DETERMINATION OF OSSEOINTEGRATION PROPERTIES OF MAGNESIUM DOPED TiN COATINGS *IN VIVO*

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Dedicated to my precious family who supported me throughout my life…

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ABSTRACT

DETERMINATION OF OSSEOINTEGRATION PROPERTIES OF MAGNESIUM DOPED TiN COATINGS *IN* ViVO

Bone implantations have been a mandatory investigative subject in cases with traumatic bone defects within the recent years. A successful implantation relies on well osseointegration and implant interface strength. Titanium implants with its alloys have been a decisive area to challenge other metallic implants for gaining an improved osseointegration. In this study, the aim is to provide a better osseointegration capabilities through the addition of the magnesium doped titanium nitride coatings onto Ti6Al4V based plates and screws. *In vivo* experiments were held on the femur of 16 male rabbits. A fracture was made on the femur of the animals, and then TiN and (Ti, Mg) N thin film coated plates were implanted for six weeks to observe the regeneration differences between the two implant groups. After six weeks of implantation, bone samples were dissected and evaluated with Micro CT-Scan analysis, X-ray imaging, histological analysis, scanning electron microscopy (SEM), and von Kossa staining, in order to examine the *de novo* bone formation around fracture site and bone responses to the implant's surfaces. Micro CT-images and histological analysis results indicated that both TiN and (Ti, Mg) N coated plates enhanced callus formation around the fracture line. Thus, (Ti, Mg) N implants led not only to bone formation but also complete regeneration of the bone. Moreover, SEM and von Kossa stain evaluations showed that Mg coated surfaces influenced intensive osteoblast formation and mineralization of bones. EDS analysis revealed the elemental characterization of the plates and it was observed that hydroxyapatite was formed only on (Ti, Mg) N coated plates. This ensures that the addition of Mg significantly increased the osseointegration rates with accelerated healing process. In conclusion, higher remodeling process was obtained from the addition of magnesium into the coatings, which clarifies that (Ti, Mg) N thin film coated implants can be used in further clinical applications as a hard tissue implant for bone.

ÖZET

MAGNEZYUM KATKILI TiN KAPLAMALARIN *IN VİVO* KOŞULLARDA OSTEOENTEGRASYON ÖZELLİKLERİNİN BELİRLENMESİ

Kemik implantanları son yıllarda travmatik kemik hasarları için oldukça önemli bir araştırma konusu olmuştur. Başarılı bir implantasyon, iyi osseointegrasyon ve kemik-implant ara yüz bağlanma gücüne bağlıdır. Titanyum ve alaşımları ile geliştirilmiş implantlar, diğer metal implantlara göre daha iyi bir osseointegrasyon sağlayarak önemli bir çalışma alanı oluşturmuştur. Bu araştırmadaki amaç, titaniyum nitrit plakalarını ve vidalarını magnezyum ile kaplayarak, bunların osseointegrasyon kapasitesini arttırmaktır. *In vivo* deneyler, 16 erkek tavşanın uyluk kemiği üzerinde yapılmıştır. Hayvanların uyluk kemiğinde bir kırık meydana getirilmiş ve sonrasında TiN ve (Ti, Mg) N ince film kaplı plakaları kemiğe implant yapılmıştır. Altı haftalık gözlem sonucunda, iki implant grubu arasındaki rejenerason farklılıkları gözlenmiştir. İmplantasyondan altı hafta sonra, kemik örnekleri dissekte edilerek; mikro tomografi analizi, X-ışını ile görüntüleme, histolojik analiz, taramalı elektron mikroskobu (SEM) ve von Kossa boyama tekniği ile kemiğin *de novo* oluşumu ve implant yüzeyindeki kemik cevapları incelenmiştir. Mikro CT-görüntüleri ve histolojik analiz sonuçları, TiN ve (Ti, Mg) N kaplı plakalarının kallus oluşumunu kırık hattı çevresinde tetiklediğini göstermiştir. Bu sonuçlar çerçevesinde, (Ti, Mg) N kaplı implantlar sadece kemik oluşumu değil aynı zamanda kemik rejenerasyonunu da sağlamıştır. Ayrıca, SEM ve von Kossa boyama değerlendirmeleri, Mg kaplı yüzeylerin yoğun osteoblast oluşumunu ve kemik mineralizasyonunu arttırdığını göstermiştir. EDS analizi plakların elemental tanımlamasını ortaya çıkarmış ve hıdroksıapatıtın yalnızca (Ti, Mg) N kaplı plakalarda oluştuğu gözlemlenmiştir. Bu sonuçlar, Mg ilaveli yüzeylerin osseointegrasyonu ve iyileşme sürecini hızlandırdığını göstermiştir. Sonuç olarak, daha iyi bir kemik rejenerasyonu, kaplamalara magnezyum eklenmesi ile sağlanmıştır ve bu da (Ti, Mg) N kaplı implantlarının ileride klinik uygulamalarda sert doku kemik implantasyonlarında kullanılabileceğini göstermiştir.

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arc- PVD	Cathodic deposition -Physical Vapor Deposition
BMD	Bone mineral density
CMD	Callus mineral density
EDS	Energy dispersive X-ray spectroscopy
ECM	Extra-cellular matrix
FTIR	Fourier-transform infrared spectroscopy
HA	Hydroxypatite
Mg	Magnesium
MSCs	Mesenchymal stem cells
PBS	Phosphate- buffered saline
RBCs	Red blood cells
RBM	Red bone marrow
ROI	Region of interest
SEM	Scanning electron microscopy
Ti-6Al-4V	Titatnium- 6 Aluminum - 4 Vanadium
Ti-6Al-7nb	Titanium- 6 Aluminum - 7 niobium
(Ti, Mg) N	(Titanium, magnesium) Nitride
TiN	Titanium nitride
TiO_2	Titanium dioxide
TMD	Tissue mineral density
UV	Ultraviolet radiation
XRD	X-ray diffraction

1. INTRODUCTION

The incidence of bone defects have been one of the major concerns around the world. Bone implantations have always been a source that contributes to better healing process when dealing with fractured bones. Challenges between different metallic implant materials have been observed during the recent years. Titanium (Ti) and its alloys, are considered as the main metals that provide improved regeneration of fractured bones. Basically, Ti is known with the features of being highly biocompatible, high rates of corrosion resistance, low toxicity levels, and well chemical stability [1]. However, one of the main drawbacks of Ti is that bone to implant fixation requires longer time during healing and regeneration process of fractured bones [2]. Thus, osseointegration rates are always the main concern to provide better regeneration capabilities between implant's surfaces and bones. Osseointegration is quite affected by the surface modification of implants, playing a major role in the primary events of bone healing process [3]. Osteoblast differentiation and bone matrix development are the two sources that act to form the interface between implant surface and host bone [4]. Better quality of osseointegration can be provided with the addition of elements and factors to the TiN plate's surfaces. This study focuses on upgrading the level of osseointegration with the use of magnesium- coated titanium nitride implants. To achieve this aim, Mg-coated TiN plates were prepared with arc-PVD technique and implanted on rabbit models. Later, these plates were examined with several analysis to test their functionality during healing process.

1.1 BONE

Bones are hard structures made of mineralized connective tissue that includes four type of cells: osteoblasts, osteocytes, bone lining cells and osteoclasts [5]. They also consist of osteoid which is non-mineral matrix of collagen and also inorganic mineral salts contained within the matrix. Bones are known for its ability to support the structure of the body and protect its vital organs, enable production of blood cells from its bone marrow, and act as mineral storage spot. Furthermore, bones consist of articulate cartilage, epiphyseal plate, bone marrow, periosteum and endosteum [6]. Articular cartilage is a specialized type of hyaline cartilage that provides mechanical support to bones. Epiphyseal plate is made of

cartilage which is responsible for bone growth and producing new bone cells especially in children. Mostly, these epiphyseal plates are available at the end of long bones. Bone marrow is usually present in cancellous bones [6, 7]. Red bone marrow is responsible for producing red blood cells. In addition, it aids to produce white blood cells and platelets. After aging, about half of this red bone marrow (RBM) is converted to yellow bone marrow. As RBM becomes concentrated only at the end of long bones and inside the flat bones. Yellow bone marrow is a fatty tissue that produces fat, cartilage, and bone through its stem cells. It acts to store fat in order to maintain healthy environment surrounding bones. Periosteum is made of connective tissue that is rich in collagen and it is the site of new bone formation by osteoblasts [6]. Periosteum acts as an outer membrane that protects bones by resisting trauma. It also contains nerve endings that help us sense the pain. Endosteum acts as an inner membrane that covers the bone's medullary cavities (Figure 1.1). It is also the site where bone reabsorption occurs through cells called osteoclasts.

1.1.1. Bone Tissues

Mainly, bones are composed of two types of tissues: compact bone and spongy bone (Figure 1.1). Compact bone is the hard outer layer which consume about 80 percent of an adult bone mass and called as cortical bone [6]. Compact bone consists of the osseous tissue that is made up of osteocytes. A solid matrix surrounds the bone cells of osseous tissue is composed of vital minerals, calcium, phosphorus and proteins. Hydroxyapatite (HA) is made out of calcium phosphate and is considered as one of the most important minerals in bones. It is known for its hard, solid and flexible composition that is surrounded with collagen fibers in a compact bone tissue. The basic structural units of cortical bone is called as osteons or Haversian system [8]. Haversian system consists of haversian canals that contains blood vessels and nerves, lamellae rings that surround haversian canals with a strong matrix formed from the mineral salts. Between each lamellae layer, small spaces are made of lacunae that includes osteocytes. These lacunae are connected to each other by canaliculi which basically provides the nutrition to osteocytes and gets rid of waste products. Spongy or cancellous bone, consists of trabecullae bone which includes lamellae, osteocytes, lacunae, and canaliculi [6, 7]. It acts as an important site for the exchange of calcium ions with blood and also a site for RBCs production.

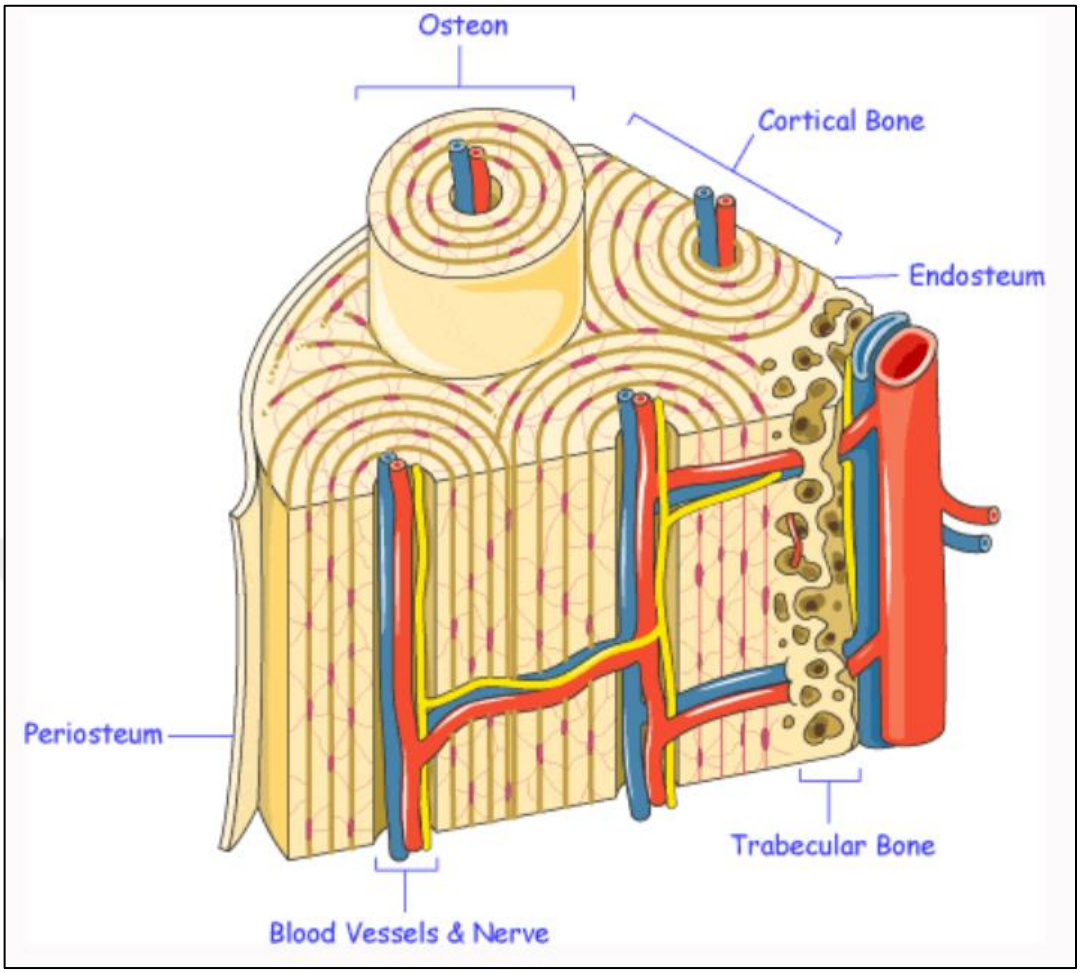


Figure 1.1. Bone structure [5].

1.1.1.1. Bone Cell Types

Four types of cell are responsible for bone maintenance, remodeling and production. Osteoblasts are considered as mononucleate cells derived from mesenchymal stem cells (MSCs) that aid to synthesize and secrete collagen matrix and calcium salts [9]. Also they produce a protein called osteoid which acts to mineralize bone. Old osteoblasts are classified as bone lining cells, and act to manage the calcium release into and out of the bone. They also activate osteoclasts by special proteins and hormones. Osteocytes are counted as inactive osteoblasts that are placed in the calcified bone matrix layer. They basically maintain the connection with other osteocytes and osteoblasts. In addition, they act to sense the changes in physical forces like cracks and transduce bone lining cells that activate the resorption by osteoclasts or formation responses by osteoblasts (Figure 1.2). Osteoclasts are

the multinucleated cells that act to digest unwanted bones by the release of specific enzymes and acids. This resorption aid in remodeling of the fractured bone [9, 12].

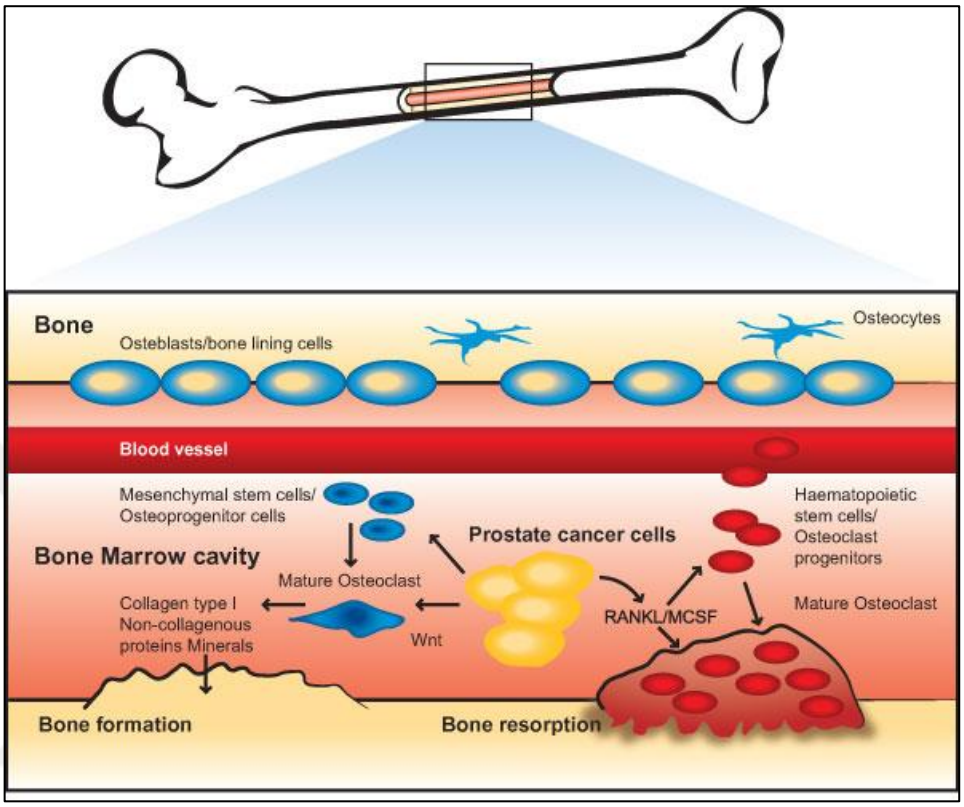


Figure 1.2. Types of bone cells and their function during resorption and formation of bone [11].

1.1.2. Bone Growth and Development

Bone development occurs when collagenous tissue is replaced with the bone tissue. This event enhances the formation of a primary immature weak bone called woven bone [10]. Woven bone is formed when osteoblasts produce osteoid. It is later remodeled and converted to a secondary mature form called as lamellar bone.

Osteogenesis or ossification is the bone tissue formation by osteoblasts [11]. It has two important processes: intramembranous ossification and endochondral ossification. Intramembranous ossification is the process where flat bones are formed, and connective tissue is replaced with bone tissue. In the meantime, endochondral ossification is involved in the formation of femur, humerus, tibia and radius bones by replacing hyaline cartilage

tissue with the mineralized bone tissue. Long bones keep on growing longitudinally in the epiphyseal plate until chondrocytes cease their proliferation [10, 11]. However, bone can grow in width in the diaphysis part of bone even after longitudinal growth is over, this process is called as oppositional growth. Increasing bone diameter happens by the reabsorption of old bone lining in the medullar cavity enhancing new bone growth underneath the periosteum layer.

1.1.3. Modeling and Remodeling of Bone

Bone modeling is needed for the increase of bone mass and shaping of the body bones [12]. It is of two forms: formation modeling by osteoblasts and resorptive modeling by osteoclasts in two different surfaces. Modeling of bones plays a vital role during longitudinal growth of long bones in endochondral ossification [12, 13]. Resorption modeling acts to remove bones present on periosteal surfaces. On the other hand, new bones are added to the endocortical surfaces by the action of formation modeling. Remodeling of bone also includes osteoblast for formation and osteoclast for resorption, but its main role is to provide an internal turnover of mineralized tissue without the need of an overall form change by substituting old bone with the new one [13]. New secondary osteon formation is provided by osteoclasts that act to open resorption cavities and osteoblasts aid to fill it with the new bones at the same surface. The main difference between modeling and remodeling is that modeling mainly occurs during maturation, while remodeling is a lifetime event [12, 14].

1.1.4. Types of Bone

Bone mainly consists of five different structures. Long bones including femur, ulna and humerus, consist of epiphysis and diaphysis that are connected through epiphyseal line where new bone forms during growth [15]. Their function is to support weight and act to facilitate movement. Short bones mainly provide stability and mobility. They include carpals and tarsals that are located in the wrist. Another type of bone is called flat bone that includes ribs and cranial bone and is responsible for protecting vital inner organs [6, 15]. It also offers areas for muscle attachment. Irregular bones such as vertebrae and sacrum, also act to protect internal organs and give the body its structural form with its complex structures. Patellae

bones or kneecaps are the known form of sesamoid bones. These sesamoid bones are embedded in tendons and their aim is to provide higher protection and shielding in cases of over stress and motion. It is also found in the soles of a feet and palm of hands.

Femur bone is considered as the longest and strongest bone in the human body [16]. It articulates from the proximal region with the acetabulum of the pelvis to form hip joints, and it articulates to form the knee joint with tibia and patella from distal region [17]. Femur bone consists of three parts: proximal epiphysis, diaphysis (shaft) and distal epiphysis. The diaphysis acts to connect the two ends of epiphysis together through metaphysis [16, 17]. In adults, diaphysis includes the medullary cavity which contains yellow bone marrow, and is mainly composed of the hard compact bone. Two layers of bone are responsible for the bone growth, repairing and remodeling. The inner layer is called as endosteum surrounding the medullary cavity, and the outer layer that surrounds the majority of the bone is called as periosteum. In addition, periosteum includes blood vessels, nerves and lymphatic vessels that aid to nourish the compact bone. On the other hand, red bone marrow fills the spongy bone contained in epiphysis. Articular cartilage covers the epiphysis ends in order to absorb shock and reduces friction. The metaphysis includes the epiphyseal plate or line in adults that is responsible for bone elongation. During bone maturation, chondrocytes in the epiphyseal plate proliferate and act to replace cartilage with bones [18]. Epiphyseal plate progression continues until it becomes as epiphyseal line where longitudinal growth stops (Figure 1.3).

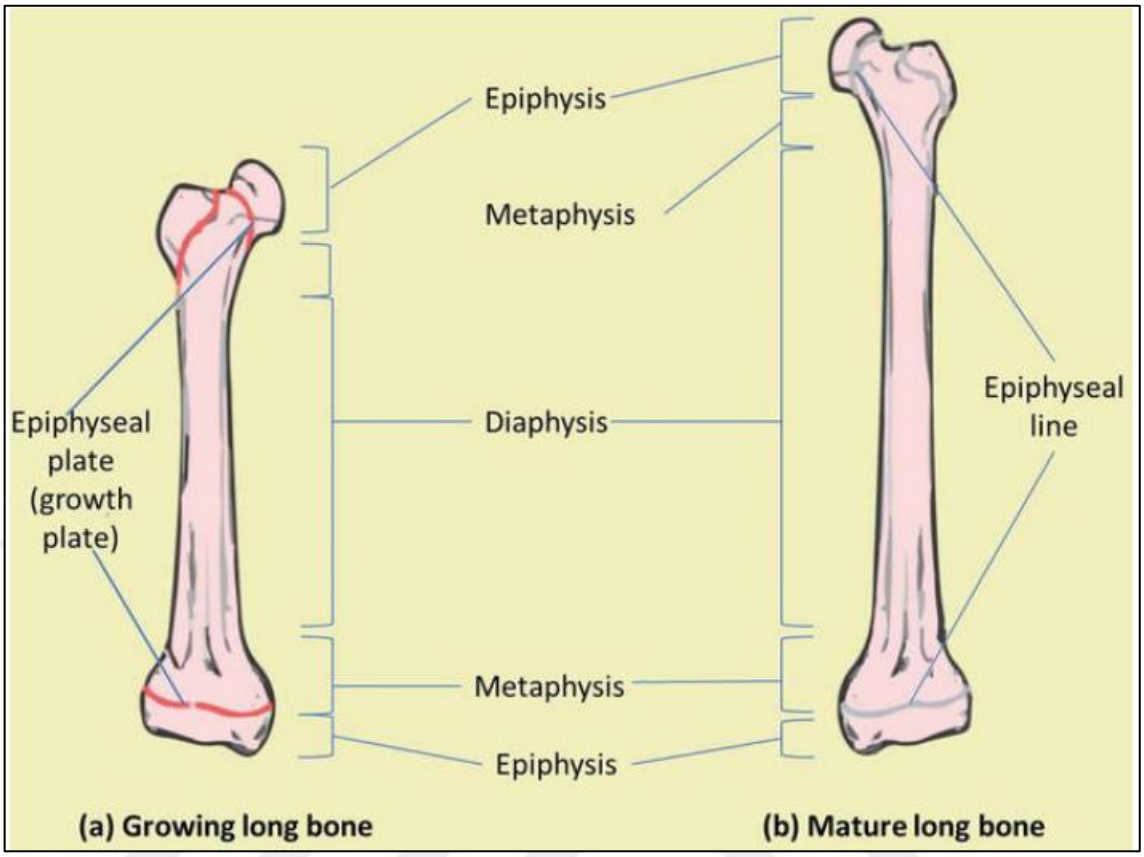


Figure 1.3. Epiphyseal plate progression during femur maturation. (a) Growing of long bone and (b) Mature long bone [18].

1.1.5. Bone Fracture and Its Treatment

Bone defects are usually caused by tumor, trauma, fracture, or infection. Regeneration of bones has been a challenging area for orthopedic surgeons [19]. Fracturing of a bone is considered as the most common bone injuries. It is caused by a strong implied force, stress, osteoporosis and few cancers. Treatments of fractured bone depends on the type of fracture and its location. Osteoporosis causes compression fracture, and this is due the compression of two bones against each other and this happens in the vertebrae of old osteoporotic patients. Hairline fracture happens due to over- stress that is usually located in the foot of athletic patients. Other types of fractures including greenstick and comminuted fractures, occur when the bone is cracked and shattered and it generally takes longer healing periods. After a bone is fractured, it should be stabilized immediately to prevent further severe injuries that might harm surrounding muscles and tissues [20]. Bone immobilization includes splints,

braces, plaster cast, pins, plates and screws. Immobilization and bone's alignment can be applied either by closed reduction where no surgery is needed or by open reduction where surgery is required. Closed reduction treatment usually undergo with the use of casts, external fixation or with percutaneous pinning. It can be applied when orthopedics can externally align the fractured bones, and there was no severe damage in the bones or even joints. Otherwise, open reduction with internal fixation is required. Fixation with plates and screws are the most common internal fixation that is used to align and stabilize the fractured bone during healing process.

1.1.6. Healing and Repair of Bone

Bone tissue is known that it does not leave any fibrous scar during healing process [21]. Bone healing process occurs under three main stages: inflammation, repair, and remodeling. During inflammation stage, blood clot forms and haematoma formation starts [22]. Phagocytes act to clear bone fragments and germs, and osteoclasts act to remove necrotic bone at the broken ends. Fibroblasts and MSCs migrate to the fracture site allowing the replacement of haematoma to granular tissue. Afterwards, repair stage takes place with the formation of soft and hard callus. During this stage, cartilage and other surrounding tissues are developed. Soft callus is formed around the fracture site allowing the formation of woven bone (primary bone formation). Periosteum and endosteum are also stimulated and osteoblasts start to form [21, 22]. Hard callus replaces soft callus and endochondral ossification undergoes allowing callus to be converted into rigid woven bone. At the end of this stage, blood vessel formation is enhanced. This process takes around 3- 4 months until remodeling phase takes place. During the remodeling of fractured bone, woven bone is slowly replaced by lamellar bone and vascularization of bone occurs. Complete remodeling takes from month to years to get the morphology of a healthy bone (Figure 1.4).

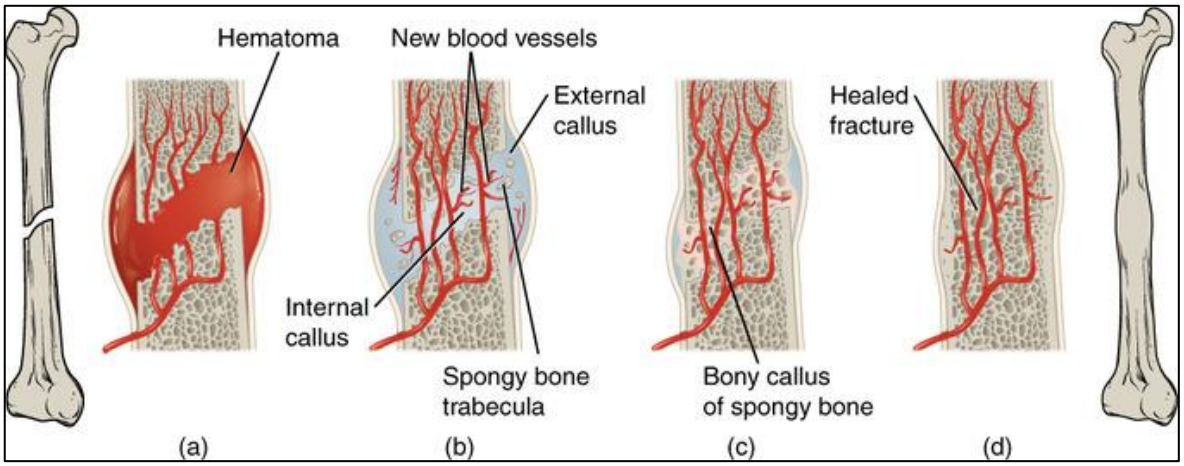


Figure 1.4. Healing process of fractured bone. (a) Hematoma formation, (b) Soft callus formation, (c) Hard callus formation, (d) Remodeling [17].

Two types of fracture healing processes are taken into consideration: indirect fracture healing and direct fracture healing [23]. Indirect fracture healing is a non-operative treatment with callus formation which follows the normal stages of healing process. Though, too much motion will result in delayed healing process. Direct fracture healing relies on internal remodeling. Thus, this type of healing is set as the last option since it needs surgical operation. It requires rigid immobilization like plates and screws and corrects anatomical reduction [24]. Basically, trabecular or lamellar bone is formed in the gap, and formation of haversian system occurs (Figure 1.5).

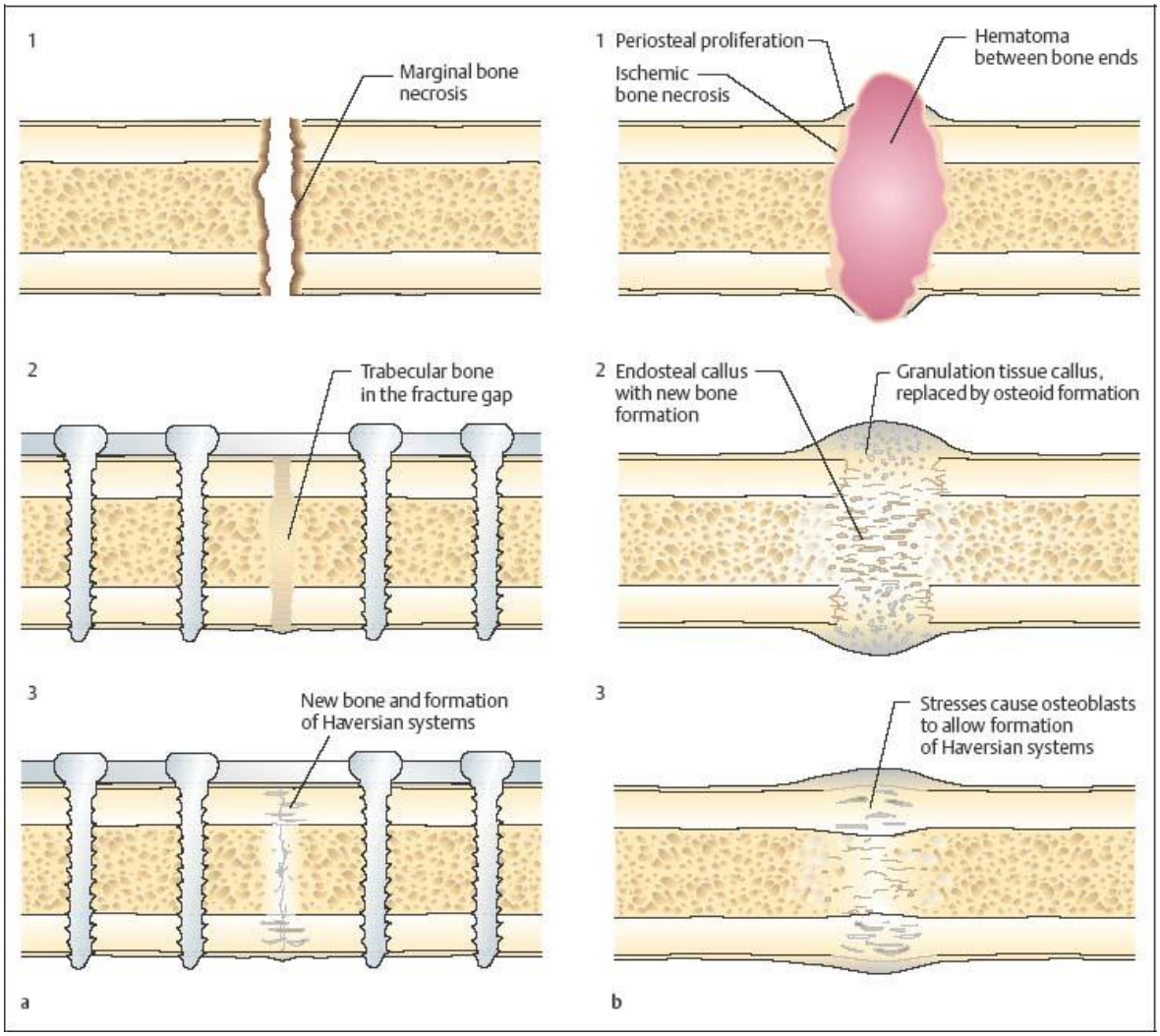


Figure 1.5. Fracture healing with (a) direct fracture healing, (b) in-direct fracture healing [24].

1.2. BIOMATERIALS

During recent years, the needs for autografts and allografts have been limited due to their limited availability, infection, donor site pain and high expenses. Instead, synthetic and natural biomaterials or scaffolds have been the ultimate substitute for natural bone regeneration [25]. Ceramics, matrices, composites and metallics are the main types of biomaterials that are used to induce cell proliferation, bone growth, and the desired environment for tissue development. An ideal biomaterial construct relies on its biocompatibility, biodegradability, mechanical strength, and porosity to enable cell adhesion and extracellular matrix formation. As bones are made up with two forms: cancellous bone

which includes porous structures and cortical bone includes dense structures that provides shape to the bones [26]. Bio-implants are made to assist and functionalize the body organs. In cases of trauma and fractures, these implants are used to stabilize and enhance tissue formation around defected site.

1.2.1. Bone Implantation And Its Types

Bone implants were first successfully introduced in the early 1900's to stabilize fractures with the use of metal plates and provide accelerated healing process [27]. However, mechanical failure, corrosion and low biocompatibility of those implants were witnessed. Until this day, implant design, material type, and their rates of biocompatibility are the three critical points to obtain better and successful implants. Orthopedics started implantations with stainless steel because of its increased corrosion resistance [28]. On the other hand, it is a heavy material and much stiffer than bones. Titanium was then introduced as being highly biocompatible, light and corrosion resistant. Orthopedics have seen that titanium was proven that it had less failure percentage as an implant than other metallics. A comparative study was made between stainless steel and titanium bone implants [29]. Through radiological and histological analysis, they declared that titanium implants had higher osseointegration rates and formation of lamellar bone was seen around the fracture. On the other hand, stainless steel implants was surrounded with giant cells and osteoid. These results resemble that titanium had better interface with bones than stainless steel. Though, the removal of titanium implants were more difficult because of bone formation around the plate. Recently, investigations around ceramics, the non-metallic-metallic, has demonstrated that they are tough and have high biocompatibility, nevertheless, they are also known with their brittleness and non-ductile nature [28]. Therefore, combining ceramics with metals like zirconia and alumina are under investigations in improving their strength. Such examples indicate that titanium is the most desirable metal in orthopedic implantation. Figure 1.6. shows the implant usage in different areas in the human body [30].

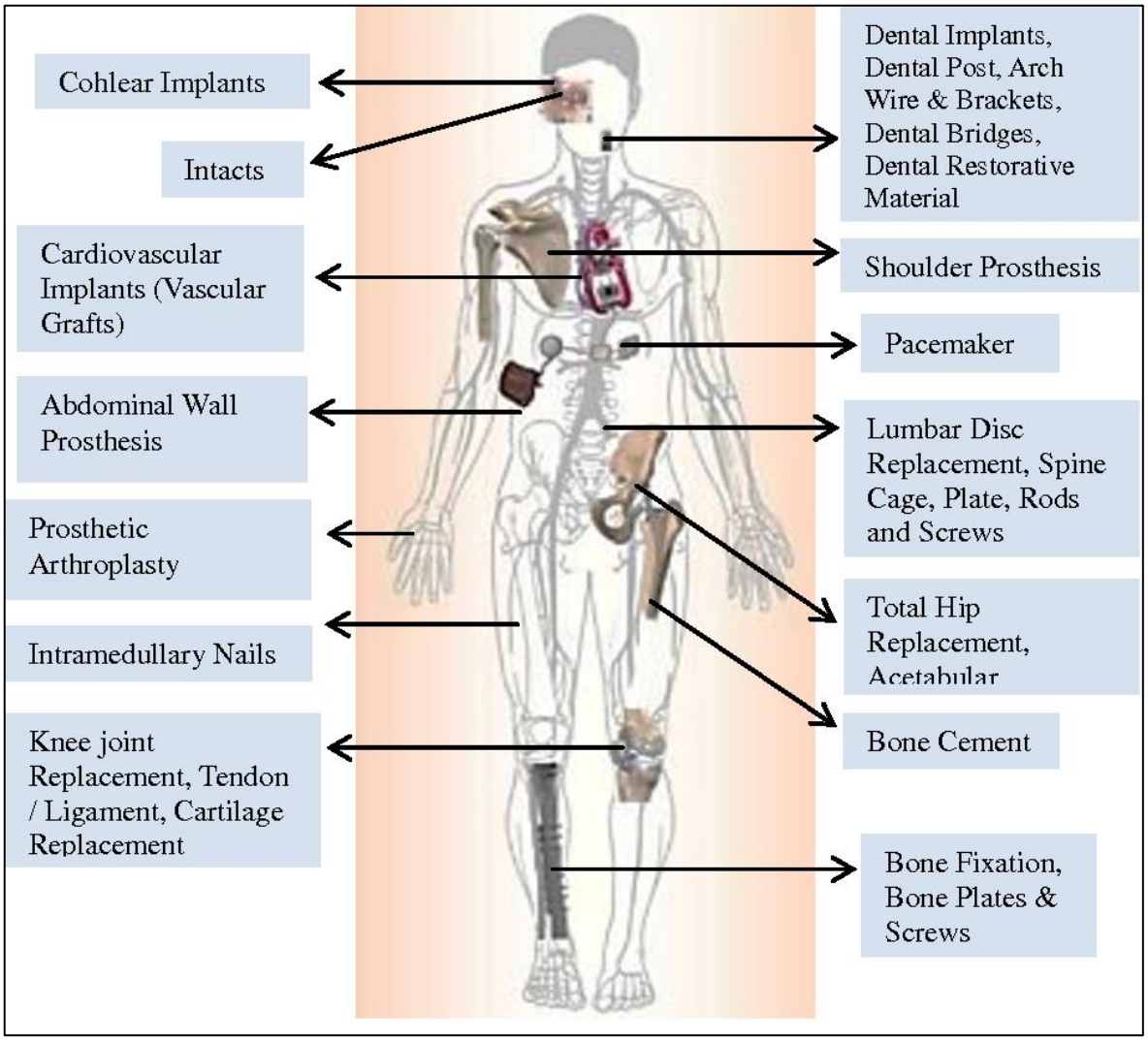


Figure 1.6. Illustrations of bone implants used on different parts in the human body [30].

1.2.2. Stainless Steel Implants

Since the 20th century, stainless steel has been introduced as the first implantable material for surgical procedures especially in orthopedics. Stainless steel implants are easily manufactured, highly ductile, and have low cost effectiveness unlike other metallic implants [31]. Generally, they are used for non-permanent implantations, due to their low biocompatibility, low strength and low corrosion rates that might be toxic and cause infections. The most common type of stainless steel is 316L, which produces higher rates of corrosion resistance [32]. These drawback could be addressed through the combination with other materials or the addition of bioactive coatings. A study was conducted to improve the

biocompatibility of stainless steel implants by titanium coating followed by micro-arc oxidation process [33]. Results indicated that stainless steel 316L implants had increased roughness and porosity on their surfaces that led to better surface characterization.

1.2.3. Titanium Implants

Implants are considered as a type of biomaterials that enter a host and act to repair any disrupted physical actions by inducing cellular activities. Titanium plates and its alloys have been proven with their high biocompatibility since the mid of 20^{th} century [27]. Titanium implants have been used in many orthopedic applications such as joint replacement, long bone fractures, and in dental applications as bone pins. Pure Ti and its alloys were introduced as a safe material with certain modulus to bones, high biocompatibility, and high corrosion resistance with their light weight [28]. Their high biocompatibility and high corrosion rates are due to the presence of thin layer oxide films [34]. Though, titanium and its alloys have the tendency to form wear debris that is released into the bloodstream causing inflammation and osteolysis. This could occur when titanium is rubbed between other materials or even between itself leading to high fractioning of the material. An orthopedic spinal cord implantation was made with titanium alloy rods and focused on the corrosion rates and metal hypersensitivity [35]. They pointed out to the fact that long term implants were subjected to micro-motion that might be caused by bacterial infection, corrosion, release of wear debris and metal ions when rubbing between the implant and tissue increases. Which further tend to cause inflammation, infection around the wound and implant loosening (Figure 1.7). Successful implants should have high wear resistance to prevent implant loosening and any surgical complications [36].

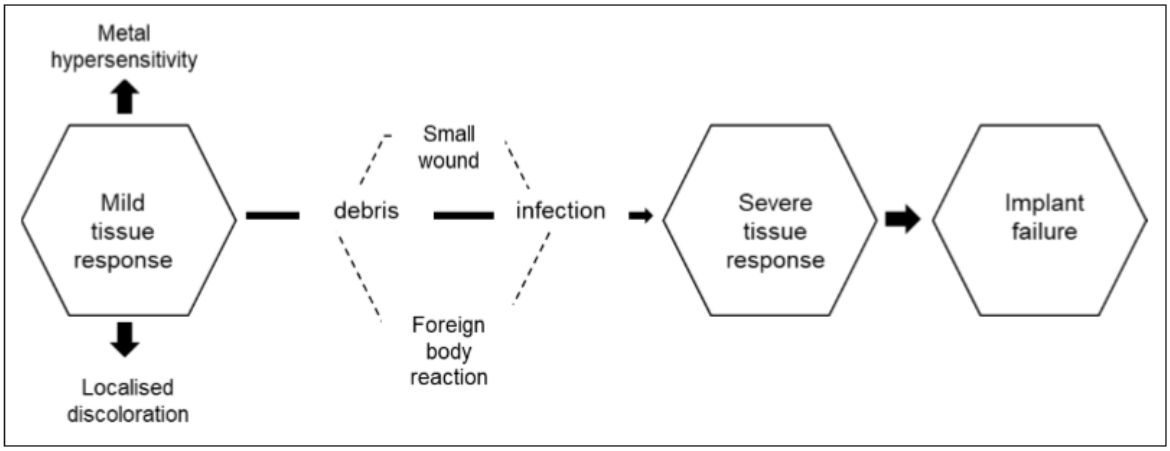


Figure 1.7. Reactions of the human body due to implant failure [31].

Furthermore, it was found that titanium alloy with Aluminum and Vanadium (Ti-6Al-4V) or Aluminum and Niobium (Ti-6Al-7Nb) had increased tensile strength and better modulus than pure titanium. A histological study was made to test the osseointegration capabilities that was provided by Ti implants during femur fracture in rabbit models [37]. They concluded that a perfect immobilization with titanium implants on the fracture site showed a high level of osseointegration, minimal callus formation, and woven bone was converted into compact lamellar bone within two months. Though, other studies showed that Ti does not form the chemical bonding with bone tissues, so the bioactivity of Ti and its alloys should be resolved to avoid implant failure [34, 38]. This happens by improving their surface modifications with either bioactive coatings or physicochemical changes on their surface. Improving surface hardness of Ti implants leads to better tribological behaviors, corrosion rates and osseointegration. However, Ti alloys have the ability to form a bonding with bone tissues which resembles better integration capabilities than pure Ti [26, 34]. Ti alloys has lower modulus that is closer to the natural bone which eventually makes then a better form of titanium metallic implants.

1.2.4. Role of Magnesium in Bone Growth and Its Uses With Titanium Implants

Magnesium is considered as one of the most abundant element in the human body [39]. It is mainly concentrated in bones. It is known that when magnesium deficiency occurs, higher risks of osteoporosis are revealed [40]. In addition, high intake of magnesium is able to prevent bone fracture. Many studies discussed the positive effect of magnesium on bone

growth by regulating the proliferation and apoptosis of osteoblast and osteoclast [38, 41]. Magnesium is also considered as a co-factor for many enzymes that participate in maintaining bone health [42]. It acts to preserve and breakdown bone by controlling thyroid and parathyroid glands [41]. Magnesium based implants show better properties when comparing to other implant types. They have lower modulus than most metals, higher mechanical strength than polymer, and higher toughness compared to ceramics [39]. In addition, tensile strength of Mg implants, close to the natural bone when compared to other implants [43]. Also, Mg enhances the formation of apatite in bone matrix. Though, Mg implants have witnessed low corrosion resistance leading to cause implant degradation and failure before achieving complete healing of defected bone. This issue could be addressed by providing better surface modification or the addition of coatings to Mg implants [39, 53]. Biodegradable silane coatings, polymeric coatings, calcium phosphate coatings, and graphene-derivative- coatings have been used as barriers to lower corrosion of Mg implants and improve their resistance rates.

Furthermore, Mg-coated zirconia and titanium implants were analyzed with XRD, SEM, and FT-IR analysis. They noticed that the presence of Mg increased the bioactivity of both zirconia and Ti implants. The release of Mg ions increased the growth rate of carbonate-containing hydroxyapatite (HAp) [44]. HA- coated TiAlV and Mg-HA coated TiAlV were tested and revealed that the presence of Mg increased the bone bonding strength [45]. Furthermore, *in vitro* and *in vivo* studies on a rat model were carried out to reveal the antibacterial properties of magnesium implants compared to Ti implants [46]. They declared that because of the degradation of Mg; reduced bacterial infection was obtained. Therefore, Ti implant infections could be prevented with the addition of Mg. In another study, porous titanium coated with Mg- doped octacalcium and hydroxyapatite thin films were implanted on the femoral bone of rabbits [47]. Results indicated that Mg- doped HA implants contributed to the increase in the volume of bones when compared to uncoated implants. All studies demonstrated the crucial role Mg in bone healing and its relation to implant's surfaces. Magnesium ions act to balance the activity between each of the osteoblasts, osteoclasts, and osteocytes during bone regeneration [48]. It also acts to enhance mineralization of bones. Figure 1.8 describes the functional processes of some important metal ions and their effects during bone healing and regeneration.

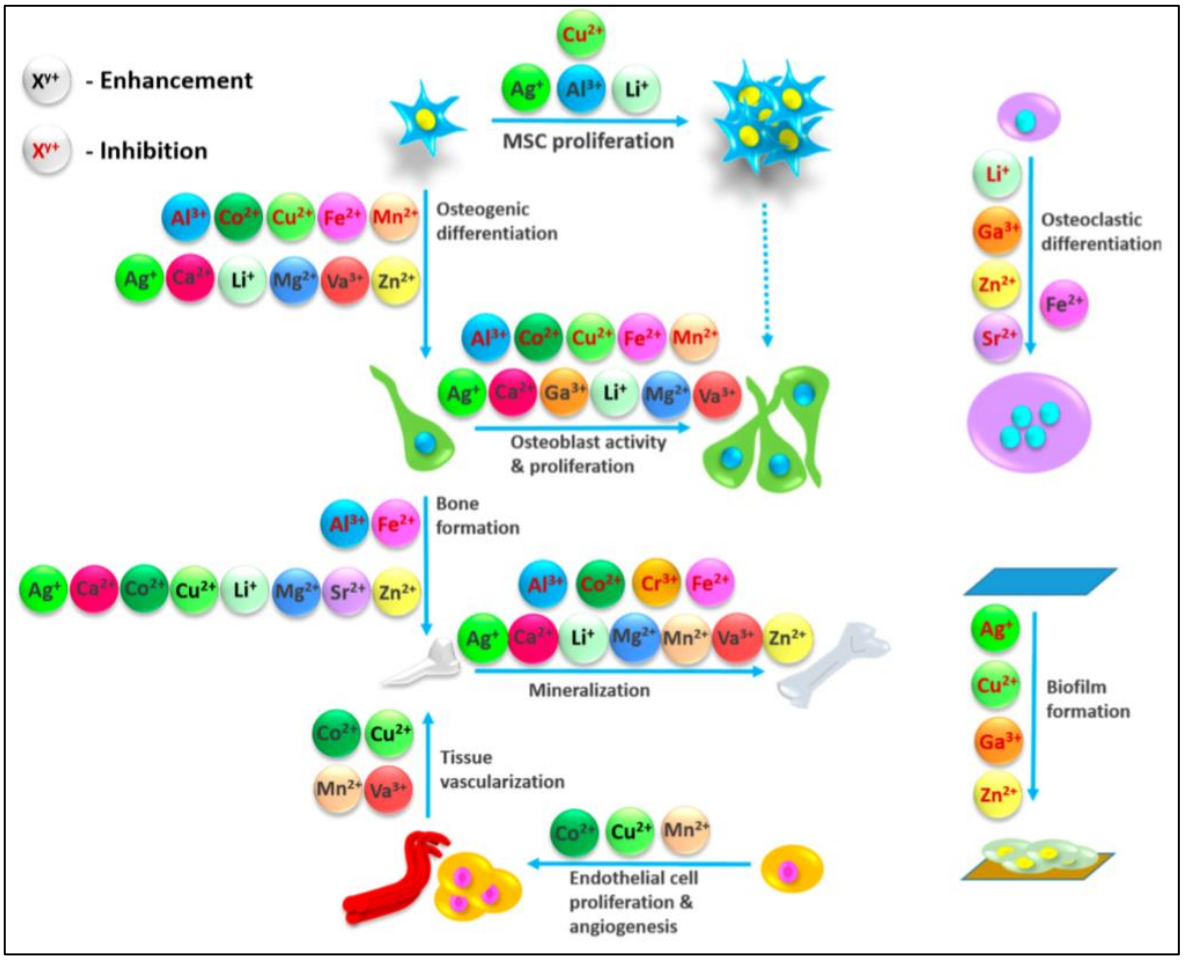


Figure 1.8. Metal ions mechanisms during bone regeneration [48].

1.2.5. Osseointegration of Implants

During the 1950s the term ‘osseointegration’ was first used [49]. Pre-Ingvar Branemark was the first to recognize the phenomenon of osseointegration when bone grows around the implants and good integration is provided between the bone and implant. Enhancement of osseointegration relies on some important parameters like high corrosion rates, surface roughness and oxidation, the porosity of the material, and the mechanical stimulation. The main concern in providing good osseointegration is the direct contact of the implant to cortical bone which provides stability and better bonding to the bone. This stimulates the expression of genes on the bone and implant interface that enhances the formation of collagenous tissue and the mineralization of bone to produce ECM. However, implant loosening and osseointegration failure might occur due to over mechanical stimulation,

failed integrity of bone and implant bonding, relative motion and wear formation. Higher rates of osseointegration can be induced by providing implants with a coating layer composed of organic or inorganic components such as calcium phosphate (CaP), type-1 collagen and some growth factors which are already available in normal ECM [50].

On the other hand, some antiresorptive and anabolic agents might be used to improve osseointegration [51]. In one of the study, titanium implants with bisphosphonate (BP)-loaded calcium phosphate nanoparticles (nCaP) coatings were implanted on osteoporotic animal models. It was found that, bone regeneration was significantly higher around the surface of the implant which tend to improve the integration between bone and implant. The understanding of bone and implant interface, osteoclast and osteoblast role around the implant surface, and the formation of soft and mineralized tissue to stabilize the implant all contribute to better osseointegration on the implant surface [52]. Ouyang et al. investigated the osseointegration of Mg-Ti composites and Ti implants. Results showed that Mg-Ti metal-metal composites had better bioactivity and increased interfacial between implant and bone than that of Ti [53]. Higher bone formation and stronger osseointegration capabilities were observed with those Mg-Ti composites.

1.2.6. Role of Hydroxyapatite in Bone Healing

Bone tissue is mainly comprised of matrix (collagen and non-collagenous proteins), organic and non-organic minerals, and water [54]. Bone collagen matrix mineralization is obtained from the presence of calcium-phosphate crystals (hydroxyapatite) that accounts for 65 percent of whole bone mass. Studies have discussed the importance of hydroxyapatite (HA) in bone regeneration. The component similarity between HA and natural bone was also revealed [55]. The osteoconductive and osteoinductive properties of HA and its porous structure have a vital role in fracture repair process [56]. Several studies proved the significant potential of HA in enhancing bone healing mechanism and blood coagulation activity due to its hemostatic properties [57, 58]. Synthetic HA is suggested as a biomaterial or implants' coating material since it has the good biocompatibility, bioactivity, osteoconductivity, and osseointegration properties [59, 60]. HA coatings made on the surface of metallic implants increased the osteoblast adhesion and provided better osteoblast activity [61]. Increased osteoblast adhesion promoted the differentiation and proliferation of

MSCs. Another study was performed to stimulate the bone-bonding relation of titanium and stainless steel implants [62]. Hydroxyapatite coatings were doped onto 316L stainless steel implants, as it is known that HA has the ability to form strong chemical bonding to bones and increase the adhesion properties of an implant.

Inserting different implants into the human body might sometimes have the risk to develop infections and chronic inflammations. One study discussed the successful usage of hydroxyapatite and natural or synthetic polymers as an antibacterial based carriers to be delivered into the bone [60]. Figure 1.9 shows the various applications of hydroxyapatite in medical field and how to serves as an antibacterial agents.

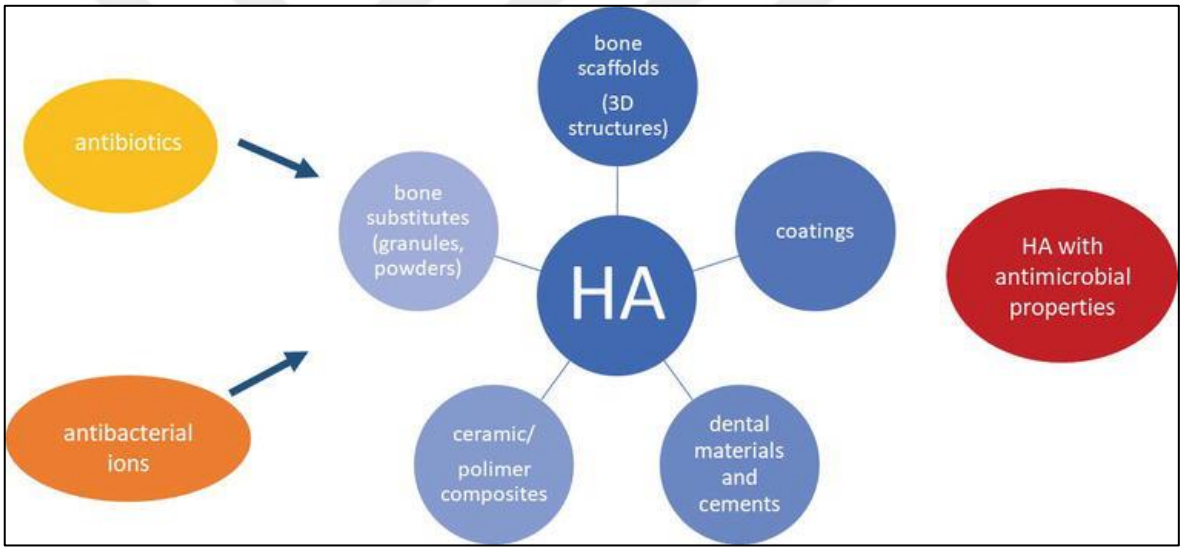


Figure 1.9. HA applications as biomaterial, coatings, bone substitutes, and antibacterial agents [60].

1.3. IMPLANT FAILURE

Demands for the use of metallic implants have been arising throughout the years. However, in some cases of implantations, implant failure has been witnessed. High corrosion rates, wear debris formation, metal allergy, and alteration in osseointegration are the major reasons behind loosening and failure of an implant [63]. Cytotoxic responses might occur due to wear formation on implants [64]. Degradation of the implant by extensive osteoclast accumalation on the implant surface is another reason behind an implant loosening.

Hypersensitivity tends to cause tissue destruction and osteolysis. Patch testing is used to diagnose metal allergy and hypersensitivity especially in the early stages of implantation and helps to predict the adverse effects of patients having a metal allergy history. The use of macromolecular imaging agents such as *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer was described in a study that shows diagnosis of peri-implant inflammation in order to prevent bone loss before the development of an implant failure [65]. Loosening of an implant can be caused by fail in osseointegration at the implant-bone interface which leads to fibrous tissue formation [66]. Failed osseointegration is the impact behind many reasons like, low strength, low biocompatibility, poor surface design of an implant, un-instability of implant and other surgical complications. That's why stimulation of osseointegration is an important key for successful implantation.

1.4. AIM OF THE STUDY

The aim of this study is to provide the hard tissue implants with a new strategy by increasing osseointegration rates and enhancing better interface between the surface of implants and bones. In order to achieve this successfully, titanium nitride coated plates and screws were doped with magnesium. The magnesium implants having low density, low yield strength and elastic modulus tend to be closest to the natural form of bone [67]. Mg ions are known for their ability to facilitate healing of tissues. Moreover, formation of calcium-phosphate crystals (Hydroxyapatite) was observed in bone fracture repair [56]. As previous studies suggested that the presence of Mg significantly enhances the formation and growth of hydroxyapatite [68].

However, Mg implants alone have high rates of corrosion which may affect bone healing process. As in a previous study, they overcame the corrosion issue of Mg implants by the addition of titanium dioxide coatings. As a result, increased corrosion resistance, higher bacterial infection resistance and better interface with bone were obtained [69]. In the current study, the coated plates were implanted on the fractured femur of rabbits for histological and biochemical assessments. Improving the characteristics of the plate's surface by surface modifications, we aimed to accelerate the healing process of the fractured bones. The addition of magnesium to TiN coated implants is a key role to improve the surface characterization which may tend to enhance better quality of bone regeneration.

2. MATERIALS

2.1. SURGICAL PROCEDURE

- TiN and (Ti, Mg) film plates
- Propofol®-Lipuro 1 per cent (10mg/ml) (Braun, Germany)
- Anaesthetic machine ANS 200 (ATESE Isoflurane, hasvet, China)
- Forane® - Isoflurane (ABBOTT)
- Rompun® (BAYER)
- Meloxicam (0.5mg/ml)
- Antibiotics (Baytril-K 5 per cent)
- Angiocuts
- Osteotome
- Sterilized surgical forceps
- Sterilized surgical scissors
- Sterilized gloves and sheets

2.2. CT-SCAN ANALYSIS

- 16 falcon tubes (50mL)
- Gauze swap
- 16 bone samples
- Bruker Micro-CT Analyser.
- Compact X-ray Micro-CT © SkyScan 2007 (SkyScan N.V. Kartuizersweg 3B 2550 Kontich Belgium)

2.3. HISTOLOGICAL ANALYSIS

- Neutral buffered Formalin (10 per cent)
- Falcon tubes 50 ml

- Distilled water
- Chloral Hydrate (Aldrich, Germany)
- Nitric Acid (ISO LAB, Germany)
- Ethanol absolute (ISO LAB, Germany)
- Toluene EMSURE® (Merck, Germany)
- Paraffin
- Thermal Scientific Machine (Heraeus, Ankara)
- Rotary Microtome (LEICA Bio 2255)
- Light microscopy (Olympus, Japan)
- Trinocular microscope (Olympus BX40, Japan)

2.3.1. Masson's Trichrome Staining

- Acid Fuchsin (SIGMA)
- Ponceau Xylidine (SIGMA)
- Distilled water
- Acetic acid glacial (Merck, Germany)
- Phosphomolybdic acid (Merck, Germany)
- Light Green (B.D.H Laboratory, England)

2.3.2. Hematoxylin and Eosin Staining

- Ehrlich's Hematoxylin (Merck, Germany)
- Absolute alcohol
- Glycerin
- Distilled water
- Acetic acid glacial (Merck, Germany)
- Potassium alum
- Eosin Y (ICN Biomedical, Ohio)
- Alcohol (96 per cent)

2.4. SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

- PBS solution
- Scanning Electron Microscope (EVO 40, Carl Zeiss, Germany)
- Gold cover (15 nm)
- Sputter Coater (Bal-tec SCD 005)
- Microscopy slides

2.5. VON KOSSA STAINING

- PBS
- Neutral buffered formaldehyde
- Silver Nitrate solution (5 per cent) (Diagnostic Biosystem, Netherlands)
- UV light
- Cell culture hood, cabinet class II (TEISTAR, Spain)
- Distilled water
- Sodium Thiosulfate solution (5 per cent) (Diagnostic Biosystem, Netherlands)
- Nuclear Fast Red solution (Diagnostic Biosystem, Netherlands)
- Plastic petri dish

2.6. ENERGY DISPERSIVE X-RAY SPECTROSCOPY (EDS) ANALYSIS

- Scanning Electron Microscope (JEOL, JSM-5410)
- Digital Control Processing (IXRF Systems)
- Energy Dispersive Spectrometer (Noran, Inc. 606M 1FSS, USA)

3. METHODOLOGY

3.1. PREPARATION OF Mg DOPED TiN THIN FILM COATINGS ON Ti6Al4V BASED IMPLANTS

Magnesium doped TiN thin film coatings were prepared by using arc-PVD technique on Ti6Al4V substrates to determine the local Mg release on bone regeneration *in vivo* [70]. Magnesium amount in coatings was checked with EDS analysis. Mg amount in the coatings was set to 8.2 percent. At that ratio, better osseointegration and corrosion resistance properties were found in the previous studies [71]. The diameter of both TiN and (Ti, Mg) N coated plates was 2x4x60 mm and the screws were 2 mm in diameter to be drilled into bones for fixation. Pre-implantation, TiN and (Ti, Mg) N coated plates and screws were autoclaved at 1.5 atm and 121˚C for 15 minutes.

3.2. *IN VIVO* EXPERIMENTS

Sixteen healthy male rabbits taken from "ABDEHAM Deneysel Hayvan Merkezi" were operated at Istanbul University Faculty of Medicine in Istanbul, Turkey. The mean weight of rabbits was around 3± 0.5 kg (Table 3.1). Rabbits were fed twice a day and water was always available. Post-operation, they were given Baytril-K 5 per cent (antibiotic), for 5 days as well as Meloxicam (non-steroid anti-inflammatory, 0.3 mg/kg) for 5 days.

Table 3.1. Summary of the plates/animal model implantation.

Total rabbit models	16
Models used for TiN group	8
Models used for (Ti, Mg)N group	8
Gender	Female
Weight	3± kg
Incision size	50-60 mm
Coated plates' size	2 x 4 x 60 mm

The rabbits were pre-anesthetized with Ksilazin HCl (Rompun®, BAYER) with 0.5 mg by intravenous injection in the jugular vein. They were later given general anesthesia with Propofol® (0.5 mg) when needed and inhalation of anesthetic isoflurane was continuously given through Anesthetic machine ANS 200 (ATESE Isoflurane, Hasvet, China). Anesthesia was maintained with 1 per cent of Isoflurane in 2 per cent oxygen. Animals were observed continuously to control that they were breathing normally.

Surgery was performed on the right femur of the white male rabbits. The surgical site was sterilized with baticon and shaved. A 50 - 60 mm incision cut was made with osteotome and fracture was made on the middle of femur. Prior to implantation, plates were washed in pure sonicated acetone, ethanol and followed by sterilization. TiN and (Ti, Mg) N coated plates were implanted on the fracture site of the femur for fixation and regeneration. Having two different implant plates, 16 rabbits were randomly divided into two groups (n = 8 per group). TiN plates (group A) and the (Ti, Mg) N plates (group B) were implanted into the femur and fixed with screws as shown in (Figure 3.1). Normal saline solution was applied on the implant site then the wound was closed layer by layer. As a control, a left femur bone of rabbit was used. No surgical complications occurred during the operations. Antibiotics were given to the rabbits intramuscularly to avoid wound infection. Bone samples were dissected and removed, also the implants were taken from rabbits after a period of six weeks of post-implantation. Rabbits were all euthanized at the same day with an overdose of Propofol® injection. Bone samples and plates were fixed with formaldehyde for further biomechanical and histological analysis.

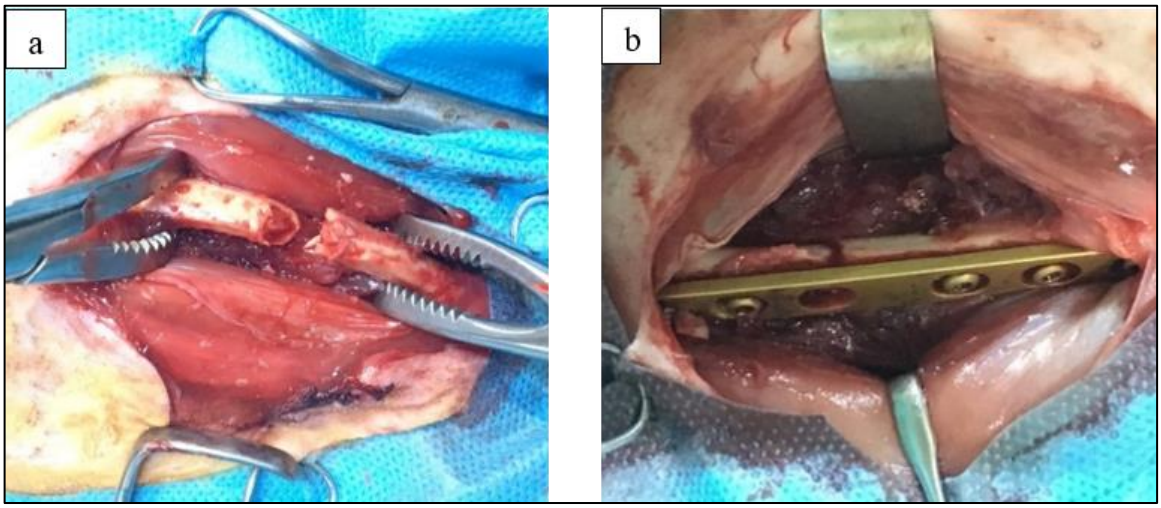


Figure 3.1. Pre-operation and fixation with plates: (a) fracture in the middle of the femur, (b) plates fixed with fractured bone.

3.3. X-RAY EVALUATION

X-ray images were recorded to check the post- operation (1st day and 6th week) of implantation for both TiN and (TiMg) N thin film coated plates and to see how bone growth was progressed within six weeks of implantation at Fatih Veteriner Capa, Istanbul/Turkey. First, X-ray evaluation was carried out directly after implantation (1st day) for both implant groups TiN (group A) and (Ti, Mg) N (group B) as shown in (Figure 3.2). Then, after 6 weeks of implantation x-ray images were also taken to check the regeneration of bones.

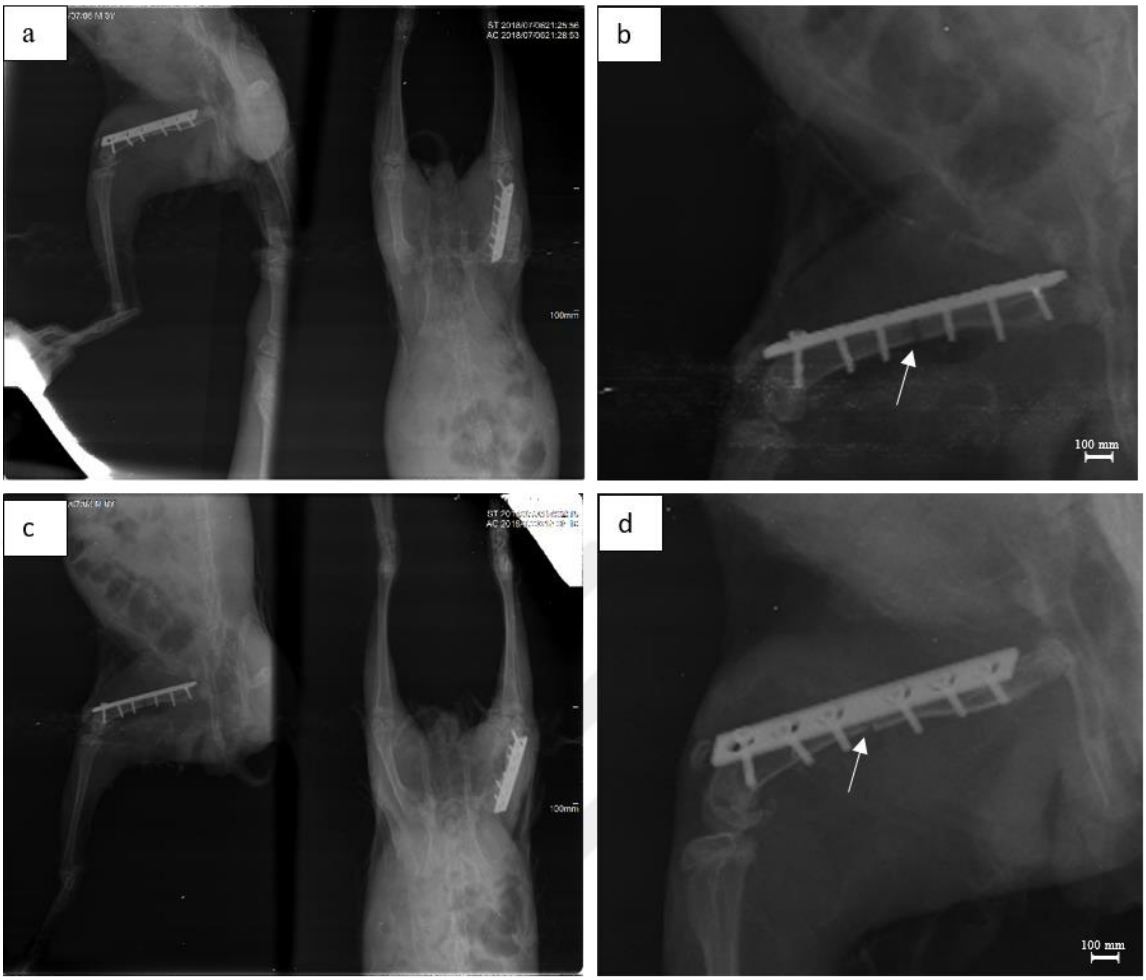


Figure 3.2. X-ray evaluation after implantation (at day 1) for TiN/(TiMg) N plates. (a, b) TiN and, (c, d) (Ti, Mg) N thin film coated plates.

3.4. CT-SCAN EVALUATION

A high-resolution desktop micro-CT system (SkyScan 1174v2; Bruker microCT, Kontich, Belgium) was used to quantify the Bone Mineral Density (BMD) and the three-dimensional micro architecture parameters in the femur. The specimens were scanned at 50 kV and 800 mA, with 0.25-mm-thick aluminum filter to adjust the contrast, a rotation step of 0.8°, three-frame averaging, and an isotropic resolution of 30.51 μm. Each image of the specimen renovated with the software (NRecon version 1.6.10.2; Bruker, Belgium), providing an axial cross-sections of the inner structures of the samples. Following renovation, the region of interest (ROI) for all bone samples were determined using CTAN (version 1.16.4.1+, Bruker MicroCT) being a 15 mm that was longitudinally centered on the callus (i.e., both sides of

the fracture line of callus tissue was 7.5 mm). Borders of the callus bone tissue was traced manually. Other calculations were adjusted with phantom size which can also be calculated from x-ray absorption, and is defined as the attenuation coefficient. The phantom was chosen as 4 mm according to normal bone size/type of rabbits (table listed in Bruker CT-scan Booklet). Old cortical bone was removed by the use of threshold delineation (global threshold >100) to allow quantification of new bone formation. Quantification of these structural parameters was applied using two grayscale thresholds for distinguishing between mineralized tissue and highly mineralized tissue.

Bone density and mineralization of bone during fracture healing were examined using CTAN software. BMD is the volumetric density of calcium hydroxyapatite (CaHA) $g.cm^{-3}$ which calculates the Bone-Soft Density or calcified bone tissue (TMD). The mineral density of the callus was presented as a grayscale index from 0 to 255 with positive correlation analysis. Tissues were classified as low-density bone when the grayscale value is between 60 -110 in the metaphyseal fracture. Grayscale values that are greater than '110' represent high-density bone. Due to some changes in the X-ray source between the scanning of samples, different thresholds were chosen for the analysis of the diaphyseal fracture group.

3.5. HISTOLOGICAL ANALYSIS

Bone specimens were fixed in formaldehyde fixative solution for at least 3 weeks in falcon tubes. After Micro-CT analysis, Decastro solution (decalcifying agent) was prepared and added to the bones for around 3 weeks, until they become softer for cutting for further processing. Decastro solution preparation included, 50 g of chloral hydrate buffer, 30 mL of nitric acid, 300 mL of absolute ethanol disolved in 670 mL of distilled water for all 16 bone samples. Later on, samples were dehydrated in graded series of alcohol (70-100 per cent) for a week. Samples were kept in 100 per cent alcohol and toluene (1:1 v/v) for an overnight. Afterwards samples were placed in toluene (clearing solution) for 1 hour before embedding in films. Paraffin wax was melted in 56°C, toluene solution was also added with 1:1 v/v ratio, and then samples were embedded. A rotary microtome grinding system was used to obtain sections with 7.0 mm and 4.0 mm thickness for further histological staining.

3.5.1. Hematoxylin and Eosin Staining

Sections were stained with Ehrlich's Hematoxylin & Eosin Y. This staining was applied to identify the bone tissue types and to understand the mechanism of bone healing during bone formation. It mainly points on compact bone and cartilage formation. For Ehrlich's Hematoxylin solution, 2 g of hematoxylin, 100 mL of absolute alcohol, 100 mL of glycerin, 10 mL of acetic acid, and 15 g of potassium alum were all dissolved in 100 mL of distilled water. To prepare stock Eosin Y solution, 1 per cent of Eosin was dissolved in 96 per cent of alcohol. Fresh Eosin stain was prepared with 1/3 of Eosin Y (stock), 1/3 of alcohol(96 per cent), 1/3 of distilled water and 0.6 mL of acetic acid. Basically, sections were hydrated gradually by alcohol (100-70 per cent) to water, stained in Hematoxylin for a couple of minutes, and washed in running tap water for 5 minutes. Then sections were differentiated in 1 per cent of acetic acid for 5-10 seconds and washed in running tap water for 3-5 minutes. Next, it was stained in 1 percent Eosin Y for 1 minute and re-washed in tap water for 1-3 minutes. Finally, sections were dehydrated through alcohol (70-100 percent), cleared with toluene and were mounted for light microscopy observation.

3.5.2. Masson (Trichrome) Staining

Trichrome stain enables the visualization of bone tissue compartments with 3 different stains during bone healing process. Trichrome stain contains Wiegert hematoxylin, Fuchsin ponceau, Phosphomolybdic acid and light green stains. The staining procedure took place first with Weigert hematoxylin. For this, 1 percent of hematoxylin and FeCl3 6H2O (4 mL) were incubated with sections for 5 minutes and then washed with tap water for 10 minutes. After that, fuchsin ponceau staining was carried out. For this staining, 2 g of Xylidine ponceau with 1 gram of acid fuchsin was incubated for 1- 5 minutes and then washed in 1 per cent acetic acid. Then, sections were stained with phosphomolybdic acid for 10 minutes, washed again in 1 per cent acetic acid, and then stained with light green for 2 minutes. Last but not least, sections were washed with 1 per cent acetic acid and dehydrated in alcohol (70-100 per cent). Finally, sections were cleared with toluene and then mounted. Furthermore, all stained sections were examined under standard light microscopy.

3.6. SEM ANALYSIS

The enhancement of osteoblast formation on the implant surface containing TiN and (Ti, Mg) N coated plates was determined by Scanning Electron Microscopy (EVO 40, Carl ZEISS, Germany). TiN and (Ti, Mg) N coated plates were washed with PBS and stored in 4°C. Following the incubation, before SEM analysis they were air dried. Eight samples of TiN and (Ti, Mg) N coated plates were randomly chosen and divided into two groups. TiN samples with the numbers of 1, 2, 7, and 10 and (Ti, Mg) N samples with the numbers of 8, 12, 13, and 16 were selected. Furthermore, plates were coated with gold with a thickness of 15 nm in 40 seconds by sputter coater (Bal-tec SCD 005) as shown in (Figure 3.3). Finally they were examined with SEM (Carl Zeiss, EVO). Examination was applied on both surfaces of the plates. Top of the plates having no bone interaction was used as a control. Meanwhile, bottom of the plates having interaction with bone was used as an experimental group.

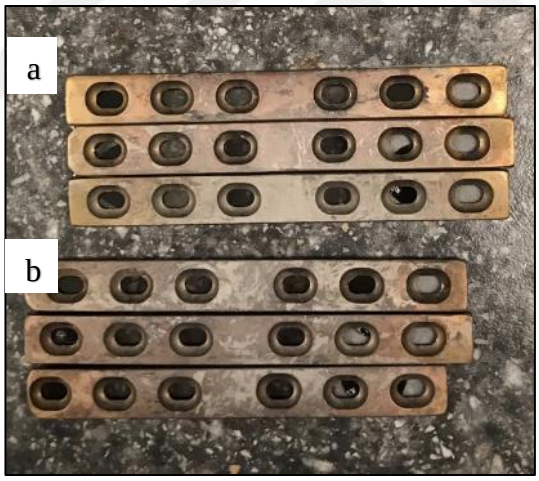


Figure 3.3. Gold coated plates for SEM (a) TiN (b) (Ti, Mg) N thin film coated plates.

3.7. VON KOSSA STAINING

Von Kossa staining was performed to show the osseointegration potentials on the surfaces of both TiN and (Ti, Mg) N coated plates. TiN and (Ti, Mg) N plates were incubated for at least three weeks at 4°C in 50 mL falcon tubes and then fixed with 4 per cent formaldehyde. Eight samples of TiN and (Ti, Mg) N coated plates were randomly chosen and divided into two groups for this staining. Plates were placed in plastic petri dishes for further staining

steps. The plates in dishes were washed with distilled water 3 times. A 2 mL of 5 per cent Silver Nitrate solution was added into each dish. Basically, silver nitrate stains calcium ions by replacing them with silver ions under the exposure of strong light. Therefore, they were exposed to UV light in a dark chamber for 45 minutes until calcium deposits were visible. Plates were re-washed with distilled water for 3 times, and then sodium thiosulphate (5 per cent) was applied on plates for 3 minutes as a treating buffer solution. Samples were washed again with distilled water for again 3 times. Followed by the addition of Nuclear Fast Red which stains the cytoplasm and nuclei, and plates were incubated for 5 minutes. Finally, they were washed with distilled water for at least 3 times.

3.8. EDS ANALYSIS

Energy dispersive x-ray spectroscopy (Noran, Inc. 606M 1FSS, USA) with the iridium altra software was used to determine the hydroxyapatite deposition on the surface of TiN and (Ti, Mg) N coated implants. Secondary and back scattering images were taken to show the topography and elemental composition appeared on the implants' surfaces. Calcium and phosphate depositions were expected to appear on (Ti, Mg) N coated plates due to the hydroxyapatite deposition. Images were taken from both surfaces of coated plates: the interfaced surface with bone (experimental group) and the non-interfaced surface of coated plate (control group) to describe the difference in elemental compositions during bone formation.

3.9. STATISTICAL ANALYSIS

CT-Scan data were given to differentiate between TiN (group A) and (Ti, Mg) N (group B) samples. Statistical analysis using unpaired (t-Test) was performed to check the statistical significance between group A and B samples. P-values were selected as 0.05 where less values should be considered as statistically significant.

4. RESULTS AND DISCUSSION

The success of the metal-implant is based on two crucial roles: the speed of the implant healing and the mechanical strength resulted between the implants to bone interface. This study basically explains the interaction between bones and implants that were prepared from either pure titanium nitride (TiN) or titanium nitride-magnesium coated (Ti, Mg) N plates and also their corresponding period of healing process which is described as osseointegration. Mainly, osseointegration is affected by the surface design of an implant along with the biological factors that increases the bone ingrowth during healing process. It is hypothesized that the chemical element magnesium has an ability to promote the interface bonding between implants and bones. A study was made on implanting degradable magnesium plates and screws on the ulna of rabbit model [72]. They found out that Mg enhanced healing of the bone by stimulating bone formation during and after degradation of implants. Though corrosion rate increased during degradation, bone formation around the implant was still stimulated. It can be concluded from previous experimental studies that the use of Mg alone as a plate can cause corrosions, therefore, addition of Mg as a coating to TiN plates may aid to influence the osteoconductivity, new bone formation and improve the implant osseointegration without increasing the corrosion rates.

At the current study, the re-generation capabilities of broken femur bone of 16 male rabbits and their response to the surfaces of both TiN and (Ti, Mg) N thin film coated plates were investigated. After six weeks of implantation, bone ingrowth around the plates (osseointegration) were observed and the effect of magnesium that was suggested to contribute to a better bone regeneration (osteosynthesis) with less time interval was tested. Figure 4.1 illustrates the healing process of fractured bone within six weeks of implantation with (Ti, Mg) N coated plate and screws. Further Micro CT- analysis, X-ray scanning, histological evaluations, SEM, and von Kossa staining were made to approve that (Ti, Mg) N coated plates can be used in subsequent clinical applications as a hard tissue implant.

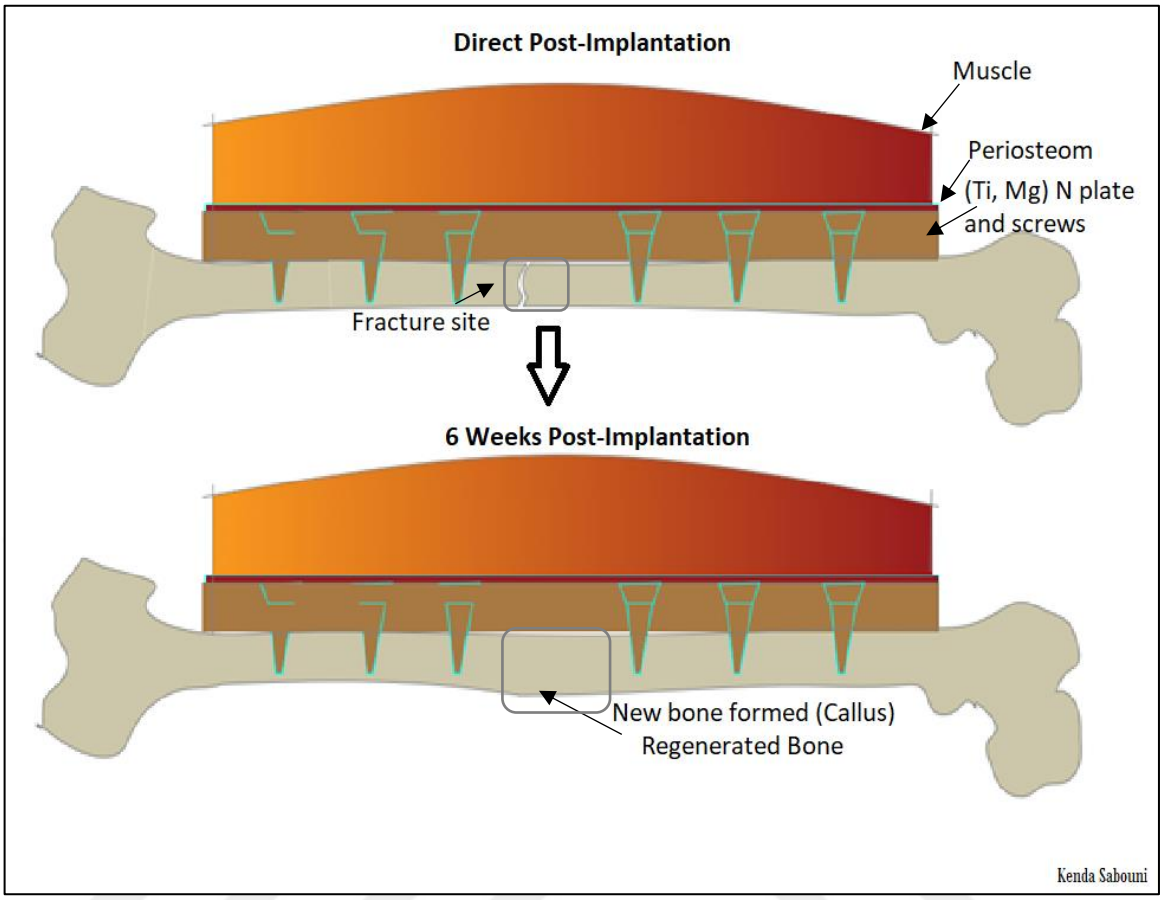


Figure 4.1. Bone remodeling during 6 weeks of implantation with (Ti, Mg) N coated plate**.**

4.1. *IN VIVO* EXPERIMENT

TiN (group A) and (Ti, Mg) N thin film coated plates (group B) were implanted and fixed on femur of rabbits (n=8). Throughout six weeks of implantation, it was observed that samples implanted with (Ti, Mg) N coated plates (group B) had greater callus density (Table 4.1). It may be due to the regeneration ability of magnesium coating.

In addition, the break line in bone specimen with (Ti, Mg)N coated plates (group B) was not appearant when compared to TiN coated plates (group A), which reveals that bone was repaired and became more like a healthy-compact bone under the use of Mg as shown in Figure 4.2. In literature, the osseointegration by implanting mesoporous TiO_2 coatings loaded with magnesium in the tibia of rabbits was investigated [73]. Post-operative results showed that the implants were well osseointegrated and the cortical bone was regenerated. This study also supports our results by showing that Mg increased the osseointegration of

plates to bone. In current study, none of the rabbits died during or after implantation. In addition, no disabilities in motion or any instability of plates to bones was observed. Figure 4.2 shows the bones after being dissected after first day and sixth week of implantation. They were compared within two groups before any further analysis. Broken bone can easily be seen in Figure 4.2 a,c at first day of implantation. At the end of the 6 weeks, bone ingrowths were observed around the plates (Figure 4.2 b,d).

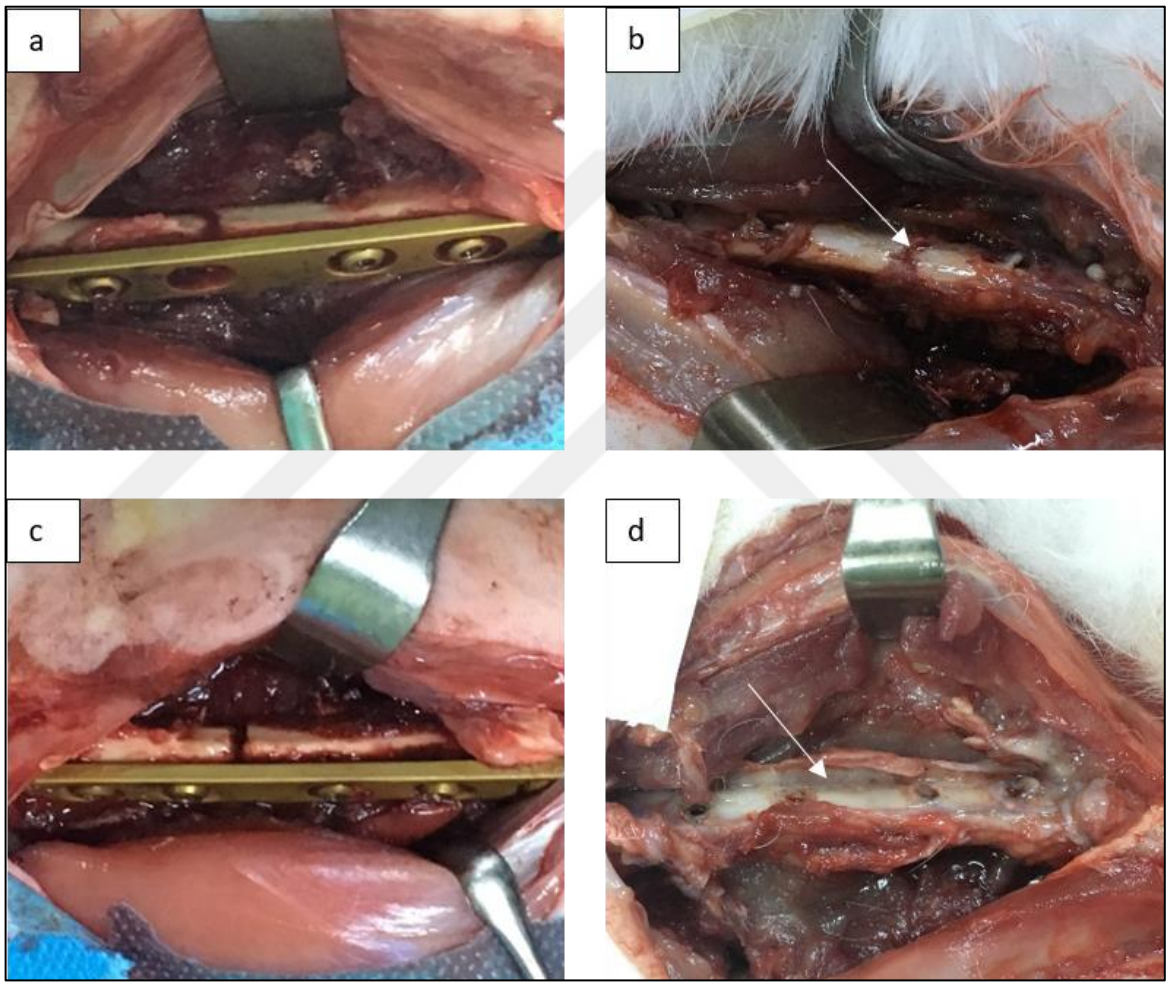


Figure 4.2. Implantation of TiN and (TiMg) N Coated Plates. (a, b) TiN, (c, d) (Ti, Mg) N coated sample. After implantation (a, c) day 1, (b, d) week 6.*Arrow line points on break site.

(Ti, Mg) N implanted bones (group B) showed significant remodeling capacity t as the control (Figure 4.3 a –i). A better osseintegration occurred at the bone and implant's interface with the use of magnesium as a coating layer as shown in (Figure 4.3 f -i). Previous studies

declared that the presence of magnesium alloy developed higher osseointegration rate and implant stability when compared to titanium implants [74].

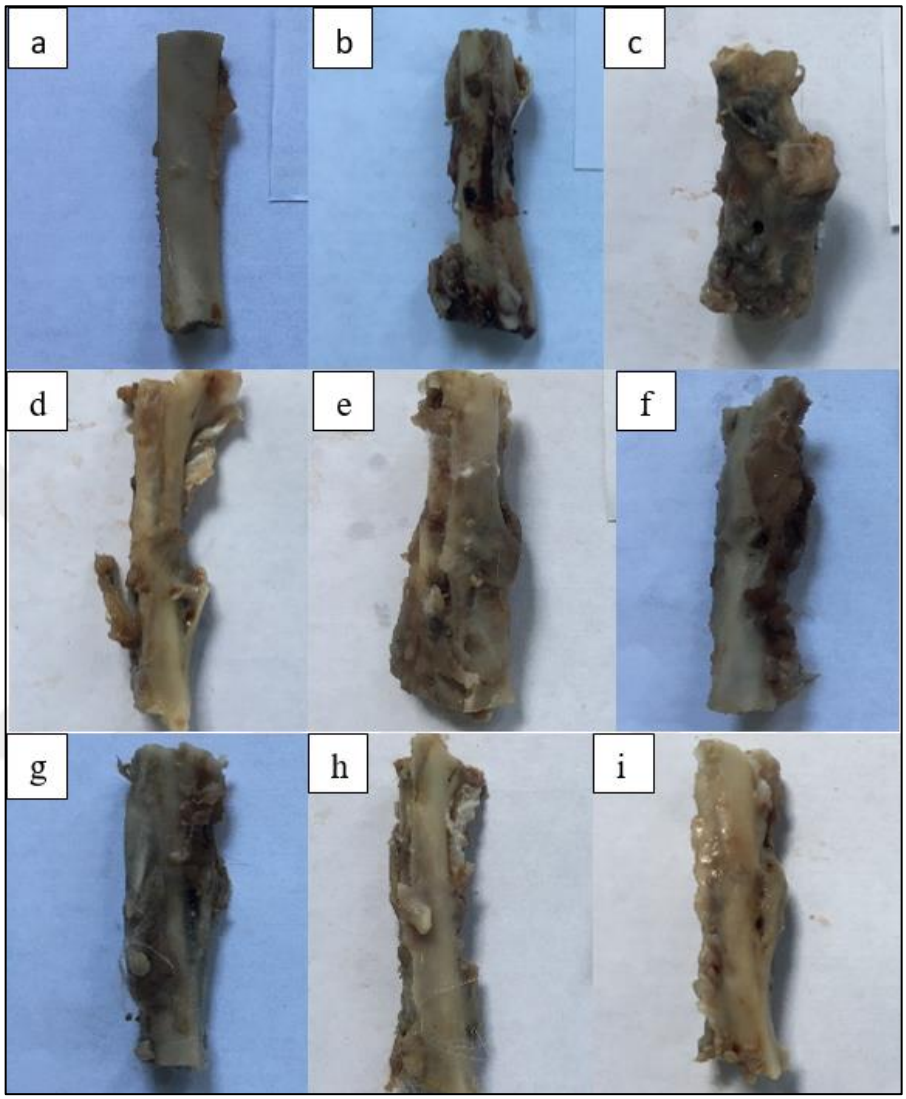


Figure 4.3. Dissected bones after six weeks of implantation for both group A and group B bone-implant samples. (a) control; bone samples that were incontact with (b -e) TiN, (f -i) (Ti, Mg) N coated plates.

4.2. X-RAY ANALYSIS

X-ray image was taken directly after the implantation one sample from each implant group (TiN or (Ti, Mg) N coated plates) to check the localization of implants. X-ray was re-taken after six weeks of implantation to assure the stability of implants. It can be seen from Figure

4.4 that there was no shifting of the implants which confirms a good fixation. It was observed that bone sample implanted with (Ti, Mg) N coatings (group B) had better regeneration capabilites when compared to the one implanted with TiN coated plates (group A). The break line in group B almost fully-regenerated and was no longer visible unlike TiN sample (Figure 4.4 a-d).

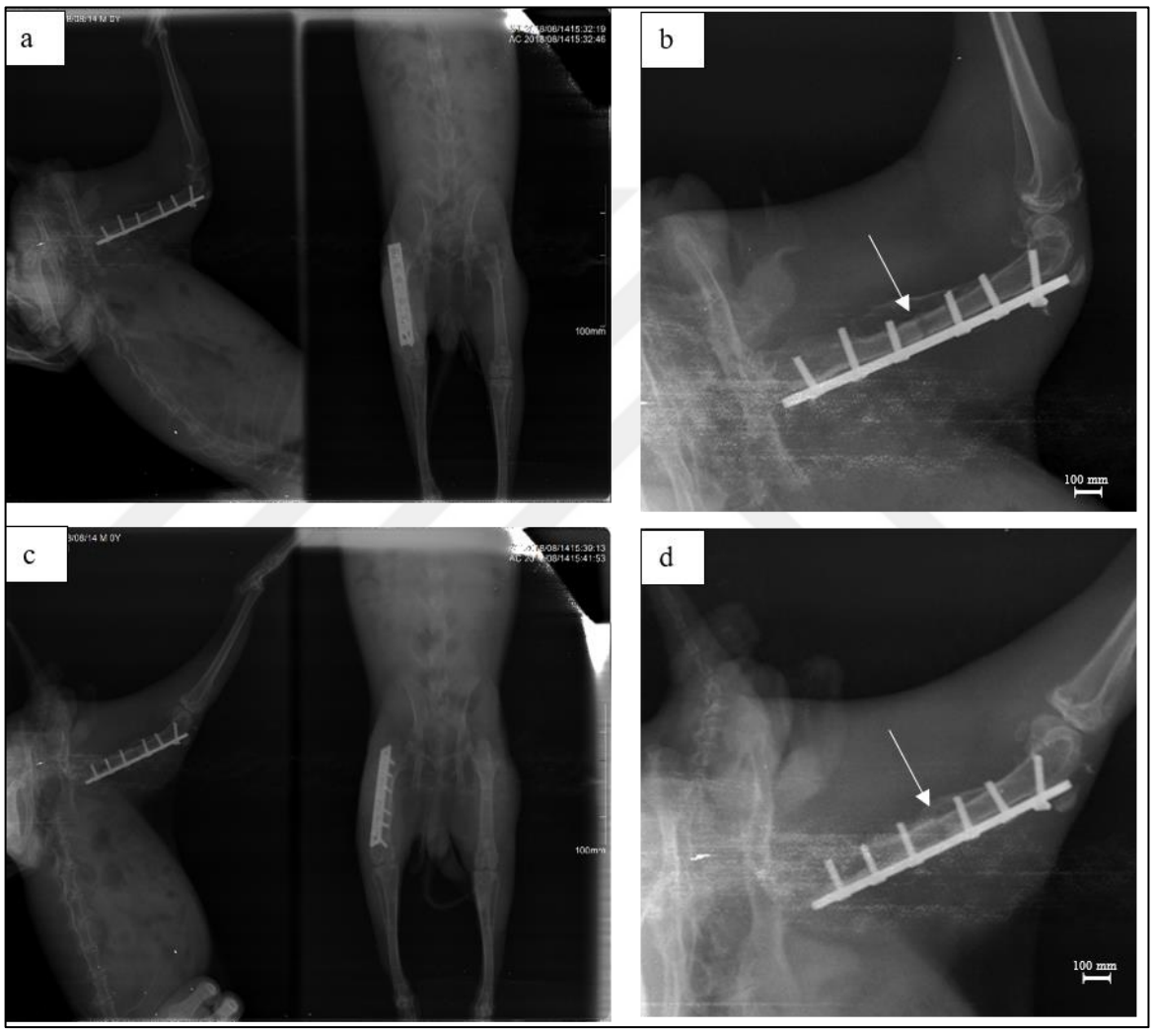


Figure 4.4. X-Ray images of plates after six weeks of implantation (a, b) TiN, (c, d) (Ti, Mg) N coated plates. *white arrow: break line.

4.3. CT-SCAN ANALYSIS

CT-Scan analysis was made to evaluate the bone remodeling and the ingrowth of new bone for all samples. This analysis was applied on all the 16 bone samples of both (TiN and (Ti,

Mg) N) groups. Through these evaluations, formation of new bone was easily visualized around the fracture site of each bone sample. This bone formation was observed in both TiN and (Ti, Mg) N coated plates. The newly formed bone tissue known as callus, grew around the fracture site and filled the defect. In addition, callus is known for its capacity to enhance bone formation which leads to further levels of remodeling [75]. After six weeks of implantation, the break line on bones that were fixed with TiN plates was still visible, whereas bones fixed with (Ti, Mg) N plates were completely fused together at the two ends of femur bone where fracture was made (Figure 4.5).

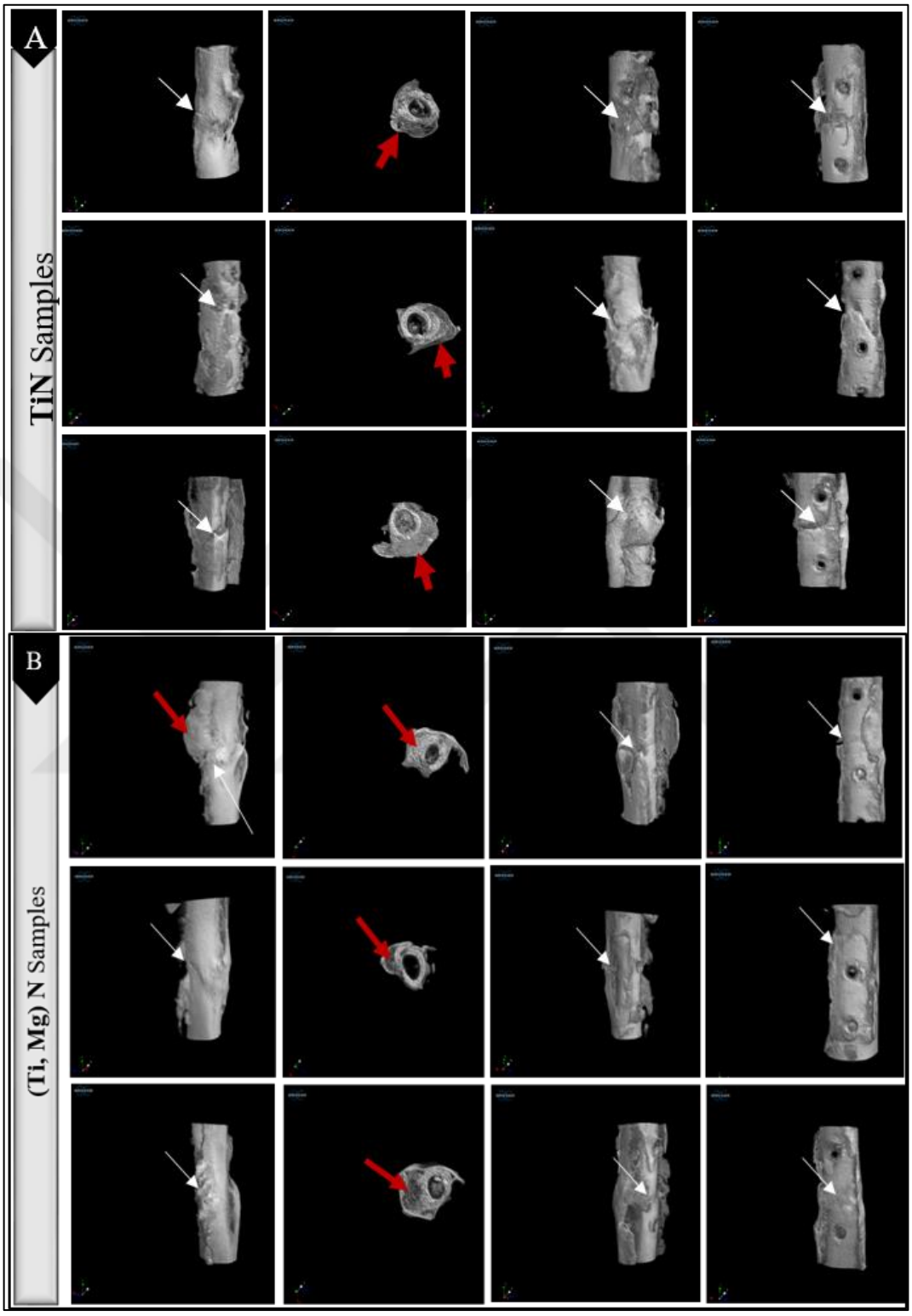


Figure 4.5. CT-Scan images after 6th week of implantation. TiN coated samples (group A), and (Ti, Mg) N coated samples (group B). *white arrows: break line. *red arrows: callus formation

A novel study sighted that Mg coated porous Ti6Al4V implants had significantly higher new bone formation through their Micro-CT evaluation, and it was statistically significant with ($P < 0.05$) value [76].

In our study, it was demonstrated that most samples of (Ti, Mg) N coatings led to the formation of denser and greater volumes of callus around fracture site than those of TiN. It clearly reveals the fact that Mg doped TiN coatings provided better remodeling potentials for bones in a way that it becomes closer to the normal-compact bone form with its tissue characteristics (Table 4.1).

Table 4.1. Callus volume/BMD and Bone volume/BMD of TiN and (Ti, Mg) N.

TiN	Callus Volume mm^3	CMD g/cm^3	Bone Volume mm^3	BMD g/cm^3	**(Ti, Mg) N**	Callus Volume mm^3	CMD g/cm^3	Bone Volume mm^3	BMD g/cm^3
1	319.70	0.43	348.93	1.221	**9**	797.61	1.22	411.00	1.168
2	229.98	0.48	317.30	1.225	**10**	471.51	0.43	409.00	1.219
3	223.54	0.42	311.91	1.224	**11**	513.63	0.40	317.01	1.217
4	933.69	0.30	364.66	1.204	**12**	443.77	0.49	396.39	1.216
5	280.98	0.40	280.71	1.226	**13**	1045.81	0.49	342.81	1.202
6	507.80	0.45	309.89	1.220	**14**	198.61	0.48	407.46	1.229
7	249.93	0.42	319.58	1.225	**15**	327.58	0.48	371.39	1.222
8	414.24	0.59	273.19	1.220	**16**	305.39	0.51	377.14	1.225
AVG	394.98	0.44	315.77	1.221	**AVG**	512.99	0.56	379.02	1.212
Control	-	-	307.29	1.211					

Moreover, some of the bones implanted with (Ti, Mg) N coated plates had less callus than the others in the same group, while having much better regenerated bone. TiN coated samples, on the other hand, mostly showed lesser amount of callus formation and lower regeneration capacity as it is shown below in (Figure 4.6 and Figure 4.7).

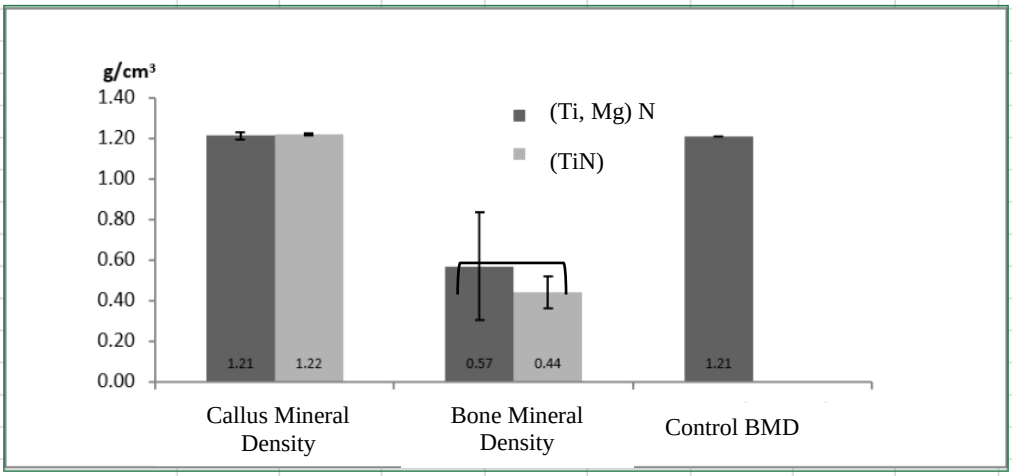


Figure 4.6. Callus density and BMD

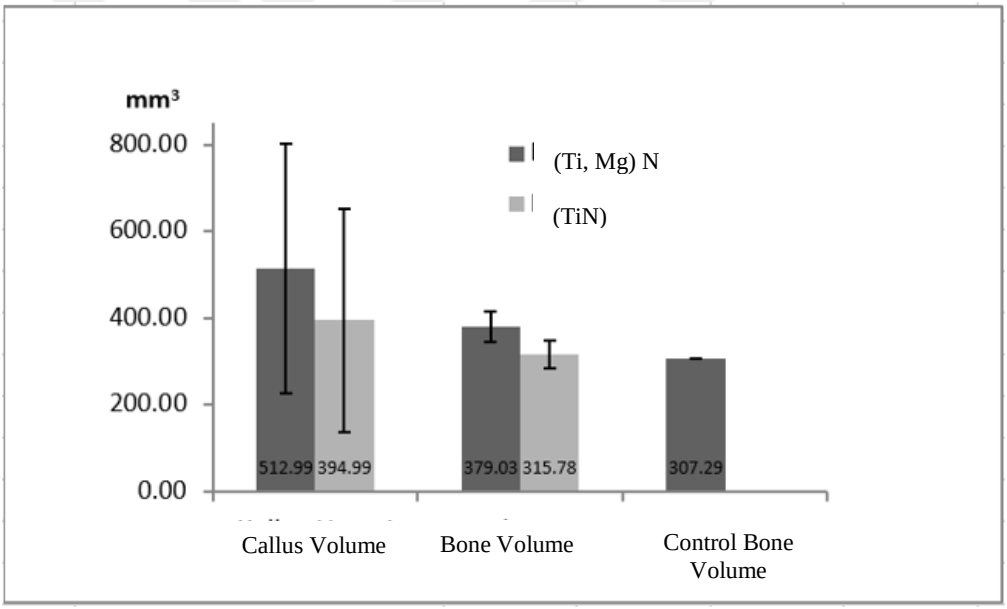


Figure 4.7. Callus and Bone volume. Statistically significant difference was labeled on the graph for $p \leq 0.05$.

It can be shown from Figure 4.6 and Figure 4.7, that (Ti, Mg) N bone samples had denser callus/bone and improved BMD which resembles increased bone strength when compared to TiN bone samples. To sum up, a better remodeling of bone was observed when magnesium coated plates was used. Mg release from (Ti, Mg) N coated plates enhanced new bone formation and accelerated healing process.

Statistical analysis using unpaired t-Test showed that there is statistical difference between TiN (group A) and (Ti, Mg) N (group B) regarding bone volume after 6 weeks of implantation (Table 4.2). This occurred in regard to better regeneration capabilities obtained with the presence of Mg. Other statistical values showed no significance difference between the two groups for callus mineral density (p-value = 0.22), BMD (p-value = 0.27), and callus volume (p-value = 0.37).

Table 4.2. t-Test analysis on TiN and (Ti,Mg)N samples for bone volume

	TiN	(Ti, Mg)N
Mean	315.7711	379.0244444
Variance	824.8011	1031.278328
Observations	9	9
Pooled Variance	928.0397	
Hypothesized Mean Difference	0	
df	16	
t Stat	-4.4046	
P(T<=t) one-tail	0.000221	
t Critical one-tail	1.745884	
P(T<=t) two-tail	0.000443	
t Critical two-tail	2.119905	

4.4. HISTOLOGICAL ANALYSIS

Four basic stages of bone healing process contribute eventually to a new bone formation [77]. Within hours blood clot formation occurs which provides the structural stability and basic framework for bone formation. Then phagocytes clear the injury from any bone fragments and germs to avoid any immune attack. Chondroblast cells basically start to form cartilage which is also called as soft callus. Soft callus act to full-fill the gap made between the two broken bones at the fracture site as immature woven bone (primary stage of bone formation). Osteoblasts later promote new bone formation, where soft callus becomes hard callus and mineralization occurs. At this stage woven bone is converted to secondary form of bone called as mature lamellar bone. Remodeling stage of bone healing continue and may last for years [77]. Histological results from a mesoporous Mg-loaded titania surfaces placed in the tibia of 10 rabbits, showed a progressed bone formation around the implant and

stronger attachment as well in the cortical region [78]. They declared that Mg enhanced osseointegration at an early stage of the healing process. Another study showed a better osseointegration in the early stages of implantation [79]. Titanium coated with magnesium-substituted hydroxyapatite implants were applied on rabbit's femur bone. Through histological evaluations, it was indicated that during the first two weeks of implantation a better osseointegration was obtained when compared to pure hydroxyapatite-titanium implants, and woven bone was formed in both implants. Thus, after 4 and 8 weeks of implantation there was no significant difference between implant and bone interface but both had mature bone formation at week 8. This indicates that magnesium supports bone healing at its early stages [79].

In this study, histology staining's' were conducted on all 16 bone samples. It was noticed after Hemotoxylin and Eosin stain that two different tissues; compact bone and cartilage were formed. TiN coated samples showed mostly cartilage tissue formation, where as those of (Ti, Mg) N coated samples showed both cartilage tissue and denser amount of compact bone formation (Figure 4.8). This highlights the known fact that magnesium acts to stimulate bone formation during early stages of new bone formation [80]. Throughout this study, it was noticed that Mg supported bone formation not only in pre-stages of healing but also in following repair and remodeling stages.

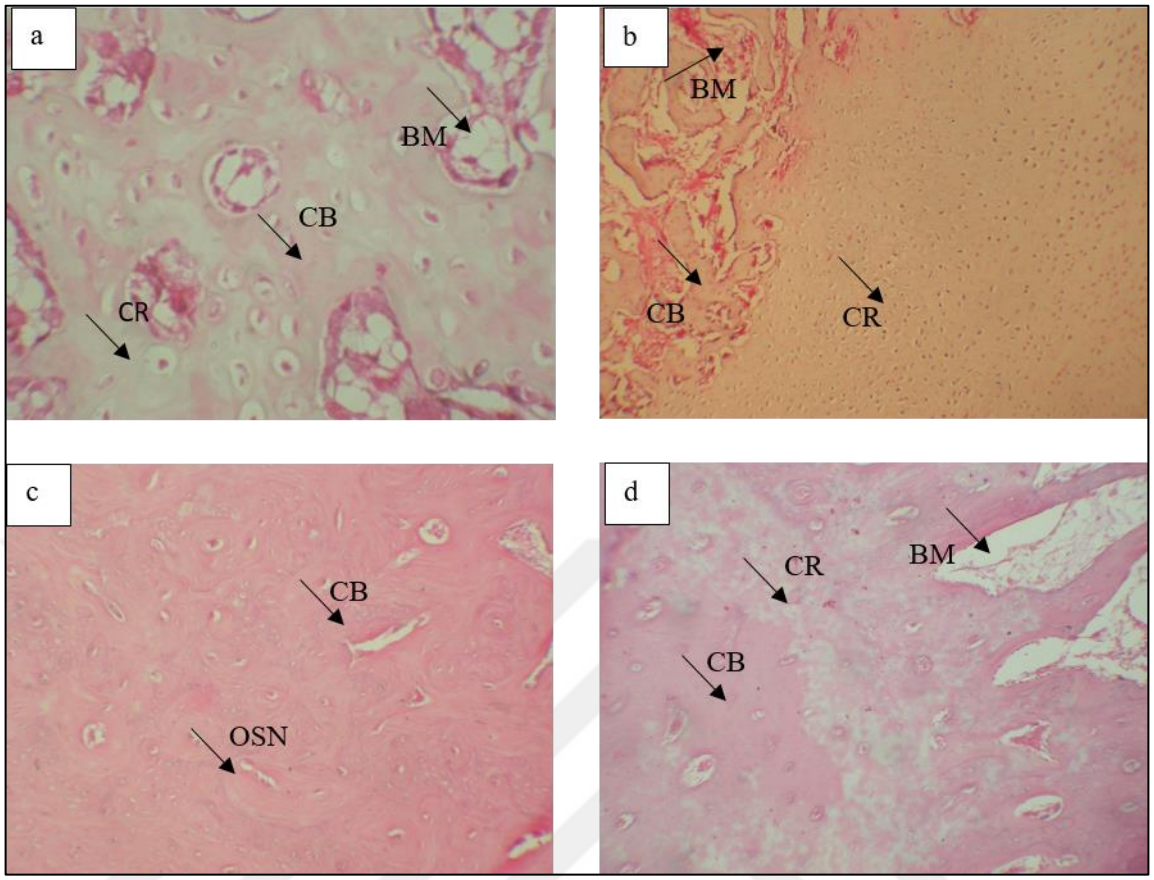


Figure 4.8. Hematoxylin & Eosin Staining of bone samples that were in contact with (a, b) TiN and (c, d) (Ti, Mg) N coated plates. (CB) Compact Bone, (CR) Cartilage, (BM) Bone Marrow, (OSN) Osteon. Objective: 10x.

Masson's trichrome staining showed that TiN coatings led to formation of many osteoids and woven bone formation, whereas (Ti, Mg) N coatings enhanced osteoblast formation, osteons, and lamellar bone formation (Figure 4.8). During lamellar bone formation, haversian canals (blood vessels) are also formed. Osteons are usually formed only in mature bone types [75]. On the other hand, immature bones are nonvascular and have less mineral content.

Through these histological images (Figure 4.8 and Figure 4.9) especially in TiN applied samples, bone marrow formation was observed. Bone marrow usually indicates the activation of blood vessels, and it resembles the beginning of new bone formation. It is later followed by the chondroblast formation (immature bone) that later contributes to the formation of mature compact bone.

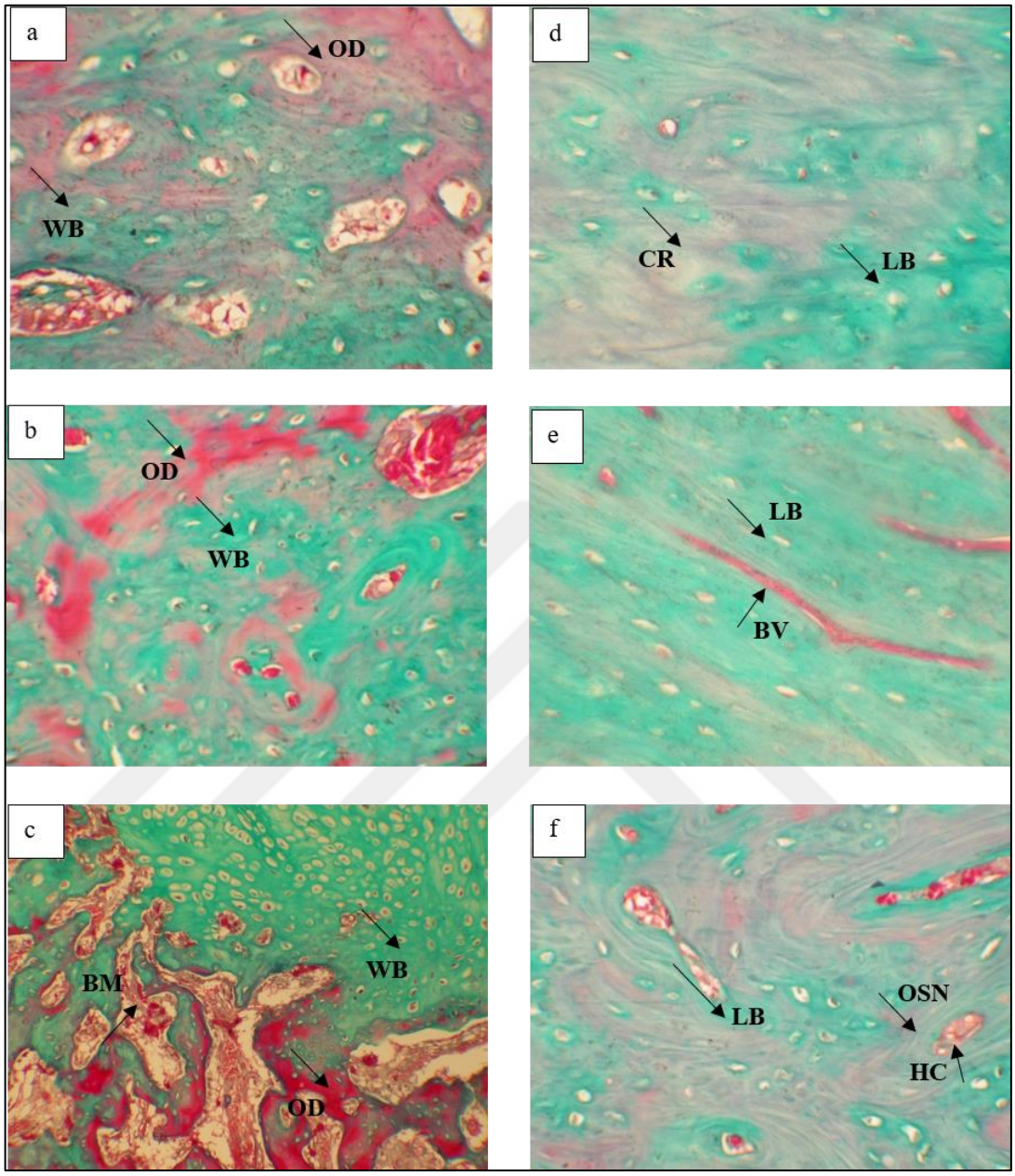


Figure 4.9. Masson's trichrome staining of bone samples that were in contact with (a, b, c) TiN and (d, e, f) (Ti, Mg) N coated plates. (WB) Woven Bone, (OD) Osteoid, (BV) Blood Vessels, (OSN) Osteon, (LB) Lamellar Bone, (HC) Haversian canal, (CR) Cartilage, (BM) Bone Marrow. Magnification objective: 10x.

Such results indicate that magnesium presence significantly enhanced osteoblast formation which led to secondary bone formation that took place in the early stages of bone healing process. In addition, vascularization of the bone apparently was provided. Getting to this stage of bone repair usually takes around 2 months. After the formation of lamellar bone,

blood vessel formation and mineralization take place [75, 77]. The results show that samples implanted with TiN coatings led to the primary bone regeneration, whereas (Ti, Mg) N coated samples provided the secondary stage of bone regeneration. In these histological evaluations it was observed that the formation of mature bone occurred successfully within only six weeks of implantations in (Ti, Mg) N coated samples because of local Mg release.

4.5. SEM ANALYSIS

Analysis of the plates coated with (TiN) and (Ti, Mg) N thin films after *in vivo* experiments was performed by SEM to evaluate the interaction between implant's surface and bone cells. 8 samples were used for this analysis, 4 from each coated plates (TiN and (Ti, Mg) N). SEM images were taken from both experimental (interfaced with bones) and control (non-interfaced with bones) groups. The presence of osteoblasts was observed on both TiN and (Ti, Mg) N thin film coated plate's surfaces (Figure 4.10 a -h).

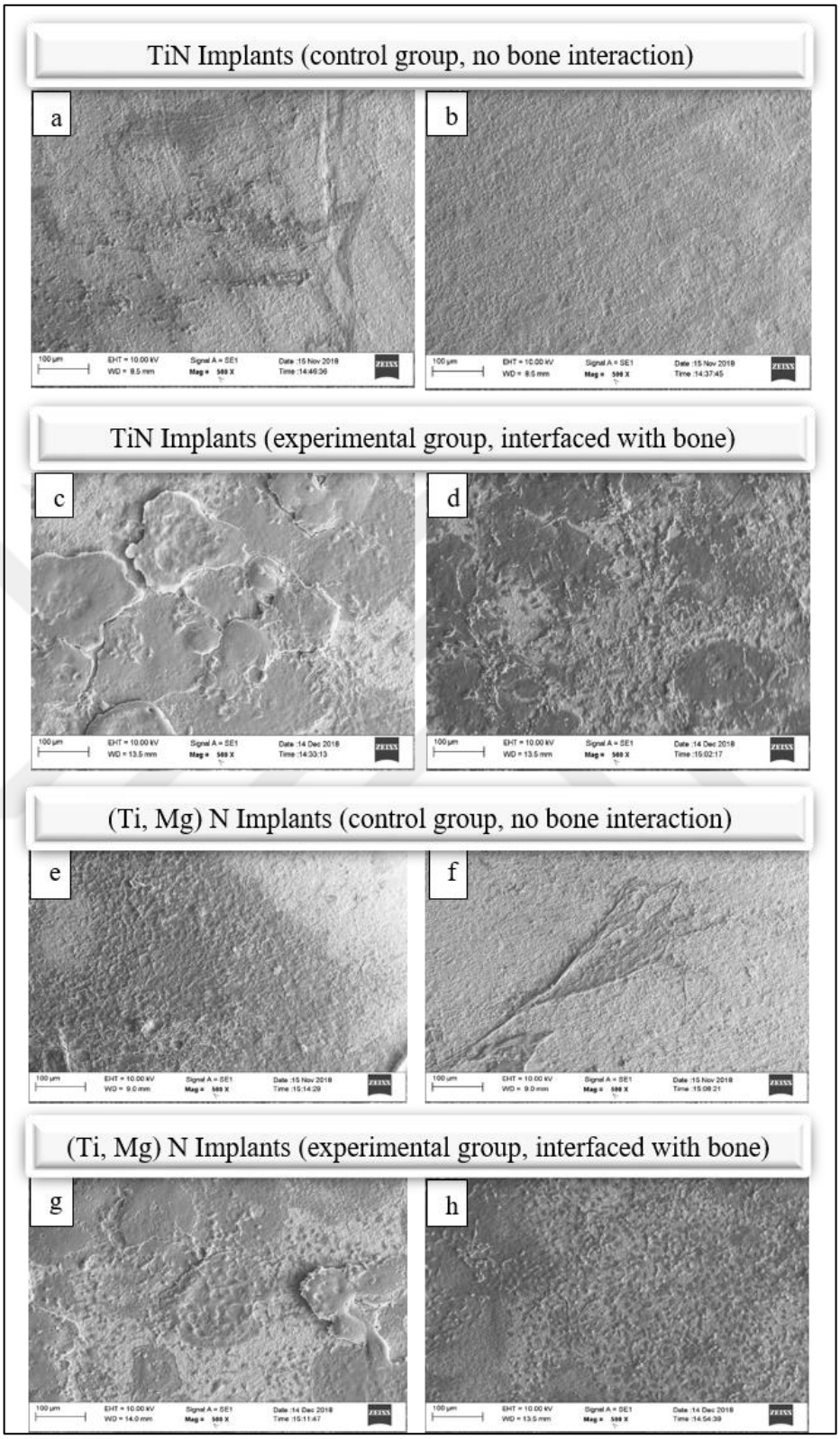


Figure 4.10. SEM images of the implant's surfaces for (a, b) TiN control group, (c, d) TiN experimental group, (e, f) (Ti, Mg) N control group (g, h), (Ti, Mg) N experimental group. Magnification: 500 x. Scale bar: 100 μm.

Through SEM images, the presence of osteoblasts was observed on both experimental groups of TiN and (Ti, Mg) N coated surfaces. Besides, (Ti, Mg) N thin film coated surfaces showed denser forms of osteoblasts than those on TiN coated surfaces (Figure 4.10 g). Magnesium is known for its role in bone metabolism and in osteoblast interaction with implant's surfaces. A study with Mg-incorporated oxide implants was performed in 2005 [81]. SEM and EDS evaluations of this study declared that these implants significantly improved bone tissue responses to implants and this was due to the addition of the biofactor Mg to the oxide implants. Bone response usually underlines the rate, quality, and quantity characterization of an implant's surface which effects initially the osseointegration [82]. Therefore, osteoblast cells were much denser and higher in number in (Ti, Mg) N coated implants than the ones in TiN coated implants (Figure 4.10). It was due to the addition of magnesium into the plates which improved the bone tissue response and osseointegration. The osteoconductivity of (Ti, Mg) N implants was observed by the presence of osteoblastic activity.

4.6. VON KOSSA STAINING

In addition to SEM, von Kossa staining was applied to check the attachment of bone to implants to get an idea about osseointegration (Figure 4.11). It was applied on 8 samples, four from each coated plates (TiN and (Ti, Mg) N) groups. Calcium deposits and stained areas showed the bone-implant interaction. Furthermore, calcium deposits declared osteogenic differentiation in both TiN and (Ti, Mg) N coated implants. In 2017, a novel study presented the use of Mg coated porous Ti6Al4V and bare porous Ti6Al4V implants on femoral condyle of rabbits [76]. Their fluorescent labelling results suggested that implants with Mg deposited higher calcification ratios than the one on pure Ti6Al4V implants at 4 and 8 weeks of post-surgery. It showed that Mg presence promoted mineralization and osteogenic differentiation.

In this study, it was observed that calcium deposits were less apparent in TiN with clusters of red stained nuclei, while calcium deposits were more apparent on (Ti, Mg) N implants. However, the reddish clusters were observed less (Figure 4.11). The control group resembles plates before applying stain on it. And negative control group of both samples describes the staining on the non-interfaced surfaces with bones. These groupings help to indicate the

difference of the staining on each surface. The observations ensures that magnesium supported osteogenic differentiation of osteoblasts as it was demonstrated in previous evaluations. It was also stated before that magnesium is involved in calcification and mineral metabolism in bones [83].

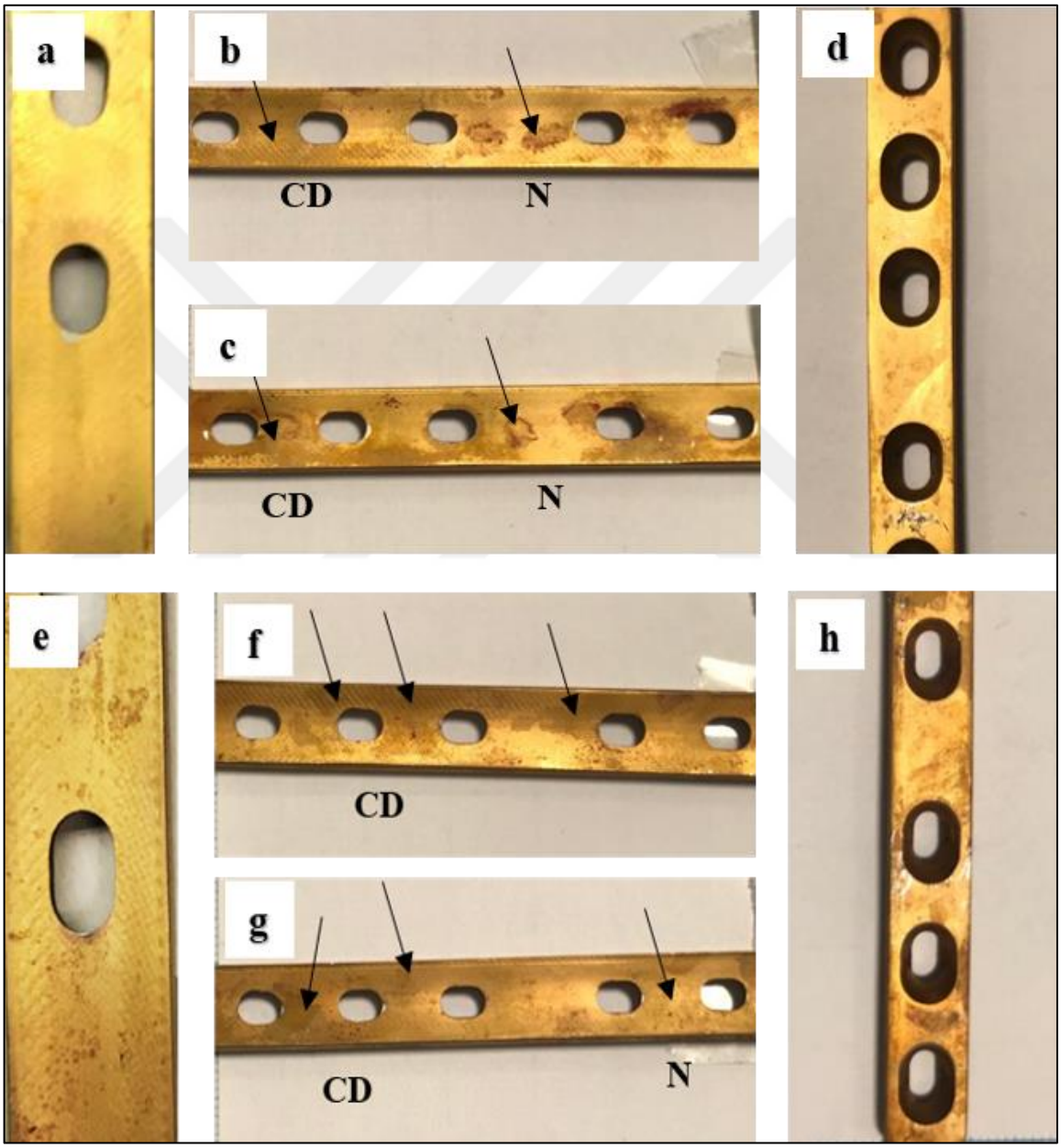
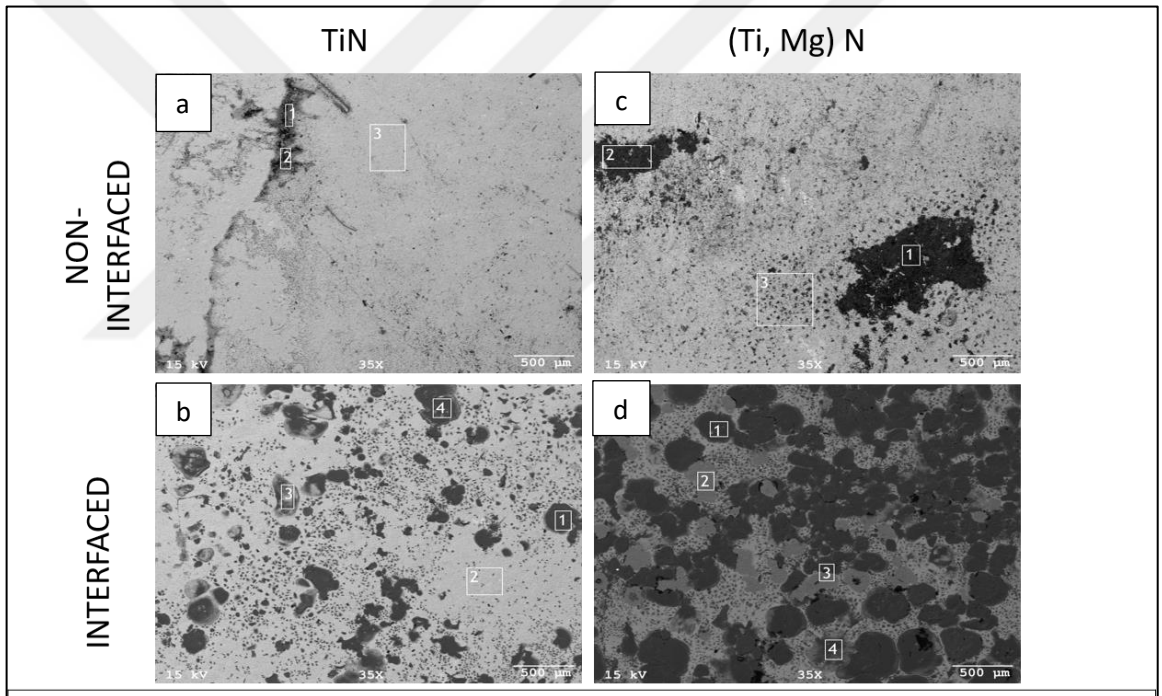


Figure 4.11. Bone-Implant interaction with von Kossa staining for TiN and (Ti, Mg) N plates. (a) TiN control for staining, (b, c) TiN experimental, (d) TiN negative control, (e) (Ti, Mg) N control, (f, g) (Ti, Mg) N experimental, (h) (Ti, Mg) N negative control; Calcium Deposits (CD), Nucleus (N).

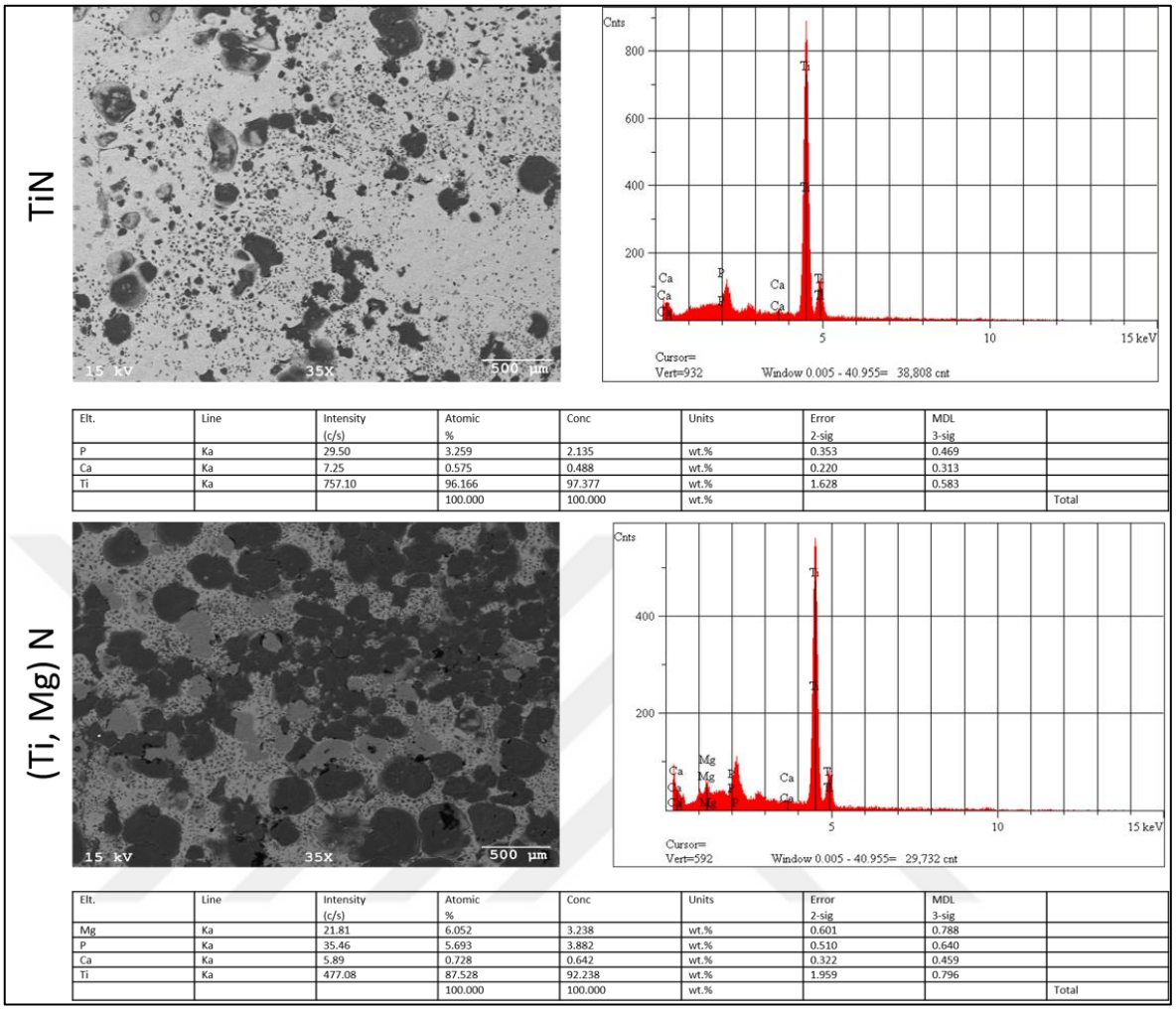
4.7. EDS ANALYSIS

EDS analysis was conducted to show the elements and chemical characterization of both TiN and (Ti, Mg) N coated plates. EDS was applied on for samples, 2 from each coated plates (TiN, (Ti, Mg) N) groups. Calcium, phosphate, and carbon deposits were frequently presented on both groups. Figure 4.12 shows that (Ti, Mg) N thin film coated plates enabled hydroxyapatite $Ca_5(PO_4)_3(OH)$ formation on the non-interfaced surface with bone and resembles that calcium and phosphate depositions were higher than those on TiN coated plates.



	TiN (Ca, Non-Interfaced)	(Ti, Mg) N (Ca, Non-Interfaced)	TiN (Ca, Interfaced)	(Ti, Mg) N (Ca, Interfaced)
	2.94	23.1	0.21	0.68
	1.01	16.84	0.65	0.2
	0.22	1.3	1.16	0.44
AVERAGE	1.39	13.74666667	0.673333333	0.44
STDEV	1.399249799	11.22437229	0.47542963	0.24
	TiN (P, Non-Interfaced)	(Ti, Mg) N (P, Non-Interfaced)	TiN (P, Interfaced)	(Ti, Mg) N (P, Interfaced)
	30.46	64.94	2.89	24.93
	12.25	54.89	9.87	3.15
	3.72	8.14	35.31	8.54
AVERAGE	15.47666667	42.65666667	16.02333333	12.20666667
STDEV	13.65889576	30.31172765	17.06346194	11.34351944

Figure 4.12. EDS evaluations of the plate surfaces. (a, b) TiN, (c, d) (Ti, Mg) N coated surfaces. 35X; 500 µm.



Elt.	Line	Intensity (c/s)	Atomic %	Conc	Units	Error 2-sig	MDL 3-sig	
P	Ka	29.50	3.259	2.135	wt.%	0.353	0.469	
Ca	Ka	7.25	0.575	0.488	wt.%	0.220	0.313	
Ti	Ka	757.10	96.166	97.377	wt.%	1.628	0.583	
			100.000	100.000	wt.%			Total

Elt.	Line	Intensity (c/s)	Atomic %	Conc	Units	Error 2-sig	MDL 3-sig	
Mg	Ka	21.81	6.052	3.238	wt.%	0.601	0.788	
P	Ka	35.46	5.693	3.882	wt.%	0.510	0.640	
Ca	Ka	5.89	0.728	0.642	wt.%	0.322	0.459	
Ti	Ka	477.08	87.528	92.238	wt.%	1.959	0.796	
			100.000	100.000	wt.%			Total

Figure 4.13. EDS evaluations of the whole plate surfaces (interfaced with bone) for TiN and (Ti, Mg) N coated surfaces. 35X; 500 µm.

More discussions were made around the interfaced surfaces of both group A and group B plates. As an overall measurement of the whole surface, it was shown that (Ti, Mg) N coated plates had more of Ca and P deposition than TiN coated plates Figure 4.13. Though, the difference could not be taken into full consideration since values are low and almost similar.

Carbon formation can be derived either from ECM of bones or from the collagen presented in bone tissue [84]. This happens because of the ultimate interface between the implant surface and bone during bone formation. In addition, magnesium presence enhanced the formation of Hydroxyapatite crystals on the implant surfaces as described by previous studies [85]. Therefore, these crystals increased the bone ingrowth and mass [86]. Also in

the present study, higher amounts of HA were available on (Ti, Mg) N coated plate surfaces (Figure 4.12. c). Hydroxyapatite formation was not clearly detectable on both experimental group surfaces, this could be due to the presence of cells on these surfaces and the binding between each of Mg, P, and Ca. Since calcium (Ca^{2+}) and magnesium (Mg^{2+}) have similar chemical composition that would allow binding of Phosphorus to Mg instead of Ca.

All evaluations declared that Mg coating had positive significant effects on the implant's surfaces. Mg not only enhanced new bone formation but also contributed to secondary bone formation. It also promoted vascularization and mineralization of fractured bones. Mg stimulated the activation of osteogenic signals and increased their expression through the protein interactions between the implant and bone. Also, Galli et al. supported this fact through his study on tibia and femur of osteoporotic rats [84]. They proposed that the titanium mini screws loaded with Mg promoted new bone formation on implants more abundantly. In addition, they supported their results with genetic expression analysis to find out that Mg promoted the activation of osteogenic signals and higher expressions of BMP2 were obtained. Also in our study, (Ti, Mg) N plates increased the quality of implants as for the hard tissue implantations.

5. CONCLUSION

Over the past years, bone fracture treatment has been improving with all the internal and external fixatives such as casts, plates, stainless steel pins, stabilizing rods and other types of implants [86]. Long bone fractures like femur and tibia, usually need to get stabilized with metallic plates and screws because of their weight and thickness. The biomaterial titanium is known for its high biocompatibility, mechanical stability, and strength among the others [87, 88]. Therefore, in order to improve the quality of these plates, new strategies are required to enhance bone regeneration and a better osseointegration capabilities for orthopedic surgeries. Accordingly, in this study, it was aimed to improve osseointegration by using magnesium doped TiN coatings on implants. Magnesium ions are known for their ability in providing proper bone growth and maintenance in bones [89]. In this study, *in vivo* experiment was held on the femur of 16 rabbits, where implantations of TiN and (Ti, Mg) N plates took place. X-ray images showed that the fracture in the bone with TiN plates was still apparent, whereas in (Ti, Mg) N implanted plates, the two ends of broken bone were firmly connected. In addition, CT-imaging and histology evaluations revealed that new bone formation was found around fracture line and bones were regenerated more likely around (Ti, Mg) N plates. Furthermore, histological analysis demonstrated that fibrocartilaginous callus, osteoids and woven bone were formed around TiN plates; whereas bony callus, lamellar bone, osteon formations were observed in (Ti, Mg) N plates. Moreover, SEM and von Kossa analysis showed that the presence of Mg influenced mineralization and osteoblast formation which eventually led to increased bone - implant bonding. EDS evaluations showed that (Ti, Mg) N coated plates enabled hydroxyapatite formation. These results ensure that magnesium ions significantly stimulated fast healing process with better bone remodeling abilities and higher quality of osseointegration. In conclusion, our implant design and components supported the previous *in vitro* and recent *in vivo* experiments, which revealed the fact that (Ti, Mg) N plates and screws can serve as a hard tissue implants for defected bones in further clinical applications.

6. FUTURE PROSPECTS

The design made on Mg-coated titanium metallic implants, showed significant results regarding bone healing processes. However, further experiments should be held to express the functionality of those (Ti, Mg) N plates. Furthermore, bone and implant interactions must be tested. Also, longer period of *in vivo* experiments must be performed to test the effects of complete bone regeneration around implant site and its adverse effects if available later. Moreover, clinical relevance and efficacy should be tested on larger animals such as dog or sheep in order to examine the mechanical loading of an implant to defected bone which is considered critical for an implant success. This helps in supporting pre-clinical trials before translating this study into human clinical trials.

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APPENDIX A: ETHICAL APPROVAL FORM

T.C
İSTANBUL ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU

Sayın Prof. Dr. Seyhun SOLAKOĞLU
İstanbul Üniversitesi İstanbul Tıp Fakültesi

Başvuru : 17.02.2017

Toplantı Tarihi : 23.02.2017

Sorumluluğunu üstlendiğiniz, aşağıda çalışma materyali belirtilen, Yard. Doç. Dr. Sakip ÖNDER'e ait "Magnezyum Katkılı TiN Kaplamaların in vivo Koşullarda Osteoentegrasyon Özelliklerinin Belirlenmesi" isimli projeniz Kurulumuz tarafından incelenmiş ve Etik Kurul ilkelerine uygun bulunmuştur.

Çalışılacak Hayvanın	Türü	Tavşan
	Cinsiyeti	Erkek
	Sayısı	32
Proje Başlangıç/Bitiş Tarihi		01.06.2017/ 01.02.2019

Prof. Dr. Alev AKDOĞAN KAYMAZ
İÜ HADYEK Başkanı

Prof. Dr. Mehmet YALTIRIK
Üye

Prof. Dr. Ufuk ÇAKATAY
Üye

Prof. Dr. İlhan İLKILIÇ
Üye

Doç. Dr. Alper OKYAR
Üye

Doç. Dr. Aygül EKİCİ
Üye

Doç. Dr. Uğur AKSU
Üye

Yard. Doç. Dr Altan ARMUTAK
Üye

Yrd. Doç. Dr. Aydın ÇEVİK
Üye

Uzm.Vet.Hek.Fatma TEKELİ
Üye

Dr. Burak OLGUN
Mak.Yük. Müh
Üye

Avukat Selma DEMİR
Üye

Tarih ve Sayı: 20/03/2017-108787

BE6P6Y20V

T.C.
İSTANBUL ÜNİVERSİTESİ REKTÖRLÜĞÜ
Hayvan Deneyleri Yerel Etik Kurul Başkanlığı

Sayı :35980450-050.01.04-
Konu :Prof.Dr.Seyhun SOLAKOĞLU'na ait Etik Kurul Onayı

Sayın Prof. Dr. Seyhun SOLAKOĞLU

İlgi :17.02.2017 tarihli proje başvurunuz.

Sorumluluğunu üstlendiğiniz **Yrd. Doç. Dr. Sakıp ÖNDER**'e ait "**Magnezyum Katkılı TiN Kaplamaların in vivo Koşullarda Osteocntegrasyon Özelliklerinin Belirlenmesi**" başlıklı projeniz Etik Kurul'umuzun 23.02.2017 tarihli toplantısında görüşülmüş olup, Etik Kurul Onayı ilişikte gönderilmiştir.

Bilgilerinizi rica ederim.

e-İmzalı
Prof. Dr. Fatma Alev KAYMAZ
Başkan

17/03/2017 B.İşl. : C.SÖNMEZTÜRK

Ayrıntılı bilgi için irtibat : Canan SÖNMEZTÜRK
İstanbul Üniversitesi Hayvan Deneyleri Yerel Etik Kurul Başkanlığı İ.Ü. Veteriner Fakültesi Dekanlık katı A 221 nolu oda Avcılar/İSTANBUL
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APPENDIX B: STATISTICAL ANALYSIS FOR TiN AND (Ti, Mg) N

Table B.1. t-Test analysis on TiN and (Ti,Mg)N samples for bone mineral density

	TiN BMD	**(Ti, Mg)N BMD**
Mean	1.220625	1.21225
Variance	5.08E-05	0.000383357
Observations	8	8
Pooled Variance	0.000217	
Hypothesized Mean Difference	0	
df	14	
t Stat	1.136807	
P(T<=t) one-tail	0.137352	
t Critical one-tail	1.76131	
P(T<=t) two-tail	0.274703	
t Critical two-tail	2.144787	

Table B.2. t-Test analysis on TiN and (Ti,Mg)N samples for callus mineral density

	TiN CMD	**(Ti, Mg)N CMD**
Mean	0.43625	0.5625
Variance	0.006598	0.071878571
Observations	8	8
Pooled Variance	0.039238	
Hypothesized Mean Difference	0	
df	14	
t Stat	-1.27469	
P(T<=t) one-tail	0.111584	
t Critical one-tail	1.76131	
P(T<=t) two-tail	0.223168	
t Critical two-tail	2.144787	

Table B.3. t-Test analysis on TiN and (Ti,Mg)N samples for callus volume

	TiN Callus volume	**(Ti, Mg)N Callus volume**
Mean	394.9825	512.98875
Variance	57089.57408	78246.78824
Observations	8	8
Pooled Variance	67668.18116	
Hypothesized Mean Difference	0	
df	14	
t Stat	-0.907282958	
P(T<=t) one-tail	0.18980456	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	0.37960912	
t Critical two-tail	2.144786688	