo hypertrophy and add more collagen and fibronectin to the matrix they synthesize. The matrix begins to calcify and chondrocytes that cannot be fed due to calcification of the extracellular matrix undergo apoptosis. Blood vessels reach the cavity and transport osteoprogenitor cells. In the body part of the periosteum called diaphysis, osteoblasts form a hardened area consisting of a compact bone called a periosteal collar where primary ossification centers are formed. As bone is replaced by cartilage, cartilage continues to proliferate at the ends of the bones, lengthening them. These areas are called epiphysis (growth) plates. A secondary ossification center is formed in the epiphysis region after birth and is ossified later (81).

2.1.4. Bone Healing

Skeletal system may be damaged due to accidents or etiological reasons. Bone healing after fracture shows properties similar to those of skin, an important difference is that the connective tissue matrix is calcified. It is a privileged tissue since it heals with regeneration rather than bone repair (82). Another important feature is that it can heal without fibrous scar tissue. Bone fracture healing is divided into two as primary (direct) and secondary (indirect). Direct healing is generally not a natural healing process. It is observed in the healing of broken segments, treated with the rigid internal fixation method, and rendered immobilized. No external tissue formation, called callus, is observed. Intramembranous ossification and remodeling occurs. Recovery occurs via osteons and can last from a few months to several years (83).

Bone repair and reorganization processes are involved in indirect healing. Inflammation, primary soft callus formation, mineralization and remodeling are observed during healing period. It occurs in methods, such as external fixation technique, where micro mobility is noticed at the broken ends, featuring both intramembranous and endochondral healing (83). It is evaluated in three phases.

2.1.4.1. Inflammation Stage

Hematoma formation occurs immediately after trauma. Inflammation occurs within 12-14 hours. The process continues with platelet aggregation, coagulation and clot formation, following disruption of the vascular supply. The clot, along with the inflammatory cells, acts as a matrix for the migration of endothelial cells and fibroblasts. The acute inflammatory response shows a peak within 24 hours and ends after 7 days (84). The first cells to be observed are neutrophils, macrophages, lymphocytes and plasma cells, respectively. Macrophages secrete growth factors and cytokines that initiate the healing process. These are TGF- β , VEGF, PDGF, FGF, IL-1, IL-6, TNF- α , BMP, IGF I-II and macrophage colony stimulating cells (85). These factors start the tissue repair by stimulating the migration of mesenchymal stem cells from the periosteum, bone marrow, circulatory system. Consequently they differentiate into cells such as fibroblast, angioblast, chondroblast, osteoblast. Growth factors stimulate granulation tissue formation (83,85-87).

2.1.4.2. Reparation Stage

After the formation of hematoma, fibrin-rich granulation tissue is produced. At an advanced stage, endochondral formation begins to form between the broken edges, and cartilage tissue creates a soft callus, acquiring a more stable structure (85). The focus of this phase is the formation of cartilage callus, which will be mineralized in the future and will be re-absorbed and replaced with bone (88). This semi-rigid callus is deprived of vasculature, but in the future it will be vasculated when it will be replaced by woven bone. Chondrocytes of mesenchymal origin multiply and synthesize the cartilage matrix. This matrix especially contains collagen II and X, and glycosaminoglycans. Hyaluronic acid, chondroitin sulfate and dermatan sulfate is essential in cartilage formation (85). With their hydrophilic properties, they draw water from the surrounding tissue and enlarge the outer matrix (89). As chondrocytes proliferate in callus, they undergo hypertrophy and form a calcified matrix structure. Liberation of TGF- β , FGF, PDGF, IGF and BMP growth factors stimulate fibroblasts and chondrocytes proliferation (85).

During fracture healing, blood circulation is essential in nourishing the defect site. In endochondral ossification, blood vessel ingrowth increases with chondrocyte apoptosis and degradation of the cartilage structure (89). Notably, VEGF, BMP, TGF- β , FGF and angiopoietins ensure angiogenesis and vascular endothelial cells migrate to soft callus (85-88). The formation of soft callus in mice, rats and rabbits is at peak in the 7th - 9th days after the injury (89).

When hard callus is formed and later replaced by woven bone, it gains increased adaptation to mechanical forces. Besides, it acts as a stimulant for angiogenesis and allows osteoblast and osteoclast migration to the fracture site (83).

2.1.4.3. Remodeling Stage

The last phase is the remodeling of the woven bone into the lamellar bone (89). Vascularisation continues at this stage. The bone gains enough strength in nearly 6 months, but the process may continue for years (86,90). As the lamellar bone forms replacing the hard callus, the bone cortex and medulla develop gradually (85).

Osteoclasts are responsible for the resorption of calcified bone. Cytokines and growth factors such as IL, TNF- α , BMP and TGF- β stimulate osteoclastogenesis (83). Osteoclasts resorb hard callus cartilage tissue thus osteoblasts may synthesize osteoid and involve in mineralisation (83,85).

Alveolar bone portrays a different healing process compared to axial and craniofacial skeleton. Craniofacial skeleton may repair minor lesions without bone loss however, alveolar bone repair depends on two variables resulting in volume loss. The first is the exhibition of osseous matrix in the extraction cavity. It can be compared to physical process observed after bone fracture. The second variable is bone/ volume proper. It is observed in the case of avulsion with vestibular bone fracture. Bone healing is observed but vestibular defect and bone volume loss are inevitable. An external wall impairment results in increased volume loss compared to internal wall impairment. According to studies two parameters uncover these results: First, periosteum is a site specific signaling tissue. Second, regardless of origin all osteogenic cells necessitate a support as a means to reconstruct the original anatomy. Healing of extraction socket includes three stages. First, growth factors and cytokines are released with clot formation in response to trauma. Next stage is osteoblastic differentiation to form woven bone. Final step is the remodeling stage in response to local stress and strain (91). The presence of two different ossification types does not necessarily mean that existing bone is either membrane bone or endochondral bone. Hence the succeeding remodeling stage would involve appositional bone growth and which is same in both cases (44).

2.2. Factors Affecting Bone Formation

Numerous local and systemic factors affect the healing process. Local inhibitory factors involve the existence of a fracture gap, loss of blood flow, presence of infection and soft tissue injury. The surgical procedure, the type of fixation and the success of fixation are the factors affecting fracture healing. Inadequate mechanical stability may result in increased or decreased callus formation leading to non unions. Metabolic and nutritional state of the patient along with the age, gender, smoking and the presence of pathologies may postpone the healing process. Another crucial factor on the fracture healing mechanism is the administration of various pharmaceutical agents (92).

2.2.1. Approved or Experimental Bone Catabolic Agents

It was shown that anti chemotherapeutic drugs such as doxorubicin, cyclophosphamide, adriamycin and methotrexate reduce bone formation (93).

Corticosteroids cause osteoporosis and steroid intake causes osteoblasts to undergo apoptosis (94). Conversely, an experiment showed that it has no effect (95).

Studies suggest that some antibiotics have negative effects. Gentamycin and tetracycline use could prevent new bone formation in rat models (96). Catabolic effects of quinolones on growing cartilage is controversial (97). The use of ciprofloxacin, levofloxacin and trovafloxacin may impair early stages of healing period (98).

Anticoagulants affect bone formation. Heparin is reported to increase resorption and decrease bone formation. It is stated that new generation anticoagulants such as low molecular weight heparin have less effect on bone (99).

Non-steroid anti-inflammatory drugs (NSAID) inhibit cyclooxygenase (COX) activity and prostoglandin synthesis. Conflicting research is present about its effects on bone (100). Prostoglandin E-2 (PGE-2) effects BMP regulation and stimulates bone formation when released with Prostoglandin F-2a (PGF-2a) during bone fracture healing (101). According to a study on rabbits, a COX-2 inhibitor was given for the first 2 weeks after the fracture and bone formation was not affected. However a decrease in bone formation was observed when it was continuously given for 6 weeks (102). Similarly, recovery was not affected in patients in the spinal fusion stage, when short-term low-dose ketorolac, celecoxib and rofecoxib were administrated (103).

According to a study, antiepileptics such as phenytoin and phenobarbital increase the release of the cytochrome p450 enzyme and cause diseases related to vitamin D deficiency such as osteomalacia. Hence, researchers claim that antiepileptics independent of enzyme interaction such as valproate can also reduce bone density (104). Although the calcium level of those receiving carbamazepine, phenytoin and valproate was lower than those receiving lamotrigine, no significant difference was found (105).

Proton pump inhibitors are frequently used in patients with gastrointestinal problems. Mineral absorption is thought to be affected and bone remodeling is impaired due to increased gastrin production and hypochlorohyrdia. Many studies suggest that PPI intake increases the risk of fractures, but contrasting researches exist (106).

Cyclosporine used in autoimmune diseases, is associated with mineral loss. Similar effects are reported in organ transplant patients, receiving tacrolimus (107).

Medroxyprogesterone acetate, a derivative of progesterone hormone, is used as a contraceptive and in the treatment of estrogen-related diseases. Literature states that it causes bone mineral loss due to the suppressed estrogen levels (108).

Loop diuretics are used to treat cardiovascular diseases and fluid accumulation disorders. In a study, elderly female patients receiving loop diuretics were more prone to bone loss than those who were not receiving (109).

Research states that high doses of vitamin A and synthetic retinoids cause osteoporosis, bone loss and fractures (110).

Selective serotonin reuptake inhibitors (SSRI) are used in depression treatment. According to the studies, SSRI are associated with increased fracture risk and reduced bone mineral density (111).

Studies report that antiretroviral agents provoke osteoclastic activity and bone resorption due to the stimulation of proinflammatory cytokines in patients with HIV infection (112). In a study on HIV infected patients, vitamin D was significantly decreased, in patients receiving efavirenz, a reverse transcriptase inhibitor (113).

Thiazolidinediones are prescribed in the treatment of diabetes mellitus. Their effects on bone are studied thoroughly. Agents, such as rosiglitazone and pioglitazone, are thought to cause adipogenesis instead of osteoblastogenesis. Studies state that they decrease bone density and increase sclerostin release by causing the death of osteocytes (114).

2.2.2. Approved or Experimental Bone Anabolic Agents

Parathormone (PTH) is a polypeptide hormone which is excreted from parathyroid glands. It plays a role in regulating extracellular calcium levels by reabsorption of renal calcium and phosphate, and excretion of calcium from bone. It is revealed in osteoporosis simulations that intermittent application of PTH enhances the osteoblastic activity. Meanwhile its continuous application results in catabolic changes. The synthetic polypeptide hormone called teriparatide is used in osteoporosis treatment and prevention of bone fractures (115).

Osteogenic growth peptide (OGP) is produced by stroma originated cells such as osteoblasts. It regulates the osteogenic response mechanism and stimulates the bone formation. It is proven that systematically given OPG stimulates the bone healing by promoting the transition from cartilage to bone tissue (116).

Statins lower the risk of cardiovascular disorders and regulate cholesterol levels with their anti-lipidemic properties. It is observed that high dose level oral application of simvastatin enhances bone healing in fracture models in mice. Studies support the observation of anabolic activities. Even though some contradictory results exist in osteoporosis patients; they are attributed to the low doses of administration (117-118).

Daily intake of vitamin D helps the maintenance of healthy bone structure and, despite its potential side effects, it promotes the bone healing. It is proven in the studies that vitamin D intake increases the strength of the callus after a bone fracture (119).

Calcitonin gene related peptides present in bone marrow and at the nerve endings in metaphysis, stimulates the osteoprogenitor cells. CGRP is shown to improve bone healing when it is applied to the damaged bone tissue (120).

Thiazide diuretics used in hypertension treatment are believed to decrease bone loss after menopause by decreasing the excretion of calcium in urinary system. Strontium ranelate is shown to enhance osteogenesis activity in literature. According to the animal studies on osseointegration around titanium implants, systemic application of strontium ranelate mediates moderate level bone formation (121).

Biphosphonates are prescribed to prevent bone loss caused by osteoclasts in osteoporosis. It is also preferred in patients with low density bone structure. However, intravenous biphosphonates such as zoledronate and pamidronate cause osteonecrosis of the jaw. Hence, the prescription of these agents needs to be thoroughly planned (122).

2.3. Critical Size Defect Model

Bone loss in large defects of maxillofacial region complicates the physiologic process of healing. New biomaterials and techniques are developed in order to improve the healing process (123). Even though in vitro studies introduce basic biological mechanisms, they do not completely reflect the clinical outcomes. Animal studies offer clinically effective information on physiological and pathological conditions (124).

Critical size defect model (CSDM) is a crucial part of the in vivo study plan. CSDM is defined as a defect that is not able to heal without an external intervention. Alternatively stated, it is a defect that does not spontaneously heal during the experiment or throughout the lifespan of the experimental animal (125). CSDs have a deficient blood flow, therefore bone healing process is hindered. This is an appropriate condition for the investigation of fracture healing and new techniques to improve bone formation (126). Calvarial defects and segmental or long bone defects are widely used to study cortical and cancellous bone defect models (127).

Calvarial model is suitable for examining complex materials and bone regeneration. Commonly used animals are mice, rats, rabbits, dogs, sheep and pigs. Rodents exhibit bone growth and remodeling throughout their lifetime. Mice and rats have limited trabecular bone mass and Haversian remodeling does not take place in their bone structures. They rather present cancellous bone formation. They are preferred subjects due to ease in handling and manipulation (128).

Calvarial bone promotes the radiological and histological studies, providing the formation of a uniform, repeatable and standard defect. Its anatomy and size are suitable for surgical manipulation. Its dura and skin layer support the the stability of the materials. If the physiological response to biomechanical stresses is to be examined, load bearing areas such as femur or tibia must be chosen for application (128).

The decision on the size of calvaria defect depends on species, gender, age, anatomical site and form. Even though different sizes of defects in CSDM are investigated, the process of healing is not completely understood (128). Honma et al. examined defects having 3.8 mm and 8 mm diameters for 24 weeks (129). They observed that a fibrous connective tissue is developed in the spaces where no healing took place. They concluded that bone healing takes 24 weeks and this period is independent from the defect diameter. Bosch et al. observed minimal bone formation at the borders of the defects in rats with 5mm CSDM in 12 weeks (130). Porto et al. investigated 5 mm diameter defects in rats (131). They stated that bone formation had not taken place up to 15 days and at the 30th day bone formation finally occurred. Vajgel et al. conducted a systematic review and they concluded that considering the percentage of bone formation, 5 mm defects can be accepted as CSDM (123).

2.4. Bone Grafts

Bone defects may occur as a result of trauma, infection, surgical procedures, or congenital malformations. Techniques such as bone grafting, guided tissue regeneration, distraction osteogenesis, growth factors and stem cell therapy are resorted to reconstruct these defects. Bone grafts are of natural or synthetic origin and provide bone formation when placed over the defect. As per their origin, they are:

Autografts, derived from the patient's own bone, do not possess antigenicity.

Isografts are derived from the same species with the same antigenic properties.

Allografts or homografts are harvested from a different person of the same species which require the elimination of their antigenic properties.

Xenografts or heterografts are obtained from different species.

Synthetic bone graft substitutes are aimed to mimic the natural bone tissue (132).

Graft materials have different regeneration capacities and properties. These are:

Osteogenesis: Osteoblasts, residing in the graft material, provide bone growth.

Osteoinduction: BMPs inside the graft material stimulate osteoprogenitor cells, inducing osteoblast formation.

Osteoconduction: The graft material provides a scaffold to allow the migration of precursor cells into the defect site in order to induce bone formation (132).

An ideal graft material should be osteogenic, osteoinductive, osteoconductive and hydrophilic. It should stimulate neo-angiogenesis as well. It should not be antigenic, teratogenic and carcinogenic, while it must be mechanically supportive and stable and economically affordable. It should also be easily obtained in adequate amounts (133).

Integration of the graft to the recipient area involves several steps; inflammation, revascularization, osteoinduction, osteoconduction and remodeling. Bone grafts must have three fundamental properties for regeneration: Firstly, there must be osteoprogenitor cells, specifically osteoblasts. Secondly, they must include growth factors that aid regenerative process. Lastly, a framework is necessary to mechanically support the adhesion of cells, leading to their proliferation and growth (134).

Autografts are accepted as gold standard due to their osteogenic, osteoinductive and osteoconductive properties. They do not cause any immune response since they present the same biological properties with the host organism. However, a second surgical site compromises the overall health and includes the risk of morbidity (132).

Allografts are obtained from alive or cadaver donors. They need to be processed to eliminate the risks of immune response and infectious diseases. Fresh frozen bone (FFB) does not contain cells. BMP content in FFB gives high osteoinductive and osteoconductive ability. However, it is no longer applied due to the infection risk. Freeze-dried bone allografts are not processed through demineralization; hence they present low antigenic properties. FDBA are only osteoconductive. On the other hand, demineralized freeze-dried bone allografts (DFDBA) are both osteoconductive and osteoinductive because of their BMP including entirely organic content (132).

Xenografts are osteoinductive materials with decreased resorptive capacity. These include bovine, porcine, equine, algae and coral substitutes. They are usually combined with growth factors or other graft materials (132).

Alloplastic grafts are produced synthetically as calcium sulphates or calcium phosphates. Their osteoconductive structure resorbs to provide a formation site.

Growth factors such as TGF- β , ILGF-I, II, PDGF, FGF and BMP are advanced bone grafts obtained by recombinant DNA technology (132).

2.5. Bone Morphogenetic Proteins

Despite the presence of type 1 collagen in both skin and bone, only bone collagen is mineralized. Urist demineralized the rabbit bone chips and implanted them into the thigh muscle to examine the remineralization potential of bone derived collagen. He observed a new bone formation which was highly vascularized and remodeled. He described this phenomenon as bone formation by auto induction. In the aforementioned case, acellular demineralized bone matrix was implanted in the subcutaneous tissues, leading to proliferation of fibroblast-like mesenchyme stem cells within 24-72 hours. Later, they differentiated into chondrocytes in 5-7 days. The osteoblasts lay down the extracellular matrix, leading to the mineralization process on 9th - 11th days. With the formation of osteoclasts, the new bone was remodeled as of the 14th day and the bone marrow was formed in 18-21 days, including red and white blood cells. Thus, it was proven that the bone matrix could perform ectopic bone formation owing to its morphogenic feature. In this regard, new studies have been conducted to gain insight into the role of morphogenic proteins in bone formation. (135)

Even though the presence of bone morphogenetic proteins (BMPs) was stated, the difficulties in isolation delayed the experiments. Demineralized bone matrix is in a solid state that can not be dissolved. Around 2 - 3% of the proteins could be dissolved with 4M guanide hydrochloride or 8M urea or 1% sodium dodecyl sulfate application at pH 7-4. Dissociative extraction of demineralized bone matrix with these solvents resulted in a loss of osteoinductive ability. The biological activity was restored when the extracted soluble components, containing proteins <50 kDa, were reconstituted with their respective inactive insoluble residue. The study stated the need of a collagenous matrix to provide a suitable substratum for attachment of mesenchymal cells (136).

In order to scale the purification process, demineralized human, monkey and bovine bone matrices were reconstituted with biologically inactive rat residue. The matrices did not depict osteoinductive properties in rats, implying immunogenic reactions. Then the extracts were partially purified, before they were implanted into the rats. Molecular size chromatography results revealed that partially purified extracts, with molecules < 50 kDa, depicted osteoinductive properties. Hence, it was concluded that soluble osteoinductive proteins were homologous, regardless of species (135).

Therefore, amino acids in protein fragments, isolated from bovine bone and genes were identified, revealing BMP-1 BMP-2 BMP-3 and BMP-4 . BMP-2, BMP-3 and BMP-4 belong to the TGF- β family, while BMP 1 is a tolloid proteinase derivative and is responsible for the processing of collagen and some TGF- β family proteins. At first, 7 different BMPs were isolated. It was stated that highly purified bovine osteogenic protein fractions were composed of osteogenic protein 1 (OP-1 or BMP-7) and BMP-2 homodimers (137).

According to the studies, BMPs are dimeric molecules with two polypeptide chains that are held together by a single disulfide bond. Each monomer is synthesized in the form of a polypeptide chain, including over 400 amino acids and each with a BMP precursor (138).

Northern blot analysis and in situ hybridization studies demonstrated that they were detectable in tissues such as embryos and adult organisms other than bone. In the light of this evidence, it was concluded that BMPs properties were not limited to bone development but, it also had other functions. For example, while BMP-2 has a developmental role in cardiac tissues, BMP-4 plays a role in lung development and BMP-7 plays a role in kidney morphogenesis. Loss of BMP-6 causes hemochromatosis, while excessive amounts can cause anemia (137).

Pleiotropy is the ability of a single gene or gene products to feature different characteristics. BMPs are true pleiotropic morphogens as they regulate the steps of chemotaxis, mitosis, cartilage differentiation and bone replacement. In addition, BMPs balance hematopoiesis and extracellular matrix synthesis, affecting cell viability and apoptosis stage (135).

Human cDNA and genomic library led to the discovery of genes based on BMP sequences, using oligonucleic probes. These genes are named as growth and differentiation factors (GDF). BMPs and GDFs are members of the TGF- β super family and are responsible for the development of various organs during embryogenesis and morphogenesis in post natal life. BMPs constitute a large subgroup of the TGF- β superfamily, with over 15 members identified so far (139).

BMP functions by binding to its unique Serine / Threonine kinase receptor complex (140). These complexes consist of type 1 receptors such as ALK-2, ALK-2 and ALK-6, and type 2 receptors such as BMPR-II, ActRII-A and ActRII-B. These kinases are enzymes that phosphorylate and activate the substrate Smads. Smads are cytoplasmic substrates for BMP type 1 receptors associated with the cell membrane. Type 2 receptors, on the other hand, provide phosphorylation of type 1 receptors (141). Ligand receptor complex provides phosphorylation of intracellular transcription factors that stimulate Smad 1/5/8. Phosphorylated Smad 1/5/8 stimulates co-smad 4. These provide activation or inactivation of the genes involved in tissue preservation, repair and regeneration by settling in the nucleus (142,143). Binding of BMP to the receptor complexes is provided by endogenous antagonists called, noggin, follistatin, sclerostin, twisted gastrulation, gremlin, DAN and USAG-1 / Wise / SOSTdc1 in the extracellular medium. Intracellular signaling also regulates the relationship between P-smad-1/5/8 and anti-smad-6/7, followed by anti-smad ubiquitination via smurf1 and E2 / E3 ligase. Besides, BMP ligands can stimulate MAPK, ERK, NK, p38, PI3K, Akt, RANK and RANKL signals as well as cross communication with Wnt, Hedgehog and VEGF signal steps (144).

During embryogenesis, the ectoderm releases BMPs that bind to the extracellular matrix and mesoderm continues to secrete them when needed. Cells that expose BMPs also expose BMP antagonists, creating the appropriate medium for ligand receptor connection to provide signal transmission (145,143).

BMPs are responsible for endochondral bone formation during development. Embryonic endochondral ossification can be regained by implanting the appropriate collagen roof with BMP in the subcutaneous region. Thus, cartilage and bone formation are observed in the postnatal period (132,136,146).

Both BMP 2 and BMP 4 are effective in bone and cartilage formation as powerful inducers in osteoblast and chondrocyte differentiation (147). As studies stated, their deficiencies were fatal in conventionally knock out mice for BMP 2, BMP 4, BMP 7 and their respective receptors. In other words, bone development and remodeling were not observed (140,147).

According to the homology of amino acid sequences, BMPs involved in bone construction are divided into subgroups as BMP 2/4 group, as BMP 5/6/7/8 group and as BMP 9/10. Other group members do not possess osteogenic features. BMP-1 is not a member of the TGF- β superfamily, while BMP 3 and BMP 13 act as BMP antagonists or inhibitors (148).

Cho et al. (2002) studied the expression of mRNA patterns for members of the TGF- β super family over a 28-day period in fracture models. According to the results, a day after the fracture, BMP 2 and GDF 8 showed maximum release. It was suggested that they could trigger early cell proliferation. BMP 2 expression was elevated again during the period of peak osteogenesis, around day 21 (149).

Yu et al. (2009) studied BMP release by immunostaining method in unstable fractures on rats. When they examined the periosteum, weak BMP 2, 4 and 5 activities were observed in the first 3 days. Only BMP 5 activity was detected on the 5th day. BMP 2, 4 and 5 activity, which was weak on the 3rd day in granulation tissue, increased to a medium level on the 5th day. At day 5, moderate levels of BMP 2, 6, 7 were observed in chondrocytes and a low level of BMP 8 was noticed. Whereas increased immunoreactivity was observed in all BMPs from day 5 to day 10. On day 7, strong levels of BMP 2, 4, 5, 6, 7 and 8 was observed in chondrocytes. On the 14th day, BMP immune reactivity reduced. BMP 2 expression was the strongest in chondrocytes during repair, whereas BMP 8 was the strongest in osteoblasts. While all BMPs may regulate osteoblast differentiation, it is suggested that BMP 2, 6 and 9 can provide osteogenic differentiation of mesenchymal stem cells in the early stages of healing (150).

2.5.1. Bone Morphogenetic Protein 2

According to studies, BMP 2 is the most effective growth factor in osteoblast differentiation and regeneration of pure bone defects (151). Although the amount of BMP 2 that can be obtained from the bone is limited to the ratio of 1-2 μg / kg cortical bone, Wozney et al. rhBMP 2 developed a method to produce recombinant BMP (152, 153). Thanks to the development of genetic recombination methods, the researchers succeeded in producing the N-glycosylated active form of BMP 2 using Chinese hamster ovarian cells and make it commercially available (154).

The protein synthesis system of rhBMP 2s produced from these cells is the same as that of humans, but production costs have limited the clinical use of the latter. Owing to the studies conducted on *E. coli*, it is possible to produce a greater amount at a more affordable cost, but the rhBMP 2 obtained has low biological activity since it is in a non-glycosylated form (155). However, in the light of current preclinical and clinical studies, rhBMP 2 obtained from *E. coli* shows successful results in bone regeneration and augmentation (156-157).

In bone healing, platelets synthesize PDGFs in the inflammatory phase, stimulate chemotaxis and involve in proliferation of the cells. Cytokines such as IL1, IL6, and TNF- α are then synthesized and inflammatory cells are expressed. VEGF and PDGFs stimulate angiogenesis to perform angiogenesis in the proliferative phase. With the formation of vascularization, osteogenesis begins to occur in the defect region, and BMPs, including BMP 2, play a role in the differentiation of osteogenic osteoprogenitor cells to osteoblasts and mineralization. Considering the order of factors in recovery, it is important to use an effective delivery system with proper planning, as BMP 2 is stated to be a late-acting growth factor (Figure 2.1.) (158,159).

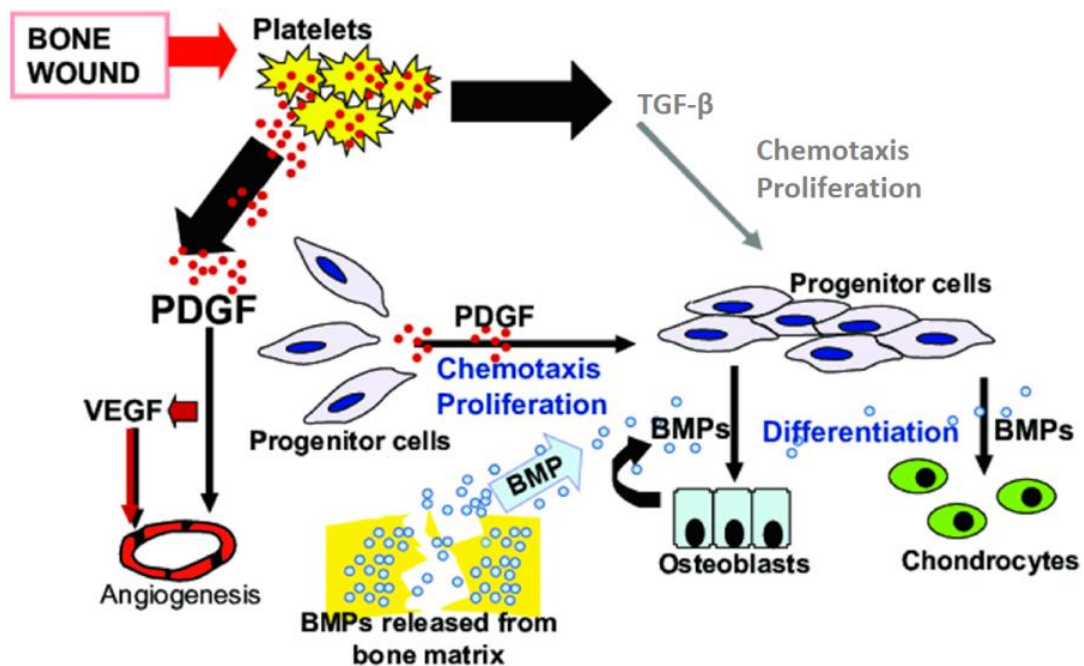


Figure 2.1. Growth factors related to bone healing

According to the studies, the greatest issues with rhBMP 2 are due to dose-related side effects. Protein-based growth factors have a short half-life and a high clearance rate. Since BMP is a late acting growth factor, it is expected to be effective in high initial dose applications. In addition, dose changes should be considered depending on the carrier system and host. Studied doses ranged from 0.75 to 2mg/ml (160-162). As stated in results, when applied over the physiological dose, increased edema, seroma formation, cystic bone lesions and cancer formation were observed (163). Ponyton and Lane stated that there is the possibility of excessive bone growth, cancer, systemic and local toxicity, immune response, osteoclast activity in overdose (164, 165).

Following various animal and clinical trials Food and Drug Administration (FDA) permitted the sale of bovine collagen sponge with the trade names Infuse Bone Graft (Medtronic, United States) and InductOs (Medtronic, United Kingdom) together with rhBMP-2 in 2014 (151). It was tested on 450 patients and approved for orthopedic use. High union rate, good wound healing, low infection rate and decreased need for second surgery in open tibial fractures were observed (161). Then, rhBMP-2 was started to be used for the maxillary sinus elevation, implant surgery, retention of extraction socket and alveolar ridge, and guided bone regeneration in the field of oral surgery (151).

The use of rhBMP 2 other than the indication accepted by the FDA is considered off-label (166,167). These uses include cleft palate repair, reconstruction of atrophied jaws, augmentation of segmental defects after tumor resection, bisphosphonate induced osteonecrosis, osteoradionecrosis, and total reconstruction of serious atrophic jaw (168-172).

Even though it is depicted as an alternative to autogenous grafts with its osteoinductive properties, there are some concerns about its safety. Carragee et al. stated that when it was used in spinal surgery, they observed a complication rate of 10-50% among patients, resulting in excessive bone growth, retrograde ejaculation, and life-threatening airway obstruction and cervical edema. In addition, the risk of malignant tumor formation is mentioned (163). Woo stated 83 side effect reports in a study that presented the side effects of rhBMP 2 used in the maxillofacial region and reported that 66.3% of them were due to off label use (173).

In a systematic review, Fernandes et al. investigated the adverse effects of rhBMP 2, following its clinical application on bone reconstruction. Similar success rates and side effects were observed in both on-label and off-label uses of rhBMP 2. It was reported that edema was not a major problem for the patient, but it was serious enough to block the airway after cervical spinal fusion surgery. In addition, although ectopic calcification and excessive bone formation have been reported before, these side effects have not been reported after dental applications. As a result, it was reported that rhBMP 2 could be an alternative to autogenous grafts considering the five-year follow-up (174).

Literature reports that some pharmacological and herbal agents such as statin and tarakasterol induce the release of BMP 2 (175,176).

Kazanawa et al. studied the differentiation and mineralization of osteoblastic MC3T3-E1 cells following the use of metformin, an antihypertensive agent. According to the results, metformin provides differentiation and mineralization of the cells by activation of AMPK and provides eNOS induction and BMP 2 expression (177).

2.6. Delivery Systems

The main reasons for the fail of in vivo growth factor applications are their high solubility and short half-life. Consequently, carrier materials, in order to increase concentration and activity, are needed. The delivery system is required to release in the desired time period without disrupting the biological activity of the factor (178,179). In addition, an ideal carrier is expected to be a biodegradable material with an adequate porous structure which is suitable for angiogenesis and cell infiltration (166).

Studies have been conducted on different biodegradable materials. Polyglycolic acid and polylactic acid and copolymers, polyethylene glycol, alginate, gelatin, collagen are used as synthetic and natural polymer carriers. The carriers may be in the form of porous sponge, microsphere, micro or nanocapsule and hydrogel (180). The release pattern can be adjusted by changing the polymer properties or physical and chemical properties of the carrier (181). Care should be taken not to denature protein factors, especially when using organic solvents and crosslinkers (180). Synthetic polymers run the risk of creating an inflammatory response due to degradation products (182).

Collagen sponges can be easily applied to the defect site, providing a scaffold for cells and bone formation (183). Collagen sponges can gradually release proteins, preventing tissue rejections, however animal-derived collagen and carrier ceramics can cause inflammatory cytokine release and produce an immune reaction (183,184).

It is suggested that a sustained release preceded by a burst release of factor is desirable. Boussein et al. tested the release rate of radiolabeled rhBMP2, implanted with an absorbable collagen sponge, from the rabbit ulna osteotomy site. It was stated that approximately 70% of the BMP2 was retained after three hours and approximately 37% of the initial dose at the end of a week. Consequently, it was slowly released over 14 days. On the other hand only 3% remained for bolus injection at 7 days. As a result, the buffer delivery was not efficient in terms of prolonged retention (185) (Figure 2.2).

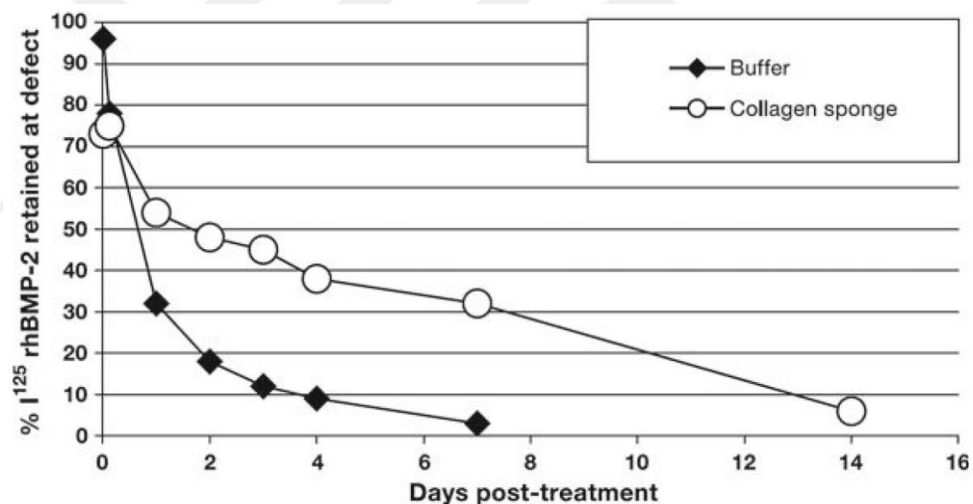


Figure 2.2. Retention of rhBMP 2 in rabbit ulna osteotomy model

2.6.1. Gelatin Sponge

Gelatin, a denatured collagen, can be produced in the form of biodegradable polymer (36). Animal skin gelatin is whipped and baked into its sponge form to prepare gelatin foams. Gelatin sponge provides a remarkable hemostatic action. It serves as a matrix for the clot formation and is often used in bleeding control (186).

Gelatin features better biocompatibility than collagen and has lower host rejection and infection risk. The fact that gelatin does not show immunological properties is attributed to the absence of aromatic radicals (187,188).

Gelatamp® is an absorbable sterile gelatin sponge containing colloidal silver ions. It provides hemostatic and antibacterial properties, yet it was reported to cause compressive complications. Thanks to its porous structure, it is reported that it could absorb approximately 55-75 times its own weight. Its large surface area contributes to the clot formation by providing a suitable medium for platelet aggregation (189).

Gelatamp® has 95% gelatin sponge and 5% colloidal silver content. It is suggested that silver ions are slowly released in order to protect against oral micro organisms, without resistance development. It is stated that topical antimicrobial agents reduce dry socket incidence and it is beneficial to provide antimicrobial properties. It is completely resorbed within 4 weeks and silver ions are continuously released during this period. It is easily applied to extraction sockets and bone defects (189).

2.7. Metformin

The origin of metformin (1,1-dimethylbiguanide hydrochloride), commonly used in the treatment of type 2 diabetes mellitus, reaches back to the discovery of a plant called *Galega officinalis* (Goat's rue or French Lilac). It is noted in Culpeper's Completely Herbal that the latter was used in medieval Europe in the field of traditional medicine against maggots, epilepsy, high fever and epidemics (190). In 1772, John Hill recommended the *Galega* plant to treat patients with the complaint of constant thirst and urination; so that, today, two of the three cardinal findings of diabetes are defined as polyuria and polydipsia (The third finding is defined as polyphagia.) (190,191,192). In the chemical analysis conducted in the mid 1800's, it was observed that *G. officinalis* was rich in guanidine and its derivatives (190).

Metformin was synthesized in Europe in 1920 and was used for its glucose-lowering effect, but its use was stopped due to toxicity (250). In 1949, while trying to produce guanidine derived antimalarial drugs, proguanil was modified to metformin and it was observed to be beneficial in local influenza outbreak.

Thus, metformin hydrochloride was started to be used as an anti-influenza agent called flumamine, and it was reported to decrease blood glucose sugar in some influenza patients. For the first time, in 1957, Jean Sterne reported that metformin can be used in the treatment of diabetes provided it had glucose lowering effects. However, it did not attract attention as it was deemed less potent than other biguanides; phenformin and buformin. Although metformin was safer, the use of guanides was avoided due to the risk of lactic acidosis following phenformin and buformin administration (190). In the 1980s, the concept of non-insulin-dependent diabetes became evaluated in terms of insulin resistance and of beta cells failure (193,194). In the light of new information, it was concluded that metformin induced insulin dependent and insulin independent reactions, affecting insulin, glucagon and metabolic pathways in different doses, respective of various organ tissues (190).

The use of metformin with its success in counteracting insulin resistance and reducing hyperglycemia in adults without the risk of weight gain or hypoglycaemia has become accepted. and was approved by the FDA in 1994 (190,195).

2.7.1. Chemical and Physicochemical Properties of Metformin

Like other biguanides, metformin is a guanidine derivative. Compared to phenformin and buformin, monosubstituted compounds with a long chain, metformin portrays a bisubstituted structure with a short chain. Due to their high basic characteristics, all biguanides are positively charged under physiological conditions. In addition, the non-polar hydrocarbon chain determines the lipophilicity of the molecule and has effects in binding to surfaces, such as membrane phospholipids (196).

Featuring a mono striated long chain, lipophilic phenformin is metabolized in the liver by aromatic hydroxylation. On the other hand, hydorphilic metformin, with a bisubstituted short chain, avoids metabolism and is eliminated by renal excretion. These pharmacological differences determine the liability to the risk factors such as lactic acidosis (197) (Figure 2.3.).

Chemically called as N, N-dimethylimidodicarbonic acid dimide hydrochloride, metformin hydrochloride consists of an almost white crystal structure.

Its molecular formula is $C_4H_{11}N_5 \cdot HCl$. Metformin has a molecular mass of 129.16 g/mol, while Metformin HCl has a mass of 165.6 g/mol. It is free soluble in water (198).

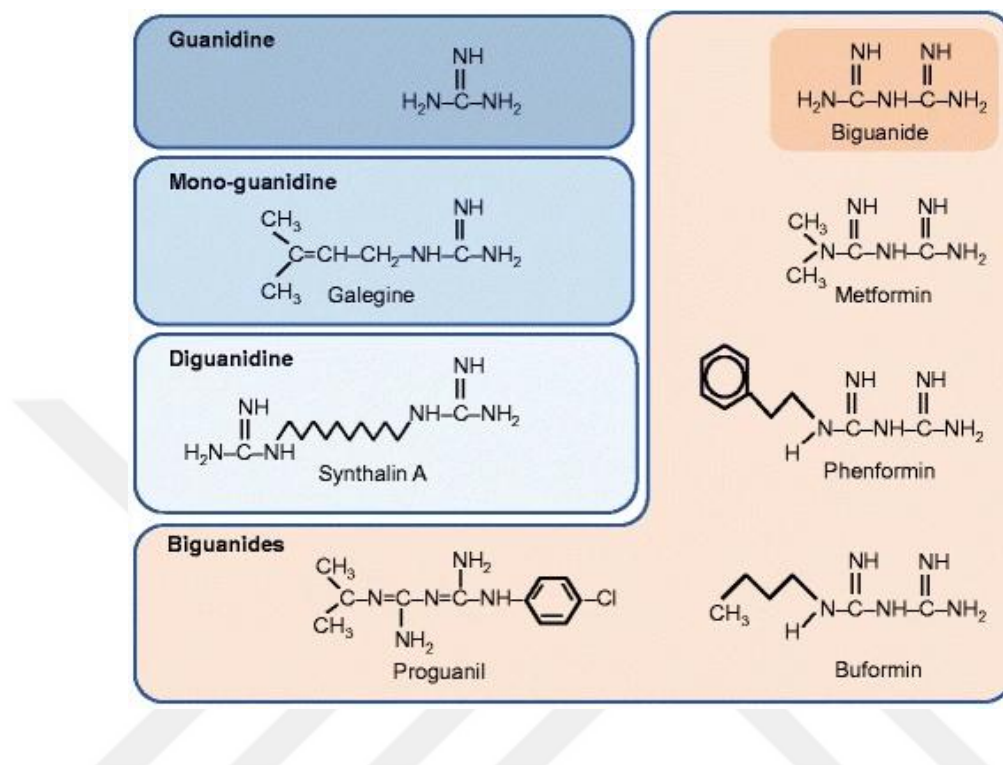


Figure 2.3. Chemical structure of guanidine and derivatives

2.7.2. Pharmacokinetics of Metformin

The gastrointestinal absorption of metformin is deficient since 20-30% of an oral dose is recovered during defecation. Metformin doses of 0.5 - 1.5 g show an absolute oral bioavailability of 40 - 60%. The absorbed rate and the available rate difference is assumed to be due to presystemic clearance or binding to the intestinal wall. Absorption is completed approximately 6 hours after ingestion. There is an inverse relationship between the the amount ingested and its bioavailability. Consequently, lower doses are absorbed faster, implying a saturable absorption process (197). The maximum plasma concentration of 1-2 $\mu\text{g}/\text{mL}$ is reached approximately in 1-2 hours after ingestion (199).

Distribution of metformin is swift, but its transfer to the deep compartment is slow. It is retained in the oesophagus, stomach, duodenum, salivary glands and kidneys.

It does not bind plasma proteins but increases in the blood. Following oral doses of 1,5 g it was detected in the plasma over a day, implying a slow binding to erythrocytes (197). Steady state plasma concentrations are achieved within approximately 24-48 hours and are above 1 microgram/milileter (200).

It does not undergo liver metabolism and its metabolite is not observed (198).

It undergoes prompt renal excretion (197). Renal clearance is 3,5 times greater than creatinine clearance. Tubular secretion is the main route for metformin elimination. After oral administration 90% of the absorbed drug is eliminated through the renal route and the plasma elimination half life is around 6.2 hours. The elimination half life is around 17.6 hours in the circulatory system (200).

2.7.3. Metformin and Diabetes Mellitus Treatment

Diabetes mellitus involves certain metabolic ailments which progress with a picture of chronic hyperglycemia, which depends on insulin release, insulin effect, or both. Some patients may not show symptoms in early years, while others may show signs of hyperglycemia; including polyuria, polydipsia, polyphagia, weight loss and blurred vision, especially in children. In uncontrolled diabetes, fainting and coma may occur and ketoacidosis-related death may be observed (201). In addition, ulcers, retinopathy, kidney failure and heart diseases can be observed (202).

Type 1 diabetes is due to insulin deficiency as a result of the breakdown of β cells. While previously referred to as insulin dependent or juvenile diabetes, it is associated with the immune system's attack on insulin producing beta cells. Daily insulin intake is necessary for patients to control the blood glucose level (203).

In type 1 DM treatment, insulin is the first drug of choice (203). Metformin is sometimes used in type 1 DM to limit the need for an insulin dose (204). According to the American Diabetes Association, using metformin can reduce weight gain and improve lipid levels, but it is not very effective in terms of HbA1c level (205). According to studies, it also reduces the development of atherosclerosis and the risk of developing cardiacular disease (206,207).

Type 2 diabetes is a chronic defect that is not previously insulin dependent or results in the body not being able to use insulin adequately due to insulin resistance under the name of adult diabetes (203). The need for high insulin due to dysfunction of β cells cannot be met (208). It has fewer symptoms than Type 1 DM and can remain hidden for years. The greatest risk factor is high weight (201). In addition, factors such as little physical movement, malnutrition, ethnicity, family history of DM, and advanced age are related (203).

A four-stage treatment protocol is recommended for the European and American Diabetes Association guidelines under Type 2 DM control. Firstly, metformin treatment is applied. If the desired HbA1c level is not reached within 3 months, a second drug such as DPP-4 inhibitor, thiazolidione, sulfonylurea, SGLT 2 inhibitor, GLP-1 receptor agonist or basal insulin is prescribed. At the third stage, after three months, if the HbA1c level is not at the desired level, a second drug is added. At the last stage, if HbA1c level is high after 3 months, a combination of metformin, basal insulin and mealtime insulin or GLP-1 receptor agonist therapy is recommended (203).

Gestational diabetes is a type of diabetes that can occur in the 2nd or 3rd trimester of pregnancy (209). Mothers and their babies diagnosed with diabetes during pregnancy are susceptible to have type 2 DM at a later time (203).

Metformin is not the first drug of choice in gestational diabetes. If the glycemic target is not achieved, insulin treatment is recommended to decrease the glucose level; if it is insufficient or insulin insufficient, metformin can be supported (210).

2.7.4. Metformin Mechanism of Action

The main antidiabetic effect of metformin occurs when it blocks the mitochondrial respiratory chain (complex 1), causing oxidative phosphorylation decomposition and increased AMP / ATP ratio. Increased AMP / ATP ratio causes 5'adenosine monophosphate activated protein kinase (AMPK) activation and allows other enzymes to be regulated by AMPK. With the activation of insulin receptors, insulin stimulates insulin receptor substrate 1 (IRS1), allowing glucose transporter (GLUT) to be released into the cell membrane and increased glucose uptake (211).

AMPK increases IR and IRS1 activation allosterically and increases insulin sensitivity. While AMPK increases the sensitivity of insulin, it ensures the intake of nutrients, it does not act exactly like insulin and inhibits anabolic pathways. AMPK puts body metabolism into a catabolic state by producing energy and ATP to maintain normal cell function. It also increases insulin sensitivity, glucose and lipid metabolism, while reducing gluconeogenesis, especially in the liver. In addition, metformin reduces hepatic lipids by inhibition of lipogenesis and increased lipid metabolism. In general, metformin stimulates cell growth, proliferation and apoptosis through some signaling pathways (211) (Figure 2.4).

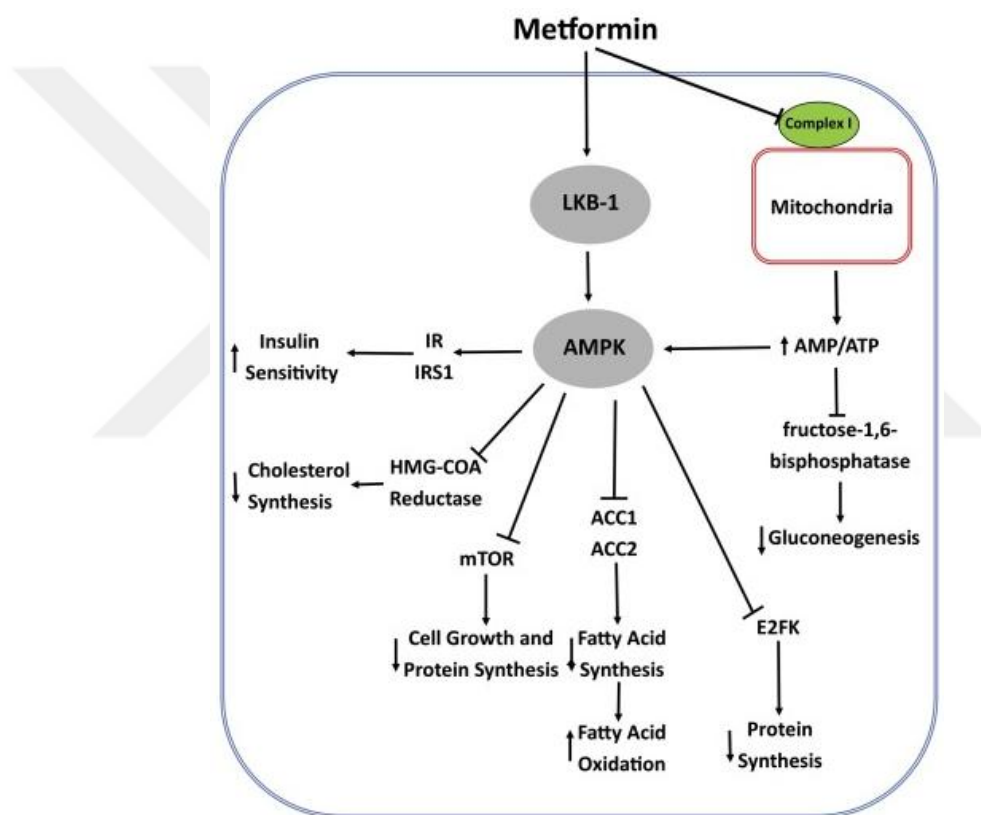


Figure 2.4. The multiple pathways of metformin metabolism

2.7.5. AMPK and Bone Metabolism

AMPK is an important homeostasis and energy regulator for cellular metabolism (212). AMPK activation is affected by the AMP/ATP ratio. Once activated, it stimulates catabolic pathways and inhibits anabolic pathways within the cell (211).

There are two mechanisms for AMPK activation. The first is the increased AMP/ATP ratio. The second is the activation of the AMPK's subunits that receive and regulate the signal. AMPK has 3 subunits as α , β , γ and they are activated when AMP binds α subunit. The catabolic process is regulated by α subunit. β subunit provides glycogen binding domain, while restraining α ve subunits. γ domain is responsible for the binding of regulatory nucleotides. AMPK limits energy expenditure in order to keep the cell alive; stops protein synthesis and cell growth (211) (Figure 2.5.).

The AMPK α 1 subunit is the main catalytic isoform exposed in bone. According to studies, AMPK signals are crucial in bone physiology (212).

According to a study, AMPK can stimulate osteogenesis in MC3T3-E1 cells with osteoblast precursor and suppress the adipogenesis of 3T3-L1 cells that have the potential to convert into lipids (213).

Mesenchymal stem cells can differentiate into osteoblasts by stimulating the Runx2 and Wnt / b-catenin pathways with AMPK (214).

Peroxisome proliferating activated receptor PP (PPAR γ) is a ligand-activated transcription factor responsible for the release of genes associated with fatty acid metabolism, adipogenesis and insulin sensitivity (215). PPAR γ -2 suppresses osteoblast differentiation and causes mesenchymal cells to turn into adipocytes. PPAR γ -1 isoform triggers osteoclastogenesis by increasing RANKL and c-FOS signals. According to studies, AMPK can reduce adipogenesis and suppress PPAR by phosphorylation of b-catenin (211).

According to the studies, AMPK activation directly inhibits osteoclastogenesis and reduces the RANKL release of osteoblasts by OPG stimulation and is indirectly effective (216,19,217).

The nuclear factor of activated T cells c 1 (NFATc1) is an effective transcription factor in differentiation of T cells, cardiac valve morphogenesis, osteoblast differentiation and osteoclastogenesis and is particularly effective in the differentiation of osteoclasts by RANKL (218).

According to the studies, NFATc1 is suppressed with AMPK activation and bone resorption is prevented (211).

Lee et al. (2010) reported that AMPK may suppress RANKL-induced osteoclast formation (219).

According to the studies, the mevalonate pathway, which is effective in the priming of regulatory proteins such as Ras and Rho GTPase, negatively affects bone formation. AMPK can suppress the HMG-CoA reductase pathway and prevent the negative effect of the mevalonate pathway (220,221). Xi et al. (2016) reported that AMPK should be activated in order to activate IGF-I in the early differentiation of osteoblasts (222).

AMPK tries to stop protein synthesis and cell growth to prevent the energy waste of the cell. For this reason, it stops the cell cycle by lowering the release of the mammalian target rapamycin (mTOR), that is overactive in many tumor cells (223,224).

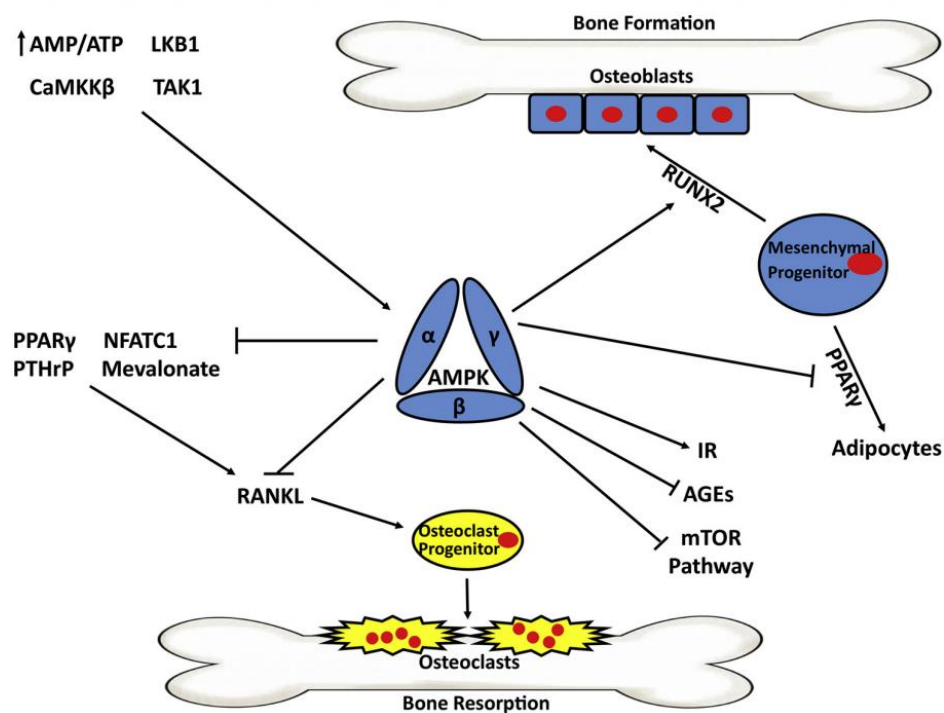


Figure 2.5. Different effects of AMPK subunits

2.7.6. Metformin and Bone

According to the studies, metformin plays an osteogenic role in osteoblastic cell differentiation, bone matrix synthesis and osteoblast proliferation due to its AMPK stimulating effect (15,177,225). It is also noted that metformin enables Runx 2 and AMPK activation in non-diabetic rats in oral use, thereby enabling differentiation of progenitor cells (18).

Metformin was suggested to be effective in osteoblast promotion by inhibiting adipogenic differentiation factor PPAR (226).

According to both in vivo and in vitro studies, metformin increases type 1 collagen synthesis and osteocalcin release. In addition, metformin ameliorates the healing process of bone lesions in rats with and without diabetes (18).

Reactive oxygen species (ROS), the radical forms of oxygen, are reported to affect age-related bone changes (227,228). ROS stimulates osteoblastic apoptosis. Bone regeneration cannot occur due to excess ROS formation (229,230).

Metformin can prevent apoptosis due to decreased osteoblastic cell proliferation, decreased ALP activity, decreased calcium accumulation and ROS production (231).

Runx 2 and IGF-1 release are also affected by metformin (232). By suppressing metformin, osteocalcin and osteoprotegrine, it improves the antiproliferative effect of high glucose in osteoblastic cells (233).

Decreased bone density in diabetic patients is reported to be an important risk factor in terms of susceptibility to fractures. The bone is affected by tissue cycle, cell activity, oxidative stress, and advanced glycation end products (AGE). Proteins with high half-life, such as collagen, which are important components of the bone matrix, tend to be more glycosylated due to their lateral chains. AGEs are released as a result of non-enzymatic reaction between protein-bound amine residues and decreased sugar. The released compounds act as agonists for AGE receptors, suppressing bone formation (234).

The destructive effect of high glucose and AGE on osteoblasts can be prevented by metformin use (235). Tolosa et al. stated that metformin treatment reduced the negative effect of diabetes on osteogenesis by increasing the Runx2 / PPAR γ ratio in diabetic rats without insulin and reduced the release of AGE receptors (236).

Nitric oxide (NO) is a bioactive free radical that plays a role in neurotransmission, vascular function, host defense and immune regulation, and is synthesized by nitric oxide synthase (NOS). NOS has three isoforms. While neural NOS (nNOS) plays a role in neural signals, endothelial NOS (eNOS) is involved in vasodilation and regulation of blood pressure. While nNOS and eNOS are constantly being synthesized, inducible NOS (iNOS) is synthesized only by proinflammatory cytokines and bacterial lipopolysaccharides when the cell is stimulated. iNOS synthesizes NO to protect immune system (237,238). On the other hand, bone loss due to cytokines and inflammation develops. It is reported that the eNOS isoform manages osteoblast activity and bone formation (239).

The osteogenic effect of metformin on cells was thought to be due to kinase (ERK) activation regulated by extracellular signal and stimulation of nitric oxide synthase (e / iNOS) (234). According to the studies, cells with osteoblast precursors such as MC3T3E1, which depend on the administration of metformin, are proliferated and differentiated with increased type 1 collagen production and ALP activity (15).

According to in vivo studies, metformin is thought to preserve bone density over core binding factor $\alpha 1$ and LDL receptor-associated protein 5, which are osteoblast markers in estrogen deficiency in ovariectomized rats (240,19).

As stated in the studies, the risk of fractures among those using metformin is higher among patients with diabetes than those who do not (241,242).

2.7.7. Potential Uses of Metformin

Metformin is thoroughly investigated for novel uses. Researchers stated that it features anticancer, anti aging, cardiovascular and neuroprotective properties. It was reported that it can be used as an alternative drug in polycystic ovary syndrome (243).

According to the studies, metformin can prevent the formation of prostate, breast, pancreas and colon cancers and reduce the risk of tumor formation in patients with diabetes (244-246). It was suggested that metformin could prevent the proliferation of osteosarcoma cells via the AMPK / mTOR / S6 signal path (247). On the other hand, it was stated that the efficacy of metformin decreases in hypoxia (248).

In a study, it was reported that second generation antipsychotic drugs cause weight gain in patients and the use of metformin in these patients is supported for weight loss (249). In another study, it was stated that it may be the first drug of choice due to weight loss and glycemic control effects in diabetic obese patients (250).

2.7.8. Adverse Effects of Metformin

The most common side effects associated with metformin are those related to the digestive system. Nausea, diarrhea, vomiting, discomfort in the abdomen and anorexia were observed in patients, but none of these symptoms reached a serious state. They disappeared by dose adjustment or ceasing the treatment (251).

Since the organ that metformin acts primarily is the liver, it is thought to be the organ that causes the development of lactic acidosis (252). When the conversion of pyruvate into glucose is inhibited and the mitochondrial electron transport chain does not function, lactic acidosis levels increase instead of oxidation. Lactic acidosis complication is extremely rare and the rate has been reported to be less than 1/10000, but it is fatal. The possibility of developing complications is thought to occur with diseases such as renal insufficiency, hepatic disease or septicemia, and when the dose specified by the FDA is exceeded (251).

The presence of pernicious anemia due to B12 deficiency was reported in patients using metformin for a long time (253). In the presence of diabetic polyneuropathy, cognitive impairment, subacute combined degeneration and blood diseases, vitamin B12 deficiency may be observed due to metformin (251). The patients, treated with high dose metformin for a long time, should be checked periodically in order to prevent potential vitamin B12 deficiency (253).

Taste alterations can be observed depending on the accumulation and release of metformin in the salivary glands (254).

Although very rare, cholestatic hepatitis due to metformin use was observed, but liver enzymes returned to normal when the drug was discontinued. It was reported that metformin use may be associated with pancreatitis when administered in high doses or in case of renal insufficiency (251).

Hypoglycemia may occur after high doses of metformin use, as liver-related gluconeogenesis and glucose uptake would decrease (251). According to a meta-analysis study, the risk of developing hypoglycaemia due to metformin is lower than that of sulfonylurea (255).

2.8. Histology and Histomorphometry

Bone histologic study is invaluable to evaluate tissue level mechanism of bone physiology and it is necessary to gain information about bone tissue structure. It can also be used to assess histopathological changes such as infection and necrosis (256).

Histomorphometry is a technique which is used in the quantitative analysis of bone architecture, aiming to obtain information about the amount of bone and its metabolism. It requires histological sections to assess cellular and structural variables. These sections are bidimensional depictions of three dimensional (3D) forms and the main goal is to evaluate the 3D reality through 2D measurement (256,257).

Histomorphometric analysis involves certain steps starting from the specimen collection to slide preparation. Then they are analyzed digitally on computer using a software which obtains a high resolution image through the microscope. Later, various measurements are studied depending on sources and referents (257).

Bone volume and fibrosis volume are area type of measurements in bone histomorphometry. New bone formation, a strong indicator of osteogenic activity, is observed as cancellous constructs and fibrous tissue, a sign of poor osteogenic activity, can be recognized by composition of loose bundles of collagen fibers (257-259).

3. MATERIALS AND METHODS

This study was conducted by the approval of Experimental Animals Ethics Committee, Yeditepe University, Istanbul on 18.02.2020 (Appendix-1). Animal experiments were performed at Experimental Research Center, Faculty of Medicine Yeditepe University (YÜDETAM). Histological and histomorphometric examinations were carried out in Department of Tumor Pathology and Oncological Study, Institute of Oncology, Istanbul University.

The sample number of the study was calculated with the program named G * Power 3.1.9.2. based on the study of Bastos et al. (260). 7 rats in each group were found to be suitable for the experiment with 42 rats in total, with a high effect size and coefficient 0.60, 80% power and 5% Type I error margin, based on the percentage of means (BA) % change in inter-group correlation.



Figure 3.1.Cages sort by respective study groups

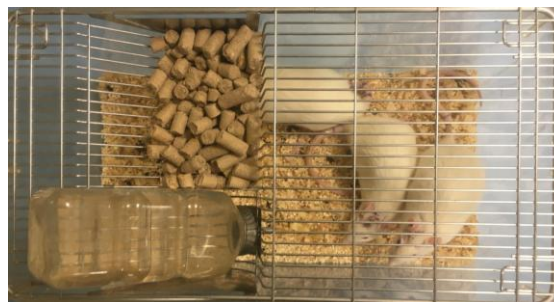


Figure 3.2. A standart cage housing three rats

3.1. Study Groups

The subjects were obtained from Laboratory of Experimental Animals, Yeditepe University. All groups were provided 12 hours light / 12 hours dark cycle. Peleted diet and fresh water were provided ad libidum. The room temperature was kept constant at 22 ± 2 °C and the relative humidity was kept between $50 \pm 10\%$. They were housed in cages covered with sawdust on the base. (Figure 3.1.) No more than 3 or 4 rats were placed in the same cage during the experiment. (Figure 3.2.)

Forty two, twelve-month-old male Sprague Dawley rats, weighting 280 ± 20 g, were randomly divided into six groups. Agent concentrations were based on previously published studies (24,261). A 5.0 mm critical size calvarium defect was created in the right parietal bone of each rat. All rats were sacrificed on day 28.

Group 1 (C) (n=7) The defect site was left empty.

Group 2 (G) (n=7) Gelatin sponge was implanted into the defect.

Group 3 (G+BMP) (n=7) 2.15 µg of BMP 2 impregnated gelatin sponge was implanted into the defect.

Group 4 (M) (n=7) Systemic metformin hydrochloride (approximately 255mg / kg) was administered after the operation once daily for the first 14 days.

Group 5 (G+M) (n=7) Gelatin sponge was implanted into the defect. Systemic metformin hydrochloride (approximately 255mg/ kg) was administered after the operation once daily for the first 14 days.

Group 6 (G+BMP+M) (n=7) 2.15 µg of BMP 2 impregnated gelatin sponge was implanted into the defect. Systemic metformin hydrochloride (approximately 255mg/ kg) was administered after the operation once daily for the first 14 days.

3.2. Material Preparation

Gelatin implants were 5 x 7 mm (diameter x length) cylinders cut from 14 x 7 x 7 mm Gelatamp® (Coltene/Whaledent, USA) sponge. 14 pieces of Gelatamp® of 10 mg were used to obtain 28 cylindrical sponges of 2 mg (Figure 3.3).

30 µg of BMP2 (B3555-Sigma Aldrich, USA) powder was mixed with 0.35 ml of saline for a minute. The solution was drawn into a 0.5 ml syringe. 14 pieces of cylindrical sponges were put on petri dish. 0.025 ml (25 µl) of solution containing 2.15 µg of BMP 2 (B3555-Sigma Aldrich, USA) was dropped onto each one of them. Sponges were left to soak for 15 minutes before implantation. They were implanted on rats in Group 3 (G+BMP) (n=7) and Group 6 (G+BMP+M) (n=7) (Figure 3.4).



Figure 3.3. Preparation of sponges to match to the diameter of the trephine bur

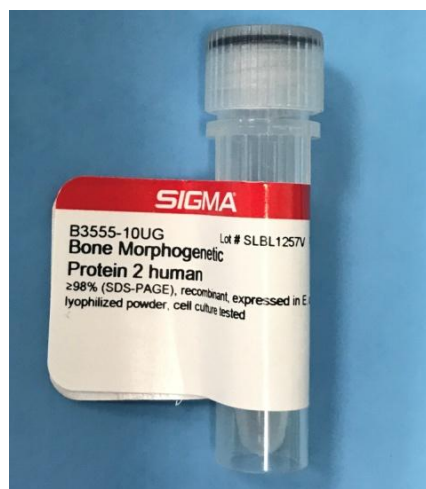


Figure 3.4. Bone Morphogenetic Protein 2

No additional procedure was performed on the other 14 cylindrical sponges. They were implanted on rats in Group 2 (G) (n=7) and Group 5 (G+M) (n=7).

Glucophage® 1000mg tablets (Merck, Turkey) were used for metformin applications at a dose of approximately 255mg/kg. The tablets were crushed and powdered (Figure 3.5). 1500 mg of Metformin HCL powder was mixed with 21 ml of sterile saline for a minute to achieve a daily dose for 21 rats (Figure 3.6). It was administrated by oral gavage for 14 days. Each rat in Group 4 (M) (n=7), Group 5 (G+M) (n=7) and Group 6 (G+BMP+M) (n=7) received 71.4 mg/day of solution.



Figure 3.5. Powdered metformin tablet and isotonic saline solution



Figure 3.6. Vortex mixer

3.3. Surgical Procedure

All rats were anesthetized, administering ketamine hydrochloride (Ketalar ®, Pfizer, Turkey) at a dose of 100mg/kg and Xylazine hydrochloride 2% (Rompun ®, Bayer, Turkey) at a dose of 10mg/kg intraperitoneally. Anesthesia was checked by skin and finger pinching. The head fur of the rats, within the boundaries of the operation site, were shaved. Later they were transferred on the operating table. Povidone iodine (Batticon ®, Adeka) solution was applied to the operation site (Figure 3.7). Using scalpel, a small incision down to the periosteum over the scalp from lambda to bregma was performed to visualize calvarium (Figure 3.8).

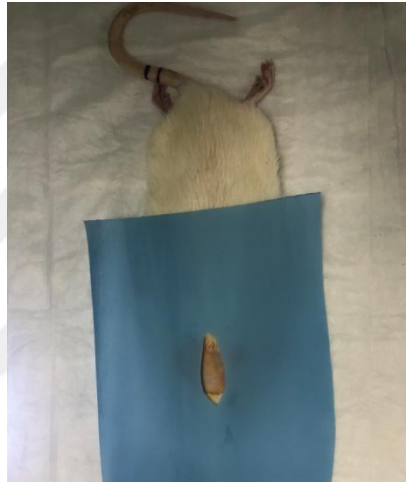


Figure 3.7. Transfer on the operating table and antisepsis of the calvaria



Figure 3.8. Incision down to periosteum after antisepsis procedure

Subcutaneous tissues and periosteum were bluntly dissected and elevated to expose calvarium. The middle of right parietal bone was scored with the trephine riding at 1500 rpm under sterile saline irrigation. Care was taken to prevent dura perforation as calvarium was approximately 1 mm thick. As the defect neared the appropriate thickness, the elevator blade was placed into the defect margin to lift the freed calvarium. (Figure 3.9) Surgical splinter tweezer forceps were used to remove the freed circular bone segment. (Figure 3.10) The same surgical procedure was performed on all rats until material implantation.



Figure 3.9. Preparation of the defect under saline irrigation



Figure 3.10. Removal of cylindrical bone segment

The defect was assessed for any remaining bone fragments. Circular bone segment was compared to the circular sponge to check their proportion (Figure 3.11). In Group 1 and Group 4 defects were left empty. In Group 2 and Group 5 each defect was implanted with a sponge. In Group 3 and Group 6 each defect was implanted with a BMP 2 loaded sponge. Attention was paid in order to refrain from excessive pressure to dura while molding the material.

The periosteum and the skin were closed over the implant using an interrupted 3.0 synthetic absorbable suture. (Pegelak ®, Doğsan, Turkey). After the completion of surgery, the head was cleaned with povidone iodine solution (Figure 3.12).

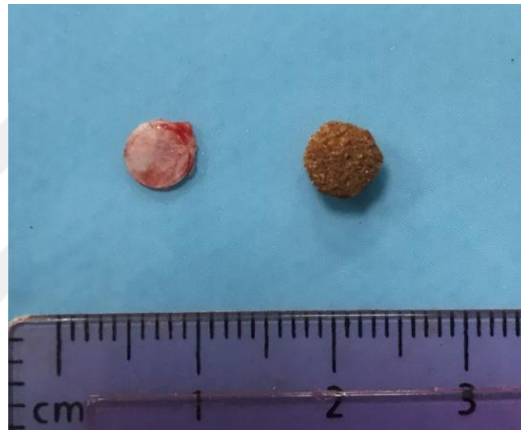


Figure 3.11. Comparison of the removed bone and cylindrical sponge



Figure 3.12. Application of povidone iodine solution after suturing

After the operation the rats were placed on warm pads to maintain their body temperature. After observation, the rats were transferred to their husbandary cages. They were housed in groups of three or four until the end of the experiment. At the end of the first day of the operation, the rats in Group 4, Group 5 and Group 6 received metformin HCL solution at a dose of 255mg/kg/day. Oral gavage procedure was carried out once a day for 14 days (Figure 3.13). Body weight of the rats was under surveillance over this period. Carbon dioxide at 40% was used for the euthanasia of all the rats on day 28 of the experiment. The mean weight of the rats was approximately 320 g.

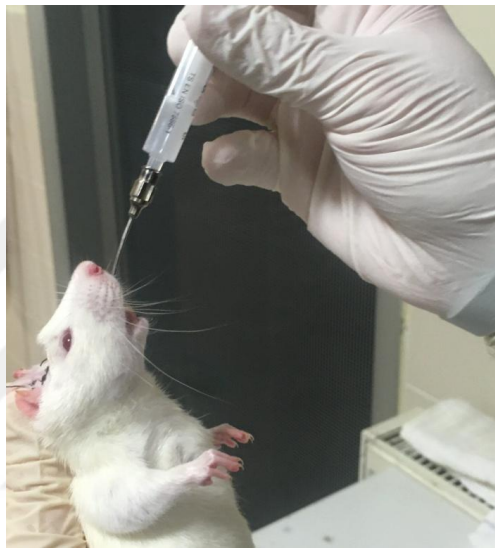


Figure 3.13. Oral gavage procedure



Figure 3.14. Calvarial bone of a rat in group 6

3.4. Histological and Histomorphometric Analysis

Histological and histomorphometric analysis was performed following the retrieval of calvarial bones (Figure 3.14). The samples were fixed in 10% formaldehyde solution for 48 hours. Meanwhile they were transferred to Department of Tumor Pathology and Oncological Study, Institute of Oncology, Istanbul University for histological and histomorphometric analysis. Later the samples were decalcified in a solution of %50 formic acid and %20 sodium citrate at room temperature for 3 weeks.

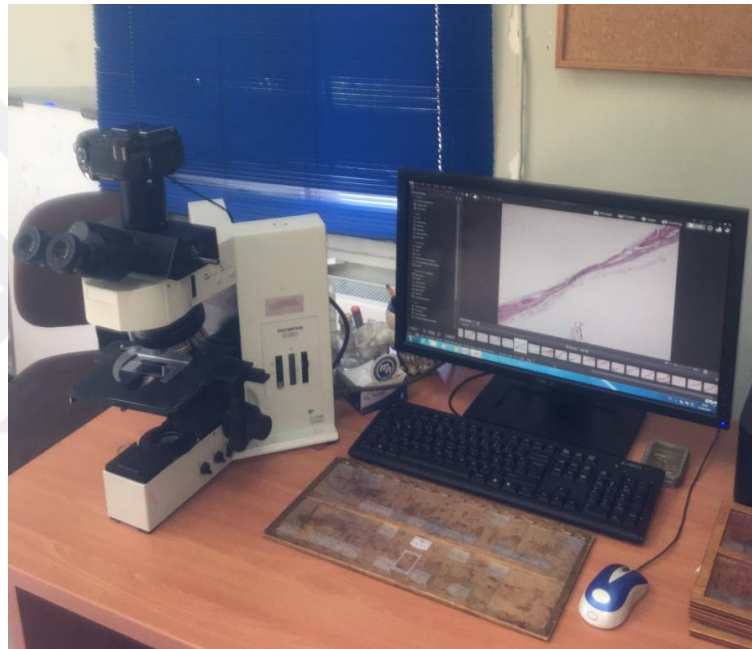


Figure 3.15. Olympus microscope camera

Parafin blocks prepared from routinely processed decalcified specimens were cut into 4- μm slices. The sections were evaluated with a light microscope (Olympus BX60) attached to a color video camera and connected to a computer. The defect sides were captured using the camera and displayed on the computer monitor. All measurements were done with Olympus Image Analysis System. The slides were evaluated at x40, x100 and 200x magnification for new bone formation and fibrosis that calculated in 1 or 2 contiguous and consecutive microscopic fields, depending on the size (Figure 3.16.).

The proportions of the area occupied by newly formed bone and fibrosis was measured and was confined to a total area. In order to scale the results, new bone formation percentage was calculated as $(\text{new bone formation} \times 100) / \text{total defect area (new bone formation + fibrosis)}$ and fibrosis percentage was calculated as $(\text{fibrosis} \times 100) / \text{total defect area}$.

Also the infection, necrosis and foreign body reaction were evaluated as ‘-’ if absent and ‘+’ if present.

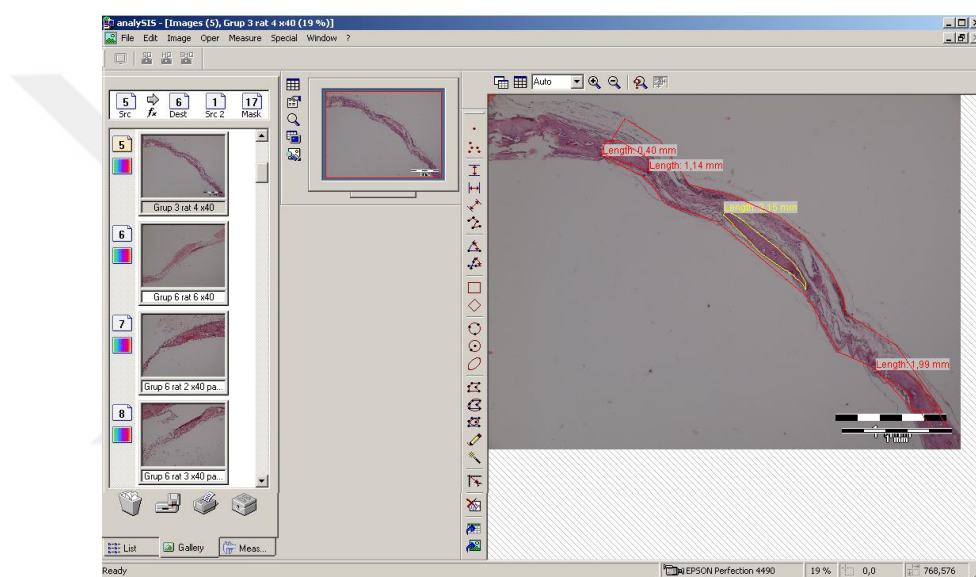


Figure 3.16. Measurement of the area covering new bone formation and fibrosis

3.5. Statistical Analysis

The data were analyzed using the SPSS 25.0 (Statistical Packages of Social Sciences) program. Explanatory statistics were presented as mean $\pm$ standard deviation for continuous variables. The compliance of the data to normal distribution was evaluated using the Shapiro-Wilks test. Homogeneity of variances was evaluated by Levene test. Comparison of the new bone percentage and fibrosis percentage of the groups was evaluated by ANOVA test. Tukey test was evaluated for paired comparisons. If $p < 0.05$, the difference was considered significant.

4. RESULTS

4.1. Clinical Results

All the procedures including the defect surgery, local carrier application with and without BMP 2 and oral gavage treatments were well tolerated by the rats.

Macroscopically, no symptoms or clinical signs of infection were observed on the rats during the experiment.

Body weights were measured in all rats at the first and the last day of the experimentation.

Table 4.1. Statistics of difference between the weight change median values of groups

Difference (Day 28-Day 1)					
Group	N	Mean	Std. Deviation	Median	P value
C	7	48,14	15,689	48,00	0,373
G	7	48,57	32,700	53,00	
G+BMP	7	40,14	8,133	42,00	
M	7	34,29	23,142	41,00	
G+M	7	47,29	9,639	45,00	
G+BMP+M	7	40,14	4,981	38,00	
Total	42	41,24	17,229	42,00	

Kruskal Wallis test

There is no statistically significant difference between the weight change median values of the groups ($p>0,05$) (Table 4.1.).

Table 4.2. Comparison of all groups in terms of the difference in weight measurements over time

Group		Mean	N	Std. Deviation	Std. Error Mean	P value
C	Day 1	277,33	7	28,516	10,778	0,000 ^{a*}
	Day 28	325,50	7	37,412	14,140	
G	Day 1	282,40	7	28,201	12,612	0,008 ^{a*}
	Day 28	322,71	7	20,560	9,195	
G+BMP	Day 1	282,57	7	24,677	9,327	0,000 ^{b*}
	Day 28	322,71	7	26,632	10,066	
M	Day 1	312,29	7	15,294	5,781	0,008 ^{a*}
	Day 28	346,57	7	24,751	9,355	
G+M	Day 1	262,83	7	26,145	9,882	0,000 ^{a*}
	Day 28	307,33	7	20,614	7,792	
G+BMP+M	Day 1	271,57	7	28,466	10,759	0,000 ^{b*}
	Day 28	311,71	7	27,897	10,544	

^apaired samples t test ^b Wilcoxon's sign test *p<0,05 statistically significant

The difference in weight measurements over time in all groups is statistically significant (p<0,05). The average weight of the rats increased after 28 days in these groups (Table 4.2.) (Figure 4.1.)

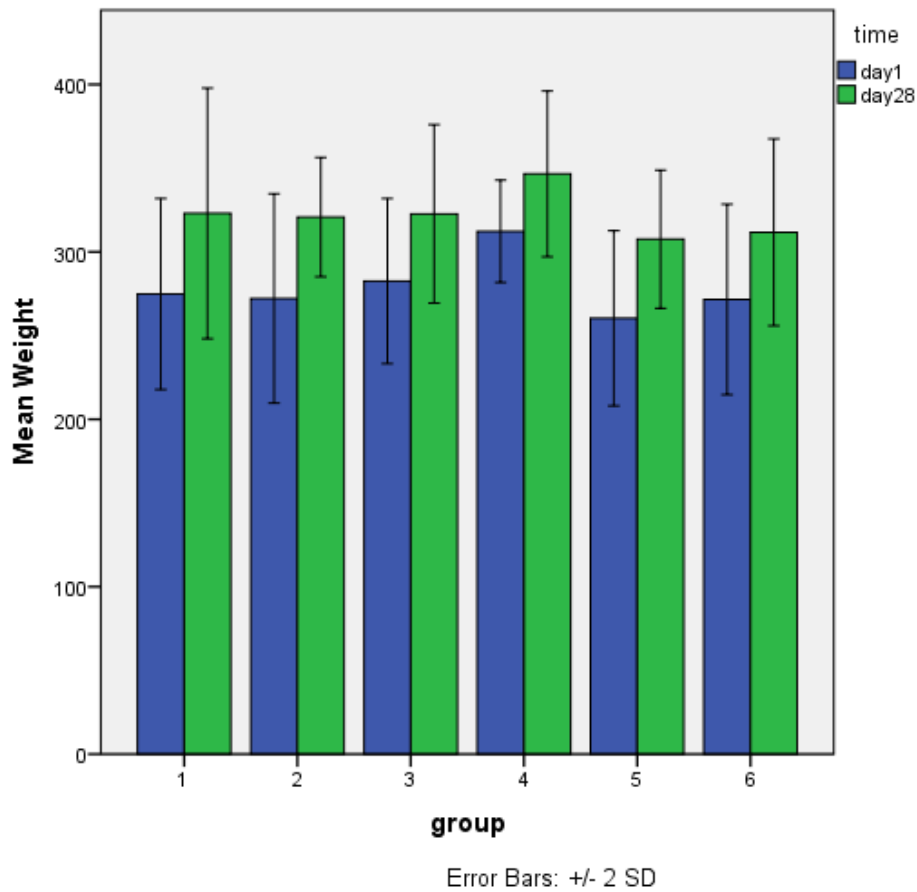


Figure 4.1. The difference between day 1 and day 28 in mean weight measurements by groups

4.2. Histological and Histomorphometric Results

No foreign body reaction, necrosis and severe inflammation were observed in the groups.

New bone formation and fibrosis in the defect area were measured for each slide under 40 x magnification (Table 4.3.).

During healing period new bone formation gained advantage over fibrosis in all groups, yet there were differences between them in terms of rate (Figure 4.2.- 4.7.).



Figure 4.2. Representative histologic HE stained image (40x magnification) from C group. 28 days post-surgery displaying the edges of the surgical defect showed new bone formation (arrows) and fibers of connective tissue (star) within the defect. (H&E x40)



Figure 4.3. Representative histologic HE stained image (40x magnification) from G group. 28 days post-surgery displaying the edges of the surgical defect showed the presence of connective tissue fibers (star) and in some amount of new bone formation (arrows) at the edge of the defect. (H&E x40)



Figure 4.4. Representative histologic HE stained image (40x magnification) from G + BMP group. 28 days post-surgery displaying the edge of the surgical defect showed dense connective tissue (stars) and new bone formation (arrows) within the defect. (H&E x40)

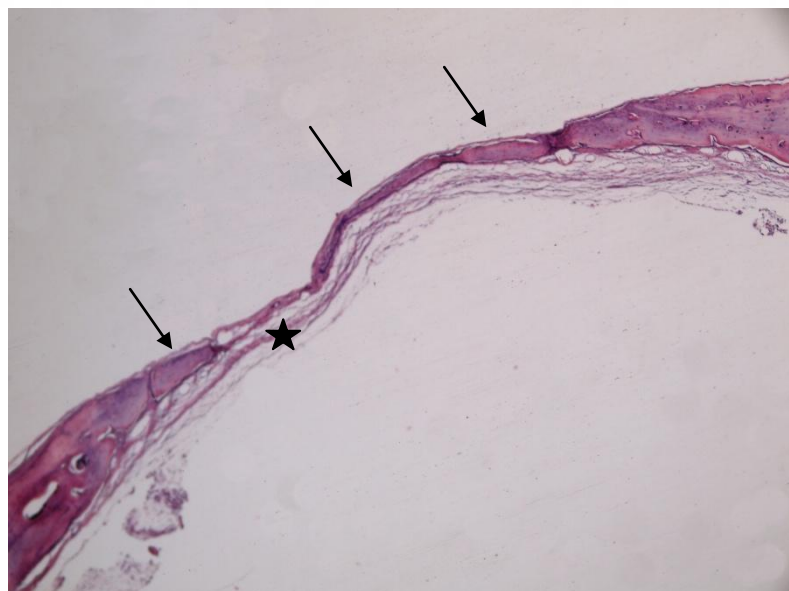


Figure 4.5. Representative histologic HE stained image (40x magnification) from M group. 28 days post-surgery the bone defect was filled by dense connective tissue (star) and new bone formation (arrows) in the middle and at the edge of the surgical defect. (H&E x40)

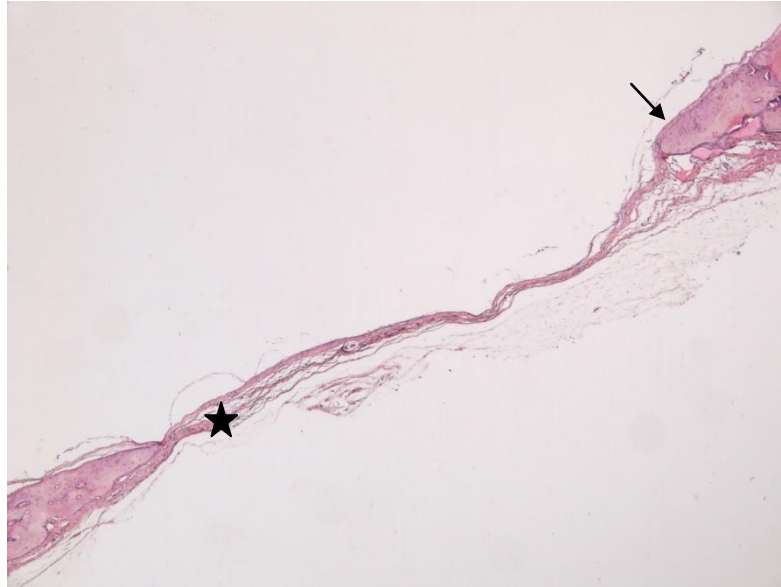


Figure 4.6. Representative histologic HE stained image (40x magnification) from G + M group. 28 days post-surgery displaying the edge of the surgical defect showed thin fibrous connective tissue (star) and a few bone formation (arrow) within defect. (H&E x40)

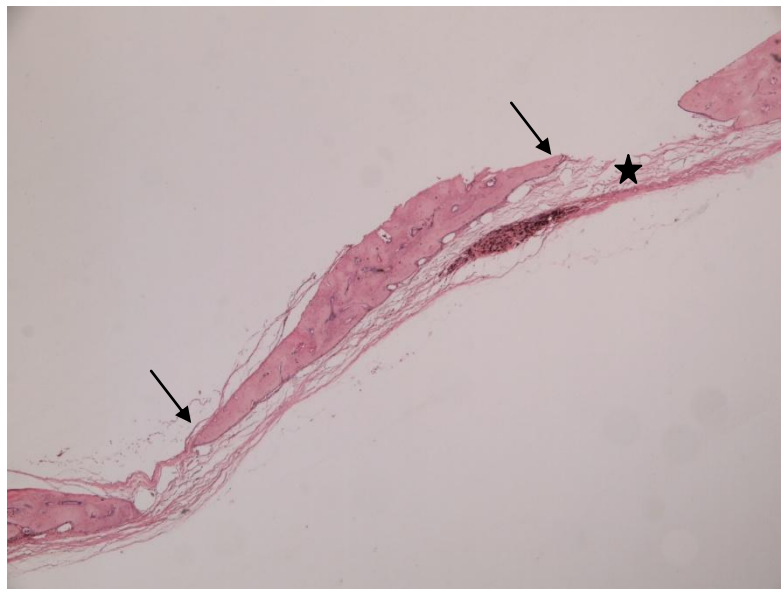


Figure 4.7. Representative histologic HE stained image (40x magnification) from G + BMP + M group. 28 days post-surgery displaying the edge of the surgical defect showed new bone formation (arrows) and fibrotic connective tissue (star) in some degrees (H&E x40).

Table 4.3: New bone and fibrosis area in each defect after 4 weeks

GROUPS	New bone (mm ²)	Fibrosis (mm ²)
C		
Group 1 rat 1	0.76	0.1
Group 1 rat 2	0.32	0.14
Group 1 rat 3	0.42	0.18
Group 1 rat 4	0.47	1.17
Group 1 rat 5	1.3	0.12
Group 1 rat 6	0.52	0.37
Group 1 rat 7	0.91	0.47
G		
Group 2 rat 1	1.13	0.75
Group 2 rat 2	0.3	0.47
Group 2 rat 3	0.63	0.24
Group 2 rat 4	0.42	1.19
Group 2 rat 5	0.41	0.1
Group 2 rat 6	0.37	0.44
Group 2 rat 7	0.79	0.43
G + BMP		
Group 3 rat 1	1.19	0.6
Group 3 rat 2	1.35	1.92
Group 3 rat 3	0.82	0.39
Group 3 rat 4	0.5	0.47
Group 3 rat 5	0.79	1.32
Group 3 rat 6	0.42	0.42
Group 3 rat 7	0.46	0.17
M		
Group 4 rat 1	1.74	0.82
Group 4 rat 2	0.6	0.38
Group 4 rat 3	0.64	0.37
Group 4 rat 4	1.06	0.65
Group 4 rat 5	0.93	0.85
Group 4 rat 6	0.68	0.33
Group 4 rat 7	0.68	0.2
G + M		
Group 5 rat 1	0.95	0.45
Group 5 rat 2	1.7	0.9
Group 5 rat 3	0.59	0.39
Group 5 rat 4	0.43	0.21
Group 5 rat 5	0.88	1.22
Group 5 rat 6	0.47	0.36
Group 5 rat 7	0.28	0.5
G + BMP + M		
Group 6 rat 1	2.03	1
Group 6 rat 2	1.5	0.26
Group 6 rat 3	1	0.69
Group 6 rat 4	2.07	0.6
Group 6 rat 5	1.15	0.22
Group 6 rat 6	0.9	0.21
Group 6 rat 7	2.01	0.3

4.2.1. Results of New Bone Formation

The new bone percentage distribution fits the normal distribution in each group $p > 0.05$ and provides the variance homogeneity $p > 0.05$.

Table 4.4. Comparison of the mean new bone percentage by groups

New Bone %					
Groups	N	Mean	Std. Deviation	Std. Error	p value
C	7	68,9429	17,89673	6,76433	0,030*
G	7	55,4843	19,35297	7,31474	
G+BMP	7	55,4886	13,67038	5,16692	
M	7	64,4857	7,65418	2,89301	
G+M	7	55,7914	12,62851	4,77313	
G+BMP+M	7	77,2800	10,41140	3,93514	
Total	42	62,9121	15,68976	2,42098	

ANOVA result * $p < 0,05$ statistically significant

ANOVA New Bone %					
	Sum of Squares	df	Mean Square	F	p value
Between Groups	2843,876	5	568,775	2,825	,030
Within Groups	7249,037	36	201,362		
Total	10092,913	41			

Comparison of the mean new bone percentage by groups is statistically significant ($p < 0.05$) (Table 4.4.). G + BMP + M group shows the highest rate of new bone percentage, followed by C group, M group, G + M group, G + BMP and G group. G group, G + BMP group and G + M group portrayed nearly identical rates in mean new bone percentage which are 55,4843 and 55,4846 and 55,7914 respectively.

Table 4.5. Pairwise comparison of all groups in terms of new bone percentage*Multiple Comparisons*

Dependent Variable: New bone %

(I) group	(J) group	Mean Difference (I-J)	Std. Error	p value
C	G	13,45857	7,58499	,494
	G+BMP	13,45429	7,58499	,495
	M	4,45714	7,58499	,991
	G+M	13,15143	7,58499	,519
	G+BMP+M	-8,33714	7,58499	,878
G	C	-13,45857	7,58499	,494
	G+BMP	-,00429	7,58499	1,000
	M	-9,00143	7,58499	,840
	G+M	-,30714	7,58499	1,000
	G+BMP+M	-21,79571	7,58499	,068
G+BMP	C	-13,45429	7,58499	,495
	G	,00429	7,58499	1,000
	M	-8,99714	7,58499	,840
	G+M	-,30286	7,58499	1,000
	G+BMP+M	-21,79143	7,58499	,068
M	C	-4,45714	7,58499	,991
	G	9,00143	7,58499	,840
	G+BMP	8,99714	7,58499	,840
	G+M	8,69429	7,58499	,859
	G+BMP+M	-12,79429	7,58499	,549
G+M	C	-13,15143	7,58499	,519
	G	,30714	7,58499	1,000
	G+BMP	,30286	7,58499	1,000
	M	-8,69429	7,58499	,859
	G+BMP+M	-21,48857	7,58499	,075
G+BMP+M	C	8,33714	7,58499	,878
	G	21,79571	7,58499	,068
	G+BMP	21,79143	7,58499	,068
	M	12,79429	7,58499	,549
	G+M	21,48857	7,58499	,075

Tukey test

Pairwise comparisons were evaluated using the Tukey test to determine the presence of difference between groups (Table 4.5.).

No pairwise comparisons were statistically significant for mean new bone percentage ($p > 0.05$).

There is a small difference between G and G + BMP groups in terms of new bone formation, but it is not statistically significant.

However it should be marked that G + BMP + M group showed the highest rates of mean new bone percentage. The results of the latter were almost statistically significant compared to G group (68%), G + BMP group (68%) and G+ M group (75%) (Figure 4.8.).

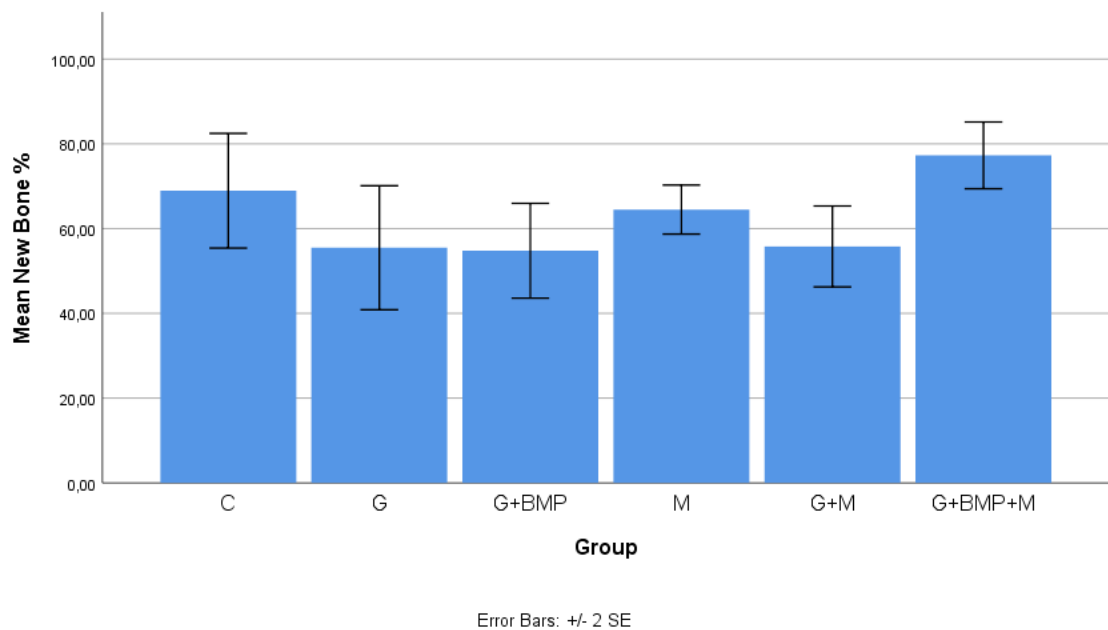


Figure 4.8. Comparison of mean new bone percentage by groups

4.2.2. Results of Fibrosis

Fibrosis percentage distribution fits the normal distribution in each group $p > 0.05$ and provides the variance homogeneity $p > 0.05$.

Table 4.6. Comparison of the mean fibrosis percentage by groups

Fibrosis %					
Groups	N	Mean	Std. Deviation	Std. Error	p value
C	7	31,0571	17,89673	6,76433	0,030*
G	7	44,5157	19,35297	7,31474	
G+BMP	7	44,5114	13,67038	5,16692	
M	7	35,5143	7,65418	2,89301	
G+M	7	44,2086	12,62851	4,77313	
G+BMP+M	7	22,7200	10,41140	3,93514	
Total	42	37,0879	15,68976	2,42098	

ANOVA result * $p < 0,05$ statistically significant

ANOVA test

Fibrosis %					
	Sum of Squares	df	Mean Square	F	p value
Between Groups	2843,876	5	568,775	2,825	,030
Within Groups	7249,037	36	201,362		
Total	10092,913	41			

Comparison of the mean fibrosis percentage by groups is statistically significant ($p < 0.05$) (Table 4.6.). The highest values of fibrosis were obtained on G, G+BMP and G+M groups. Whereas the lowest values were determined on G+BMP+M group.

Pairwise comparisons were evaluated using the Tukey test to determine the presence of difference between groups (Table 4.7.).

Table 4.7. Pairwise comparison of all groups in terms of fibrosis percentage*Multiple Comparisons*

Dependent Variable: Fibrosis %

(I) group	(J) group	Mean Difference (I-J)	Std. Error	p value
C	G	-13,45857	7,58499	,494
	G+BMP	-13,45429	7,58499	,495
	M	-4,45714	7,58499	,991
	G+M	-13,15143	7,58499	,519
	G+BMP+M	8,33714	7,58499	,878
G	C	13,45857	7,58499	,494
	G+BMP	,00429	7,58499	1,000
	M	9,00143	7,58499	,840
	G+M	,30714	7,58499	1,000
	G+BMP+M	21,79571	7,58499	,068
G+BMP	C	13,45429	7,58499	,495
	G	-,00429	7,58499	1,000
	M	8,99714	7,58499	,840
	G+M	,30286	7,58499	1,000
	G+BMP+M	21,79143	7,58499	,068
M	C	4,45714	7,58499	,991
	G	-9,00143	7,58499	,840
	G+BMP	-8,99714	7,58499	,840
	G+M	-8,69429	7,58499	,859
	G+BMP+M	12,79429	7,58499	,549
G+M	C	13,15143	7,58499	,519
	G	-,30714	7,58499	1,000
	G+BMP	-,30286	7,58499	1,000
	M	8,69429	7,58499	,859
	G+BMP+M	21,48857	7,58499	,075
G+BMP+M	C	-8,33714	7,58499	,878
	G	-21,79571	7,58499	,068
	G+BMP	-21,79143	7,58499	,068
	M	-12,79429	7,58499	,549
	G+M	-21,48857	7,58499	,075

Tukey test

No pairwise comparisons were statistically significant for fibrosis percentage ($p > 0.05$).

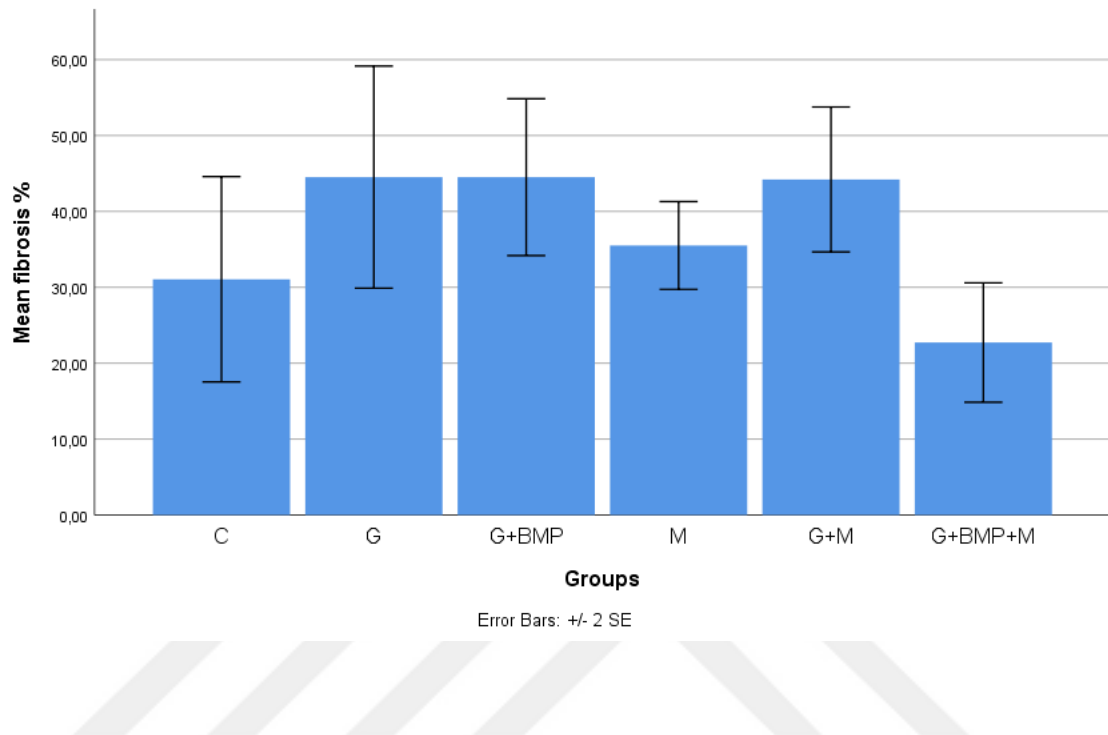


Figure 4.9. Comparison of mean fibrosis percentage by groups

There is a small difference between G and G + BMP groups in terms of fibrosis, but it is not statistically significant (Figure 4.9).

5. DISCUSSION

Large bone defects hinder the ideal healing process and complicate the treatment planning in maxillofacial region (123). Bone grafting procedures aim to act as osteoconductive and osteoinductive scaffolds during reconstruction period (262). However it is not always possible to estimate the success rate of bone grafts (132). On the other hand the bone graft materials should be cost effective and efficient at the same time. BMP 2 is an effective growth factor and it is considered a feasible option from the medical perspective, yet the use of BMP 2 in addition to the standart treatment increases the cost and causes limited accesibility (263). It should also be noted that increased doses of BMP 2 may cause adverse effects and conversely low doses may not be able to induce osteogenesis.

It is obvious that single dose application of growth factors without any carrier has limited success in bone healing, so it is mandatory to use an appropriate release system during the regeneration process (262).

Bone healing mechanism is impeded by the local and systemic administration of particular drugs such as biphosphanetes and sodium renalate (92).

Metformin, a well known agent which is prescribed for type 2 diabetes mellitus patients, stands out as an agent with the possibility to induce osteogenesis despite the conflict between numerous research groups. On account of the significant divergence in the results, there is no consensus about the ideal doses of metformin to provide proosteogenic effects.

In the present research, it is aimed to investigate the possibility of metformin administration to achieve increased bone healing in non diabetic patients. We presume that metformin increases the osteogenic effects of BMP 2. On this subject, we wanted to justify our thesis by comparing the osteogenic effects of local BMP 2 application to that of systemic metformin administration, along with their combined therapy. We preferred to use gelatin sponges as BMP 2 carriers and scaffolds, as they are used with ease during clinical practice. Our experiment was conducted on rat calvarial defects including 6 different groups for 28 days. The results were evaluated using histological and histomorphometric analysis.

Based on the increasing number of medical studies for the use of metformin and its potential contribution to the bone formation, this study assessed, for the first time in our knowledge, the comparative effects of systemic metformin administration and local BMP2 application on rat calvarial defects under non diabetic conditions.

INFUSE[®] Bone Graft (Medtronic Spinal and Biologics, Memphis, TN) is a FDA approved autograft replacement, available in kits with different sizes including sterile absorbable collagen sponge, rhBMP 2 powder and sterile water vials, for sinus augmentations and alveolar ridge augmentations. Our carriers were prepared in a similar fashion as described by the manufacturer. A certain amount of BMP 2 powder was reconstituted with sterile water and dropped on gelatin sponge. Later it was allowed to soak for 15 minutes and then applied to the defect site. Even though cross linking using chemicals such as gluteraldehyde or hexamethylene diisothiocyanate is a possible method to facilitate cell adhesion and proliferation, we avoided it. These agents tend to leak out from the carrier causing increased inflammation in terms of time and intensity. We also refrained from them as they could hinder osteoinductive capacity (263,264).

BMP released from a delivery material firstly induces cartilage tissue around the carrier. The developing cartilage tissue is periodically replaced by bone tissue and the carrier is absorbed by host tissues. The carrier acts solely to deliver BMP and has no potential to become the matrix for developing bone. Omura et al. demonstrated that dehydrothermally cross linked composite of fibrillar and denaturated atelocollagen (gelatin) sponge, as a carrier of rhBMP 2, formed compact bone without having major changes in shape and size during intramembranous ossification in rat subcutaneous pouch at the end of four weeks. In their study, twenty-four male Wistar rats with a mean body weight of 200 g received implants. A dry collagen sponge (5x5x3 mm) was soaked in 10 µl of buffer containing 10 µg rhBMP-2. As a control, similar collagen sponge was soaked in 10 µl of the same buffer without rhBMP-2. Under ether anesthesia, 24 rats received bilateral implants in subcutaneous pouches created by blunt dissection within the dorsal area. They were harvested four weeks after implantation and examined radiographically and histologically. They observed bone formation in rhBMP 2 group, however no bone or cartilage was observed in the control group. The size, shape and location of the forming bone were limited by the carrier and no inflammatory response was observed due to low antigenicity of collagen, cross linking without chemicals and presence of spongy gelatin (264). The cellular affinity of

gelatin is highest among collagen derivatives. This feature may have contributed to the vascularization of new connective tissue (265), and the lattice space of the spongy structure may be able to provide a scaffold for the adhesion, proliferation and differentiation of osteoprogenitor cells.

There is not a consensus about the optimum rhBMP 2 dosage during clinical practice. Pelaez et al. studied effect of rhBMP 2 dose on bone formation and maturation in a rat critical size calvarial defect model. They aimed to determine the most effective dose for rhBMP-2 soak loaded onto an absorbable collagen sponge carrier for bone formation using a defect model. One hundred eight male Sprague Dawley rats, age 11-13 weeks, randomized in 8 groups were studied to evaluate the bone formation process at 2, 4 and 8 weeks. rhBMP 2/ ACS were applied into 8mm critical size defects for a dose of 1.25, 2.5, 5.0, 10 and 20 μg along with sham surgery controls. According to the results, dosages ≥ 2.5 μg / defect showed a great defect closure within 2 weeks and complete resolution within 4 weeks. No adverse effects such as swelling and excessive bone formation were determined in the study. Control sites were completely closed within eight weeks. They concluded that rhBMP2/ ACS accelerated local bone formation in the rat critical size defect model once osteoinductive threshold is reached and stated that doses between 1.25 and 2.5 μg were enough to reach it. They claimed that higher doses did not create a significant difference in healing (261). In the present research, rhBMP 2 dosing was based on the latter hence the results suggested that 2.15 μg of rhBMP 2 could be accepted as osteoinductive when applied for two weeks, as in the present experiment. We also wanted to study on a lower dose in order to observe the possible synergistic effect of metformin, therefore, we wanted to reduce the risk of adverse effects, as aimed in combination therapies (266).

Zara et al. evaluated multiple BMP 2 doses in a rat femoral segmental defect model and in a minimally traumatic rat femoral onlay model to determine its dose dependent effects. Results of their study model showed a low BMP 2 concentration range (5 and 10 $\mu\text{g}/\text{ml}$, total dose 0.375 and 0.75 μg in 75 μg total volume) was unable to induce defect fusion. On the other hand, a mid range BMP 2 concentration range was able to fuse the defect without adverse effects (30 $\mu\text{g}/\text{ml}$, total dose 2.25 μg in 75 μg total volume) along with a high BMP 2 concentration range (150, 300 and 600 $\mu\text{g}/\text{ml}$, total dose 11.25, 22.5 and 45 μg in 75 μg total volume) , but with formation of cyst like bony shells filled with adipose tissue. Moreover, 4mg/ ml BMP 2 also caused tissue

inflammatory infiltrates and exudates in the defect model, consisting of increased numbers of osteoclast like cells at 3,7 and 14 days. At the end of their experiment, they stated that BMP 2 showed side effects like bone and soft tissue swelling following the use of high BMP 2 concentrations. (approaching the typical human 1500 µg/ml) (267). We prepared our experiment in respect to the doses given by them to avoid the side effects of BMP 2 and chose a relatively small yet effective dose. However increased bone formation was not observed in our study after applying 2.15 µg BMP 2 for 2 weeks.

Histopathological evaluation periods vary differently according to the literature. As stated in the studies, rhBMP2 soaked in collagen sponge showed a sudden release pattern in initial hours. However it was succeeded by a gradual loss of rhBMP2 from the collagenous implants over a 14 day period. Less than 5% of the initially implanted dose remained in the implants after 14 days (39,31) On the other hand Gelatamp is completely resorbed within 4 weeks and silver ions are continuously discharged during this period. It is easily applied to extraction sockets and bone defects. (189) De Giacomo et al. stated that in healthy rats, the periods of endochondral formation through cartilage resorption may last until 28 days, whereas the period of coupled remodeling starts around day 21 and may continue until 12 weeks (268). In this study, it was aimed to observe the effects of BMP 2 with the complete resorption of the carrier to inspect the effects of the protein and metformin in bone formation, leaving last the 14 days of the experiment without any application or administration.

Porto et al. suggested that it may be risky to study rat critical size defects of 5 mm after 4 weeks as there may be a natural bone formation to some extent and it may cause false conclusions about the efficiency of the materials (131). Vajgel et al. conducted a systematic review and they concluded that considering the percentage of bone formation, 5 mm defects can be accepted as CDSM (123). In light of these reviews, we chose to study on 5 mm defects in our trials during 4 weeks period.

Ruhe et al. studied in vivo release of rhBMP 2 loaded Ca-P ceramics and observed a retention of 20-30% after 28 days in rabbit ulna model, however Li et al. noticed a burst release of rhBMP 2 loaded α -BSM® of approximately 40% within 1 day in a rabbit ulnar defect (269,270). Uludağ et al. observed a burst release of more

than 70% within 3 h from different rhBMP 2 loaded Ca- P ceramics applied on in rats. This demonstrates that growth factor release kinetics are complicated and may be affected by different factors. The rate of rhBMP-2 loss from a gelatin sponge (Spongelt) and DBMs (both rat and human derived) was similar to that from the collagen sponges. The chemical nature of the chosen collagen-based carriers, ACS (chemically crosslinked), DHTS (dehydrothermally crosslinked), Spongelt (gelatin, heat sterilized and crosslinked), and DBM (intrinsically crosslinked), was vastly different. Eventually, the nature of interaction with rhBMP-2 was estimated to be different. Additionally, foreign-body cellular reaction to a carrier, pore size and geometry, mechanical strength, and biodegradation rate were all proposed as important carrier material properties (39). Ruhe et al. concluded that the life cycle of rhBMP 2 was no longer than 4 weeks and it was unnecessary to estimate an additional bioactivity of proteins even if they were retained for a longer time (269). In a coherent manner, it was estimated that our carrier would release the most of its content in 2 weeks, hence the study was ended in 4 weeks.

Luca et al. evaluated and compared the efficiency of two different injectable carriers for rh BMP 2 in the rat ectopic bone formation model. They compared two biopolymers with similar chemical structures but opposite charges. Increased bone formation was observed at low pH medium (4.8 ± 0.2) as compared to high pH medium (6.2 ± 0.2) for both rhBMP 2 loaded hyrdogels (271). They related the decrease of rhBMP 2 bioactivity at high pH medium to the aggregation and conformational changes of protein (272). In contrast to hemostatic agents such as oxidized cellulose, the pH of gelatin sponges is neutral (189). The low pH level may also cause inflammation and delayed healing (273). Therefore pH levels should be considered when preparing an optimal BMP 2 carrier in order to reduce the effective dose of the protein.

Many studies contain collagen sponge as BMP 2 carrier. According to the studies their interaction is based on electrostatic attraction. Moreover collagen is naturally found in the bone and it has a tendency to act as a substrate for bone regeneration. Gelatin is promising as it has been used as disks for delivery of growth factors including BMP 2. The controlled release of growth factors from gelatin is due to enzymatic degradation of gelatin. It is estimated that matrix proteinases such as collagenase and the extent of cross linking may affect its degradation (274).

Patel et al. studied on gelatin microparticles and biodegradable composite scaffolds to observe the controlled release of rhBMP 2, investigating the effects of gelatin cross linking, growth factor dose, release medium and gelatin type on BMP 2 release kinetics. They pointed out the efficacy of gelatin microparticles for having different release profiles of BMP 2 over 4 weeks. They concluded that BMP 2 discharge from PLGA microparticles resulted in a moderate burst release followed by minimal cumulative release, whereas BMP 2 discharge from gelatin exhibited minimal burst release followed by linear release kinetics. They claimed that the amount of BMP 2 dose within the gelatin achieved an equilibrium state independent of the amount of dose (274). These results suggest that some amount of early release combined with sustained delivery may enhance growth factor efficacy. As stated in the literature, rhBMP2 soaked in collagen sponge showed a sudden release pattern in initial hours preceded by a sustained release profile, which is desirable according to Bouxsein et al. (39,31,185). Even though our carrier was not radiolabeled to test the release pattern, it was aimed to achieve a similar outcome as gelatin sponges were soaked loaded with rhBMP 2.

Mumcuoğlu et al. studied injectable BMP 2 delivery system based on collagen derived microspheres in order to reduce the clinically used supra physiological dose of BMP 2 (usually 1.5mg/ml) which carries the risk of adverse effects. BMP 2 doses of 10 µg, 3 µg, 1 µg per implant (50µg/ml, 15 µg/ml and 5 µg/ml) resulted in ectopic bone formation in rats in a time and dose dependent manner as demonstrated in histological studies. Imaging of fluorescently labelled BMP 2 showed 4 weeks of retention. In the critical calvarial defect model of 5 mm, micro CT results demonstrated a higher bone volume for the 2 µg of BMP 2 per implant condition (50 µg/ml) as compared to a lower dose (0.2 µg per implant, 5 µg/ ml). Complete defect bridging was obtained with 50 µg/ml of BMP 2 after 8 weeks. The lowest dose was not efficient to heal a calvarial defect faster than the empty defect or biomaterial control group (275).

The dose of BMP 2 is an important factor in carrier material decision. When 5 µg of BMP 2 is used with 9 mm³ of β tricalcium phosphate carrier, 32% bone formation is obtained. On the other hand 5 µg of rhBMP 2 may cause 3% bone formation in a rat ectopic model. (276) According to Kim et al., 5 µg of rhBMP 2 is necessary to achieve bone formation in mice with a maximum volume of 5 mm³, using a collagen sponge of 6 mm diameter. (277) Bessa et al. noted that 5 µg of BMP 2 with 10 mg of silk fibrin

particles resulted in the formation of 2 mm³ bone at the end of 4 weeks in rats (278). Martinez-Sans et al. stated that BMP 2 doses lower than 1 µg, carried on a collagen sponge, are not enough to induce bone formation in rats (279). At the end of 4 week period, the maximum amount of bone we achieved is 1.19 mm³ and the minimum is 0.42 mm³, following the implantation of 2.15 µg of rhBMP 2 with a gelatin sponge carrier on a 5 mm defect.

De Queiroz Fernandes et al. noted high rates of success in bone regeneration prior to implant placement with rhBMP 2 use. They compared it to autogenous bone graft and suggested the advantage of not needing a second surgical site to obtain it along with the advantage of osteoinductive capacity. It brings along the risk of edema and erythema due to high chemotactic potential to inflammatory cells (174).

Even though review studies claim that edema does not pose a great threat, there are reports of airway obstruction in cervical spinal fusion surgery. Other side effects include ectopic calcification, excessive bone formation and increased incidence of cancer. On the other hand there are not many studies demonstrating side effects and complications of rhBMP 2 in dentistry (174). We did not encounter any side effects in our study groups during 4 weeks period and there was no sign of adverse effects in histological examinations.

As silver is known to be a strong antibacterial agent, clinically, gelatin sponge with silver is used to reduce complications due to extractions. Dong et al. studied on the effect of gelatin sponge with colloid silver on bone healing in infected cranial defects. Methicillin resistant *Staphylococcus aureus* infected 3 mm diameter cranial defects were created in adult female Sprague Dawley rats. One week after infection, the defects were debrided of all tissue and then implanted with gelatin sponge with colloidal silver (Gelatang) or gelatin alone. While 4 weeks after debridement, the defects of gelatin group were unfilled, those of the gelatin with colloid silver were almost closed, in coherence with the histological results. Their study has demonstrated that gelatin with colloid silver application could efficiently reduce the infection caused by the bacteria and enhance infected bone healing process (280). In terms of reduced infection, their results are in coherence with ours, therefore we did not evaluate any sign of infection during 4 week period. On the other hand, no increased bone formation was observed in

Gelatamp group compared to the control group. This may be due to the difference in healing between subcritical size and critical size defects, as gelatin sponge in larger defects may not be as efficient as it is in smaller defects in terms of enhanced healing.

Sun et al. prepared a collagen scaffold composite encapsulating silver nanoparticles and BMP 2. They aimed to observe its effects in bone healing. They concluded that they had strong antibacterial properties and augmented the differentiation of bone marrow stem cells towards osteoblasts (281). Nevertheless, these studies are *in vitro* and it necessary to evaluate *in vivo* test to investigate the antibacterial activity and biocompatibility of silver ions.

It is believed that silver-based antimicrobials attach to specific thiol groups containing sulfur and hydrogen found in a variety of bacterial proteins; however, they may differ in the reservoir form for the active silver. Studies have demonstrated that 5–50 nm size nanosilver particles are bactericidal (282). The nanosilver particles used are commonly synthesized through chemical reduction of a silver salt solution by a reducing agent, leading to residual chemical impurities and wide distribution of particle sizes that can disrupt the antibacterial effect of nanosilver particles (283,284).

Zheng et al. aimed to create BMP 2 coupled nanosilver (NS) poly-lactic-co-glycolic acid (PLGA) composite bone grafts in order to treat infected bone segmental defects. Antibacterial activity against bacteria and cytotoxicity of nanosilver coupled BMP2/NS/PLGA grafts were studied *in vitro* and *in vivo*. In the creation of a BMP-2/NS/PLGA composite graft, the effect of nanosilver particle on BMP-2 was also considered, as nanosilver particles could possibly interfere with essential cellular elements relating to BMP-2 osteoinductivity when they bind to thiol groups. They claimed that up to 2.0% concentration of nanosilver particles of 20-40 nm did not interrupt bone regeneration induced by BMP-2 either *in vitro* or *in vivo* as shown in their study. They demonstrated that BMP-2/NS/PLGA composite grafts portrayed significant antibacterial and osteoinductive activity in 6 mm critical size defect model (285). 10 g of Gelatamp contains 9,5 g of gelatin and 0,5 g of colloidal silver, in other words 5% concentration of colloidal silver. We believe that further studies are necessary to evaluate the relationship between colloidal silver and BMP 2, it was not observed an increased bone formation in BMP 2 + Gelatamp group compared to the

control group in our study. We assume that increased amounts of colloidal silver may hinder the osteogenic properties of BMP 2.

Kanazawa et al. studied the effects of metformin on the differentiation and mineralization of osteoblastic MC3T3-E1 cells along with intracellular transduction. Metformin was added after the cells were gathered on day 5 and total RNA was collected on days 7,14 and 21. They observed an increase in the expression of BMP 2 levels in mRNA in a time dependent manner until day 14 by real time PCR. They concluded that metformin could promote the differentiation and and mineralization of osteoblasts through activation of the AMPK signaling pathway and increase in eNOS and BMP 2 production (177). This study was an inspiration in our research in order to examine the osteogenic effects of metformin in a more practical scale.

Gao et al. investigated the action of metformin on bone mass in ovariectomized rats. A sham surgery or bilateral ovariectomy was performed on thirty two female Sprague Dawley rats, weighting 190-210 g. First ovariectomy group did not receive any metformin treatment but the second group had a diet of metformin 50 mg/kg/day and the third group had a diet of 100 mg/kg/day with orogastric intubation. They were sacrificed after 8 weeks and the tibiae were collected for dual energy X ray absorptiometry, micro CT and histology analysis, while the bone marrow cells were collected for measurement of the mRNAs expressions for three osteoblast cells using real time RT-PCR. They observed improved bone density and quality in metformin treated groups. The data obtained from real time PCR test indicated that metformin could prevent bone loss in overactomized rats to a certain degree, increasing bone formation through certain osteoblast genes (240).

Sedlinsky et al. investigated the in vivo effects of orally administered metformin and rosiglitazone, an insulin sensitizer drug associated with reduced bone mass and increased fracture risk, along with their co treatment and combined therapy on rat bone reossification, on the micro architecture and histomorphometry of femoral metaphysis. 40 Sprague Dawley rats weighting 190-210 g were divided into four groups. Control group received water ad libidum. Metformin treated rats received 100 mg/kg/day of metformin in drinking water for two weeks. Rosiglitazone treated rats received 4 mg/kg/day of rosiglitazone in drinking water for 2 weeks and metformin – rosiglitazone

treated rats received a combination of both. Following their sacrifice, bone resossification was calculated as the ratio between the newly reossified area and the average bone thickness in parietal defects of 1 mm. Control group values were 100 ± 8 . Metformin group values were 207 ± 15 , Rosiglitazone group values were 22 ± 3 and they were statistically significant versus control. Metformin + rosiglitazone group values were 93 ± 9 and they were statistically significantly versus rosiglitazone group. They concluded that treatment with rosiglitazone decreased bone regeneration, femoral metaphysis trabecular area, osteoblastic and osteocytic density, whereas metformin increased all these parameters versus control, inducing osteogenesis (286). In the present research, pairwise comparisons of mean new bone percentage of control and metformin group were not statistically significant ($p > 0.05$) even though metformin doses of our study were higher during 4 weeks. The size of the defect may be a factor in healing as our defect was larger (5mm) compared to the latter (1mm).

Similarly, Molinuevo et al. evaluated the effect of in vivo metformin treatment on bone regeneration in a model of parietal lesions in non diabetic and streptozotocin induced diabetic rats. Circular craniotomy defects of 1.0 mm were created on parietal bone of 40 adult male Sprague Dawley rats, weighting 190 – 210 g. They were divided into 4 groups. Control diabetic rats and diabetic rats received water ad libidum, whereas metformin treated diabetic rats and metformin treated non diabetic rats received 100 mg/kg per day of metformin in drinking water for 2 weeks. After 15 days of metformin treatment, rats were processed for histologic and quantative histomorphometric analysis. Reossification was calculated as the ratio between either the newly reossified area or thickness and the average bone thickness. During repair of the intramembranous bone, the site underwent various stages such as inflammation period followed by the formation of granulatory and fibrous tissue and osteoclastic resorption of bone and bridging of the defect by a hard callus of intramembranous bone. However they were unable to observe remodeling of the primary callus to cancellous bone due to short period of study time. In their model of bone healing, they suggested that metformin could simultaneously increase reossification and osteoclastic activity in the lesion site, indicating a coordinated increase in bone formation and remodeling (18). Surprisingly, they had observed an increase in the TRAP activity, a marker of osteoclastic activity, of non diabetic rats after 15 days of treatment with metformin.

Śmieszek et al. evaluated the pro osteogenic function of BMP 2 in differentiation of adipose derived stromal cells derived from rat adipose tissue. Additionally, they considered the effect of metformin treatment in bone formation in a cranial defect in four month old non diabetic male Wistar rats. (290 ± 30 g of body weight). Circular craniotomy defects of 1.0 mm were created in half of the rats. They were divided into following subgroups: control group (n=6), metformin treated group receiving 100 mg/kg/day (n=6) and metformin treated group receiving 250 mg/kg/day (n=6). The experiment was carried out for 4 weeks. Samples were collected for histological and CT analysis. During in vitro part of the study, they analysed secretory activity of rASC at 7th and 14th day and observed an increase in ALP activity and osteocalcin production. Metformin did not affect the secretion of BMP-2 in non-osteogenic cultures, but in osteogenic environment metformin at concentrations of 10, 50 and 100 μ M elevated secretion of BMP-2 in culture supernatants, while the highest concentrations of the drug decreased the BMP-2 concentration. As for the in vivo study, Metformin treatment in the group without a cranial defect increased BMP 2 concentration, whereas they were not statistically significant. Circulating BMP-2 level was decreased in rats with a cranial defect in relation to the group without a defect. In the cranial defect group a statistically significant elevation of serum BMP-2 level was noticed after supplementation with 250 mg of metformin. The body weight of animals receiving metformin, both in the non-cranial as well as in the cranial defect group, was reduced. Newly formed bone area/ total defect (%) and newly formed bone area (mm²) and TRAP area/ reossification area were evaluated for cranial defect groups. They observed significantly enhanced bone formation in animals receiving metformin at a dose of 250 mg/kg/day, both in cranial defect and non cranial defect group. The defect in the control groups were mainly filled by fibrosis tissue. They also noted that number of osteoclast precursors (TRAP positive cells) in the reossified surface showed that metformin reduces osteoclast activity. They noticed a significant decrease in both groups receiving metformin. They concluded that application of metformin in a dose of 500 μ M increases the osteogenic differentiation potential of rASCs in vitro. They also remarked that administration of metformin in a dose of 250 mg/kg/day promotes trabecular bone formation in a rat bilateral cranial defect model. Additionally they stated metformin caused body weight loss in animals, both in the cranial defect and non cranial defect group (24). The results do not match up with the results of our metformin group even though the doses were similar. The difference may be due to the race of the rodents, size

of the defect site, number of samples and the duration of the drug administration. Also, we observed increased body weight in all groups in our study.

As mentioned before, some researchers have contradicting opinions about the osteogenic effects of metformin. Kasai et al. studied on the role of AMPK in osteoblast differentiation using in vitro differentiation models. They demonstrated that osteoblast differentiation was remarkably inhibited by metformin. They claimed that Metformin stimulation inhibited gene expression of Runx2 along with osteoblast differentiation markers. They presumed that the different results may be due to differences of the metformin concentration during the experiments and added that long term treatment of AMPK were not observed in the reports of Kazanawa et al. (287). Similarly, Wu et al. also suggested that metformin at 1mM had no effect on rabbit bone marrow stem cells and added that it slightly compromised the osteogenic induction of β -glycerol phosphate and ascorbic 2-phosphate in their in vitro study (288). Salai et al. reported that metformin could induce osteoblastic proliferation, however it inhibited mineralisation of sarcoma osteogenic cell line, stating that in vitro drug administration studies may not be able to accurately demonstrate in vivo results (289).

Jeyabalan et al. aimed to access the osteogenic effect of metformin in vivo. They used three month old female Wistar rats (approximately 200 g) to investigate the effect of metformin on basal bone mass and 3 month old mice to investigate whether metformin could protect against the bone loss induced by ovariectomy to imitate the case of menopausal women. Mice received metformin by gavage 100mg/kg/day for 4 weeks and rats received it in drinking water at a concentration of 2mg/ml for 8 weeks. (water consumption in rats is estimated to be 10-12 ml per 100 g body weight daily.) At the end of the experiment, mice tibia were dissected for micro CT analysis, trabecular bone parameters and bone histomorphometry. Following the analysis of micro CT, they concluded that metformin did not show remarkable changes in both the trabecular and cortical compartments compared to control mice. Moreover metformin had no effect on trabecular bone volume (BV/TV), trabecular number and thickness compared to saline, along with the cortical thickness and periosteal and endosteal parameters. Following the histomorphometric analysis, they stated that metformin decreased the mineralisation surfaces and mineral apposition rate compared to control mice and remarkably reduced the bone formation rate. They added that treatment with metformin AMPK had no

effect on bone. The second experiment was planned on twenty female rats in order to investigate the effects of metformin on fracture healing. Half of the rats received metformin in drinking water (2mg/ml) for 8 weeks, while the other half had access to drinking water alone. Four weeks later, a mid diaphyseal osteotomy with a gap of 0.5 mm was performed on left femur of all the rats. Four weeks after the procedure, the rats were euthanised and radiographs of the operation site were taken and osteotomised bones were collected for micro CT analysis and histology. Mineralising surfaces were evaluated per bone surfaces (MS/BS/%) and the bone formation rate was calculated as MS/BSxMAR. Additionally, sections were stained for TRAP. Metformin did not significantly affect rat body weight at the end of the experiment. No significant effect on bone trabecular and cortical parameters were observed following micro CT analysis. Trabecular separation was decreased by metformin but it was not significant. Radiographically, they stated that not all fractures were united after 4 weeks. They suggested that metformin treatment did not affect fracture callus size or speed of healing. They concluded that metformin did not affect the endochondral ossification and fracture healing, 4 weeks after osteotomy (25). The results are in par with ours, however we preferred to use oral gavage method in our experiment, as a crucial concern in dosing with drinking water is the accurate quantification of oral delivery (290).

Bastos et al. aimed to evaluate the effects of metformin on bone healing around titanium implants. A screw shaped titanium implant of 4 mm and 2.2 mm in diameter was placed in tibia of four months old twenty male Wistar rats (weighting 310 ± 20 g). They were divided in two groups: a control group (n=10) rats without metformin treatment and metformin group (n=10) in which they were treated with 40mg/kg/day metformin by gavage. Thirty days after placement, the samples were collected for histometric and immunohistochemical analyses. They stated that metformin reduced the percentages of bone to implant connection and bone area and increased the expression of RANKL around titanium implants in non diabetic rats, hindering osseointegration (260).

Hegazy evaluated the anti osteoporotic effect of metformin in forty post menopausal diabetic women. Half of them received metformin (Glucophage® 500 mg) 1 tablet twice daily for 12 weeks. At the beginning and at the end of the experiment bone mineral density of anterior posterior bone lumbar spine was measured by dual

energy X ray absorptiometry. Additionally fasting blood and urine samples were collected in order to measure ALP, osteocalcin and deoxypridinoline once in every four weeks until the end of the study. Their results showed that the mean values for all bone turnover markers were not significantly different from those on the first day of the study. They concluded that metformin was neither osteogenic nor had an anti osteoporotic effect (291).

Even though literature mainly comprises in vivo studies including systemic metformin treatment, there are several studies which consist of local application. We preferred to conduct our experiment planning a systemic metformin treatment in order to correlate the results of the previous studies with ours, as they were higher in number compared to the studies with local treatment.

The present research has some drawbacks, such as the administration of a single amount of metformin and the observation during a single period of time. Still, we conducted our research based on similar studies in respect to the possible efficient dosage and duration. Besides it was aimed to observe new bone formation following osteoblastic differentiation, where adequate bone formation could occur after BMP 2 expression. Also, we wanted to eliminate the risk of spontaneous healing which could occur during extended period of time considering the defect size, as it could compromise our results. Additionally we preferred to evaluate our results in the light of histological and histomorphometric analysis and did not resort to micro CT scanning or radiological imaging. Even though histology is regarded as the gold standard, we are aware that it has some cons such as limitation to two dimensional information and overestimation of the measurements and risk of damage to the interface during cutting and grinding. On the other hand, even though micro CT analysis may contain more informations about bone structures in a three dimensional medium, it can not deliver information about cells and pathologic conditions, in addition to the risk of artefacts (292). The combination of both methods could give more insight into the results, yet ours was indicative.

In conclusion, we believe that metformin does not promote osteogenesis as effective as it was stated in the other studies. Interestingly, we observed the highest amount of new bone formation in our combined therapy group even if it was not

significantly different. It leads us to believe that a relationship between rhBMP 2 and metformin can not be ignored as indicated in our results and a combined therapy could be possible under different conditions. Further studies should be performed to evaluate possible effective dose and successful application.



6. CONCLUSION

1. No signs of chronic inflammation or necrosis were observed in our study. These results indicate the safety of the procedures and biocompatibility of the materials. Colloidal silver ions in the carrier may have antibacterial effects.
2. Metformin does not decrease body weight of non diabetic rats at the dose given. We stopped metformin administration for 2 weeks after daily oral gavage treatment which lasted for 2 weeks. This interval may play a role in weight gain, however the difference between the first and the last days were significant.
2. Metformin does not increase new bone formation in oral doses of 255mg/kg when administered for 2 weeks.
3. Low doses of BMP2 (2.15 μ g) are not enough to induce increased bone formation when applied with Gelatamp carriers. Higher doses of BMP2 may be more effective in bone formation.
4. Gelatamp causes increased fibrosis in all groups in 28 day healing period with the exception of combined therapy group (G + BMP + M group), where BMP2 is locally applied with the latter and metformin is orally administered for 14 days. We believe that different time periods with higher doses and different defect sizes should be studied to observe the amount of new bone formation.
5. Release pattern of gelatin carriers should be reevaluated to assess the optimum amount of the material delivered. The results should be compared to those of other sponge carriers to observe the possibility of differences due to preparation. Gelatamp may not be an ideal scaffold for delivery of certain materials due to colloidal silver content.
6. We may assume that there is a relationship between BMP2 and metformin, however BMP2 and metformin doses should be reevaluated to determine the ideal amounts. Further animal and clinical studies are needed to use metformin with BMP2 in bone healing and augmentation procedures.

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8. APPENDIX

8.1. Ethics Committee Approval Form



T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2020-824	18.02.2020	2020/02	2020/02-6	Prof. Dr. Ahmet Hamdi Arslan
'Kritik Boyutlu Defekt Modellerinde Kemik Morfojenik Protein 2 (BMP2) ve Metforminin Kemik Oluşumuna Etkilerinin Histolojik ve Histomorfometrik Olarak Değerlendirilmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü / Irkı		Toplam Hayvan Sayısı		Hayvanın Cinsiyeti
Sıçan / Sprague Dawley		42		Erkek

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	

BIOGRAPHY

Personal Information

Name	Orkun	Surname	UYGUN
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Educational Status

Degree	Field	Name of Institution of Graduation	Year of Graduation
Doctoral	Oral and Maxillofacial Surgery	Yeditepe University	2021
Graduate	Faculty of Dentistry	Yeditepe University	2013
High School	Science	Saint Joseph French High School, İstanbul	2007

Foreign Languages	Foreign Language Proficiency Exam Scores
English	YDS 87,5
French	DEL F B2

Publications

1. Arslan AH, Uygun O. Removal of a dental bur displaced into maxillary sinus: a case report. <i>Yeditepe J Dent.</i> 2020; 16(3): 250-253
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