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HISTOMORPHOMETRIC EVALUATION OF THE EFFECT OF LOCAL AND SYSTEMIC APPLICATION OF BORIC ACID ON BONE HEALING IN RATS

DOCTOR OF PHILOSOPHY THESIS

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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 01.03.2021 and numbered 2021/02-05.

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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.

Nevzat Sezer Işıksaçan

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LIST OF SYMBOLS AND ABBREVIATIONS

BSP II	Bone sialoprotein II
ALP	Alkaline phosphatase
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B Ligand
OPG	Osteoprotegerin
M-CSF	Macrophage colony-stimulating factor
TNF-α	Tumor necrosis factor-α
IL-1	Interleukin-1
IL-11	Interleukin-11
IL-6	Interleukin-6
IL-18	Interleukin-18
TNFR	Tumor necrosis factor-α receptor
VEGF	Vascular endothelial growth factor
MSC	Mesenchymal stem cells
BMP-2	Bone morphogenic protein 2
BMP-7	Bone morphogenic protein 7
BMP	Bone morphogenic protein
TGF-β	Transformation growth factor beta
NADP	Nicotinamide adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
BA	Boric acid
SPSS	Statistical Packages of Social Sciences
MC3T3-E1	Osteoblast precursor cell line derived from mouse calvaria
OCN	Osteocalcin
COL I	Collagen type

ABSTRACT

ISIKSACAN, NS (2020) Histomorphometric evaluation of the effect of local and systemic applied boric acid on bone healing on rats. Yeditepe University Institute of Health Sciences, Oral and Maxillofacial Surgery Department. Doctorate Thesis.

In this study, it was aimed to evaluate the short and long term effects of bone regeneration and healing capacity, local, systemic and combination administration as a result of applying boric acid solution locally directly and systemically to the rats with critical size defects created in rat calvaria.In this study, 5 mm diameter critical-sized full thickness defects were created with trefan burs in symmetrical position to the bregma point on the parietal bone in 28 rats. The defects to the left of the skull were left empty, and 3% boric acid solution was applied to the right defects. Boric acid was applied to half of the animals by systemic gavage method. Half of the rats were sacrificed on the 14th day and the other half on the 28th day. In this way, bone samples, which emerged with 8 different applications, were obtained. Different applications were; only systemic application of boric acid, only local application of boric acid, combination of systemic and local boric acid application, and control group. These application variety combined with the two different time periods, resulted in eight experimental groups. The bones were decalcified and evaluated histomorphologically.As a result of the study, boric acid application to the living tissues could be beneficial in preventing excessive amount of connective tissue accumulation and new bone formation. In addition, systemic application of boric acid solution in terms of preventing fibrosis formation ended a statistically significant difference in the 28-day period. On the other hand, combined (systemic and local) application has been shown to reduce systemic effectiveness. When the results are evaluated, it is concluded that boric acid is an effective anti-inflammatory agent and its bone-building effect is limited. Further studies are needed to investigate the effect of different doses on bone formation and fibrosis prevention.

Key words: boric acid, fibrosis, critical size defect, bone regeneration

ÖZET

ISIKSACAN, NS (2020) Lokal ve sistemik olarak uygulanan borik asitin sıçanlarda kemik iyileşmesi üzerine etkilerinin histomorfometrik olarak değerlendirilmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Ağız, Diş ve Çene Cerrahisi ABD. Doktora Tezi

Çalışmamızda, borik asit çözeltisinin sıçan kalvaryasında oluşturulan kritik boyutlu defektlere lokal olarak doğrudan ve sistemik olarak sıçanlara gavaj yöntemiyle uygulanması sonucunda kemik rejenerasyon ve iyileşme kapasitesinin, lokal, sistemik ve kombine olarak uygulamasının kısa ve uzun süreli etkilerinin değerlendirilmesi amaçlanmıştır.Çalışmamızda 28 sıçanın kafatasında parietal kemik üzerinde bregma noktasından geçen orta hattın her iki tarafına simetrik olacak şekilde 5mm çapında kritik boyutlu tam kalınlıklı defektler trefan frez ile oluşturuldu. Tüm hayvanların kafatasının solundaki defektler boş bırakılırken, sağ defektlere ise %3'lük borik asit çözeltisi uygulandı. Hayvanların yarısına sistemik gavaj yöntemiyle borik asit uygulandı. Sıçanların yarısı 14. günde, diğer yarısı 28. günde sakrifiye edildi. Bu şekilde 8 farklı uygulama ile ortaya çıkan kemik örnekleri elde edildi. Sadece sistemik borik asit uygulaması, sadece lokal borik asit uygulaması, kombine sistemik ve lokal borik asit uygulaması ve kontrol grubu oluşturuldu. Bu oluşturulan gruplar iki farklı sürede değerlendirilerek sekiz farklı deney grubu elde edildi. Kemikler dekalsifiye edilerek, histomorfolojik olarak değerlendirildi.Çalışmamızın sonucunda, canlı dokulara borik asit uygulanması, yüksek miktarda bağ dokusu birikiminin önlenmesi ve kemik oluşumunun indüklenmesi noktalarında faydalı olabilmektedir. Fibrosis oluşumunu önlemesi anlamında borik asit çözeltisinin sistemik uygulanması 28 günlük dönemde istatiksel anlamlı fark oluşturmuştur. Bu sonuca ek olarak kombine (sistemik ve lokal) uygulamanın sistemik etkinliği azalttığı görülmüştür. Sonuçlarımız değerlendirildiğinde borik asitin etkin bir anti-enflamatuvar ajan olduğu kemik oluşumunu arttırıcı etkisinin limitli olduğu sonucuna varılmıştır. Farklı dozların kemik oluşumu ve fibrozisin önlenmesi üzerindeki etkisini araştırmak için daha fazla çalışmaya ihtiyaç vardır.

Anahtar kelimeler: Borik asit, fibrosis, kritik boyutlu defekt, kemik oluşumu

1. INTRODUCTION and PURPOSE

Bone tissue is a specialized connective tissue forming the skeletal system that acts as body support. In addition, it also contains the bone marrow, where blood cells are formed[1]. Bone healing is one of the most debated subjects in oral and maxillofacial surgery. Although the injured bone tissue has the ability to regain its shape and function, the existing healing potential may be insufficient due to trauma, infection, pathological occurrences, congenital anomalies, or extensive damage caused by surgical interventions[2,3]. In the treatment of bone defects, different surgical techniques are applied to mechanical and bio stimulating systems to accelerate the recovery[4]. Augmentation with graft materials has been reported as a successful treatment option for bone defects[5]. The major bone grafting options are autogenous bone, allogenic, and xenogenic bone. Among these methods, autogenous bone grafts obtained from the organism itself are considered as the gold standard due to their osteogenic, osteoinductive, and osteoconductive effects. However, the disadvantages of autogenous grafts, such as a limited amount of exposure and the formation of another surgical site, led the researchers to different materials[2,3,6–8]. In order to obtain new bone tissue, various allogenic, heterogenic and alloplastic graft materials have been produced. The osteoinductive effect of allogenic bone grafts is of great importance in their use as an alternative to autogenous grafts. While it has advantages such as the absence of a second wound, lack of quantity limitation, potential rejection, rapid resorption and carrying the risk of infectious disease are important disadvantages that can be encountered in the use of allogenic grafts [2,3,6–8]. The disadvantages of allografts have led researchers to xenografts, which are a form of heterografts, bone grafts of animal origin. Xenografts have only an osteoconductive effect. Biomaterials with osteoconductive effect cannot stimulate new bone formation by transforming mesenchymal cells into osteocytes, they only create a roof in the wound area and prepare a place for new bone formation [3,6,8].

Boron shows a regulatory function in the absorption of several micronutrients such as, calcium, magnesium, phosphorous, and vitamin D. These micronutrients are related to the bone metabolism directly. The deficiency of this molecule effect the bone regeneration and development[9–11]. Boron deprivation can negatively affect bone metabolism. Hunt et. al. evaluated boron supplementation effect on growth in chickens, and reported that boron deprivation led to decrease in chondrocyte density and delaying

calcification of cartilage pieces[12]. Another study evaluated the physical resistance force of the bones between boron applied and non-applied groups, they reported that boron applied group was more resistant to the breaking force till the fracture occur. In addition, deprived group was presented reduction of bone volume and trabecular thickness[13,14]. Moreover, boron plays role on regulation of messenger RNA (mRNA) synthesis. Regulation of this molecule affects extracellular proteins and mineralized tissue proteins such as, osteocalcin, osteopontin and collagen type I [15]. In the literature research, it was noticed that local application of boron in the living tissue was not done before.

Therefore, the purpose of this study was to assess the local application of the boric acid and its effectiveness on the living bone tissue, in the terms of healing period and also to compare with the systemic application. Moreover, it was aimed to evaluate the side effects of the boron.

2. LITERATURE REVIEW

2.1 Bone Tissue

The term “bone” refers to a specific type of mesenchymal tissue or structures comprised of bone tissue[16]. Bone tissue, the main structure of the skeletal system, has the resistance to pressure, shrinkage, and bending, as well as the dynamics of continuous destruction and re-formation[17]. Also, it is very well known that, bone has an important role in the body such as, movement, support, and protection of soft tissues. Moreover, bone tissue stores calcium and phosphate and harbors bone marrow[18,19]. Developing and adult bones have a definite gross appearance composed of a mineralized organic matrix, which consists 28% type I collagen by weight, deposited in a woven or lamellar pattern, and cells that in combination are organized into compact or trabecular bone with inorganic matrix accumulation[16,20].

Bone tissue involvement to mineral homeostasis, especially calcium and phosphorus homeostasis, is vital to life, and the structural properties of bone are essential for movement, acting as leverage for muscle movement and organ protection. In addition, bones form the framework of our bodies, thereby giving it size and shape and provide a nurturing storehouse for the hematopoietic elements. Lastly, the cells of bone have been shown to show a key role in energy metabolism, the renal excretion of phosphate and male growth and maturation. Accordingly, bones have four basic functions: (1) storage for elements and minerals, (2) mechanical anatomy for movement and shelter of viscera, (3) a household for hematopoietic tissue, and (4) an endocrine organ that helps regulate important biologic processes[16].

2.1.1 Types of Bone

Histologically, bone tissue, disregarding whether it is cortical or cancellous, normal or part of a pathologic progression, is classified into woven (primary bone) and lamellar (secondary bone) forms on the basis of the organization of its type I collagen fibers which are the major structural proteins of bone tissue. In woven bone, the collagen fibers are organized in a apparently haphazard felt-work, while in lamellar bone they are accumulated in parallel design either longitudinally or circumferentially around haversian canals forming osteons or haversian systems[16].

Microscopic evaluation of bone, unrelatedly of whether it is cortical or cancellous, in cross section shows two types: primary bone (immature) and secondary bone (mature)[1]. The categorization of bone tissue is made according to the formation of the type I collagen fibers which are the main structural proteins of bone tissue[16].

2.1.1.1 Primary Bone Tissue

Woven bone is produced at the time of rapid bone growth or formation phases. It forms parts of both the cortex and trabeculae of the bony skeleton that develops during embryogenesis, and parts of the bone in the growing baby and adolescent. It might also be the major type of bone that occurs in fracture repair[16]. It is characterized by the random positioning of fine collagen fibers[1]. In addition to these, primary bone tissue has a lower mineral content and higher amount of osteocytes then secondary bone tissue[1]. Overall, this fundamental organization facilitates woven bone to struggle forces evenly in all ways and simplifies quick formation, mineralization, and resorption[16].

Primary bone tissue is the first tissue type to develop in embryonic advancement and fracture healing.

2.1.1.2 Secondary Bone Tissue

Normally, mature skeletal tissue consists only of lamellar bone. Lamellar bone, unlike woven bone, is manufactured slower, less cellular, and its osteocytes and lacunae are smaller, and collagen is distributed more systematically across lamellas. In addition, it differs from the progress of woven bone, in that the mineralization of lamellar bone progresses more slowly and continues for a long time after the organic matrix is first deposited. Furthermore, the mineral deposits are localized almost exclusively within the collagen fibers and are first accumulated within the spaces, or “hole regions,” between the ends of adjacent collagen fibers[16].

2.1.2 Bone matrix

Bone, which contains both organic and inorganic materials, is different from all other tissues in the body. Bone cells and proteins are the building block of the organic matrix, and the inorganic part consists of hydroxyapatite[16]. Hydroxyapatite is the richest, but bicarbonate, magnesium, citrate, sodium and potassium are also located in inorganic

matrix[1]. The combination of the mineral materials with the organic base specifies bone with toughness, strength, and restricted elasticity[21].

2.1.2.1 Inorganic Matrix

Half of the dehydrated mass of the bone matrix contains inorganic material, which has magnesium, potassium, sodium, bicarbonate, citrate and hydroxyapatite[1]. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], in which phosphate and carbonate replace with hydroxyl groups is the primary mature unit of bone inorganic matrix. Mature crystals contain minimal amount of water. These units are the major stock for the calcium and phosphate for the organism. It covers 85% of the body's phosphorous and 99% of the body's calcium[22]. On the other hand, there is a layer around the crystal hydroxyapatite called the hydration shell. This layer, which contains hydrated surface ions and of this crystal structure, enables the interchange of ions between the body fluids and the crystal[1].

2.1.2.2 Organic Matrix

The organic matter mass is approximately 35% in the wet weight of bone[16]. This organic form includes proteoglycan aggregates and several specific multi adhesive glycoproteins and collagen types[1]. Collagen type I, which is 90% of organic component of the bone is the primary structural protein of bone. In addition to these, type III, V, XI, and XIII are also found in bone organic matrix but in much smaller quantities. In pathologic conditions the amount of type III collagen may be increased[16,23].

The proteins which are non-collagenous are categorized bestowing to their functions as calcium attaching proteins, growth factors, mineralization proteins, receptors, cytokines, enzymes[24]. These proteins are exceptionally important to the biological continuity of the bone and regulate all features of bone cell activity[16,24]. Some of these elements are manufactured and released by osteoblasts and some others are originated and concentrated from the serum. Calcium-binding glycoproteins, especially osteocalcin, were secreted by osteoblasts to stimulate calcification of the matrix[1,16]. In addition, osteocalcin functions as a hormone that is involved in the regulation of glucose and insulin metabolism as well as male development and fertility[25].

2.1.3 Bone cells

There are four kinds of cells in the bone: osteoblast, osteoclast, osteocytes and osteoprogenitor cells. Osteoclasts differ in many respects from the other three cells. These three mesenchymal cells can be defined as different functional stages of the same cell type. Osteoclasts, on the other hand, originate from bone marrow monocytes in the circulatory blood[1,26].

2.1.3.1 Osteoprogenitor Cells

Osteoprogenitor cells are of mesenchymal origin, which have not yet differentiated into a specific tissue cell and are capable of proliferation throughout their lives. Peri-anlage tissue of fetuses, the haversian systems, and Volkmann's, medullary canals and the periosteum are sited locations for the mesenchymal stem cells. Osteoprogenitor cells have the capability to fabricate only osteoblasts with the complex development of variation and maturation. This process occurs a variety of different factors including Runx2, Osterix and Wnt signaling[27–31].

2.1.3.2 Osteoblasts

Bone forming function is the major role of the osteoblasts, which are positioned alongside the bone surface and covering 4-6% of over-all inhabitant bone cells[32]. These cells, with abundant endoplasmic reticulum and prominent Golgi apparatus, have the morphologic characteristics of protein synthesizing cells[32,33]. Synthesizing of the organic constituents of bone matrix, which comprise proteoglycans, type I collagen fibers, and numerous glycoproteins containing osteonectin are done by osteoblasts[1]. The osteoid is secreted by osteoblasts toward the bone matrix[34]. In addition, viable osteoblasts are responsible for deposition of inorganic apparatuses of bone matrix. When osteoblasts are active in matrix synthesis they have a cuboid to column shape and basophilic cytoplasm, when inactive they become flattened and cytoplasmic basophilia is reduced. The activities of osteoblasts are stimulated by parathyroid hormone[1].

The production of bone matrix ensues in two core steps: installation of organic matrix and its following mineralization. The osteoblasts exude collagen proteins(mainly type I) and non-collagen proteins such as, osteocalcin, BSP II, osteopontin, osteonectin, and proteoglycan including decorin and bi-glycan, in order to arrange the organic

medium. Subsequently, mineralization of the bone matrix takes place into two stages; the vesicular and fibrillar phases[35,36]. Matrix vesicles are discharged from the membrane of osteoblasts to the nascent bone matrix where they bind proteoglycans and other organic components. Because of proteoglycans negative charge, they immobilize calcium ions that are accumulated inside the matrix vesicles[35,37]. Osteoblasts exude enzymes that degrade proteoglycans and then proteoglycans release the calcium ions through the calcium channels which called annexins located in the matrix vesicle membrane[36]. On the other hand, osteoblasts secrete ALP enzyme to degrade the phosphate containing compounds and release their phosphate ions inside the matrix vesicle. Subsequently, the calcium and phosphate ions inside the vesicle fuse to form hydroxyapatite crystals[22]. The fibrillar stage arise when the saturation of phosphate and calcium ions inside the matrix vesicles drives to breach of these vesicles and saturated crystals expand to the adjacent matrix[38,39].

2.1.3.3 Osteocytes

90% of total bone cells are composed of osteocytes, which their half-life is projected as long as 25 years[40,41]. Formerly osteocytes were described by their morphology and location. For years, osteocytes were considered as passive cells and their functions were mistook because of the difficulties in isolation osteocytes from the bone matrix[42].

Osteocytes are individual osteoblasts, which are progressively encircled by their own secretion to form lacunae. During the alteration from osteoblasts to osteocytes the cells prolongs many long cytoplasmic processes called canaliculi, which are similar to dendrites and axons in neurons. Each lacunae is occupied by an osteocyte and the canaliculi is radiated from it[1,16]. This radiated cell processes connect the adjacent osteocytes to each other and using gap junctions to osteoblasts surfaces. The exchange via gap junctions provides nourishment, transmission of tiny molecules and biologically created electrical potentials between cells. Function of osteocytes is considered as sensor cells in bone that can mediate the effects of mechanical loading through their broad communication network. Osteocytes manufacture and release inter-cellular emissaries that aim osteoblasts, osteocytes and precursor cells, due to the exposure of physical forces. Stimulated cells respond by remodeling the bone in mass and organization according to requests of external environment[41–43].

Mineral homeostasis is another important role of widespread distributed osteocytes. Osteocytes generate and respond to micro-fluxes in ion concentrations and mediate the exchange of calcium and other ions between the bone matrix and extracellular fluid[16,44].

2.1.3.4 Osteoclasts

Osteoclasts are fatally differentiated, large, motile and multinucleated cells are carrying the load for bone resorption. Their lifecycle is only several weeks. Since they are originated from the union of bone marrow derived cells, they are large and multinucleated cells. The Howship lacunae or resorption bay, depression or crypts in the matrix, is the residential sites for osteoclasts and it is produced with the digestion of bone tissue. In functioning osteoclasts, the surface against the bone matrix is folded into irregular projections to form ruffled border[1,16]. The part of the cell membrane loaded with αVβ3 integrins adheres to the bone and seals it. In order for osteoclasts to attach to the cell surface, the integrin molecule binds to specific extracellular bone matrix proteins (vitronectin, osteopontin and bone sialoprotein) previously deposited by osteoblasts[33]. This creates a micro-environment between the osteoclast and matrix at the adhesion site followed by bone resorption. The actual bone digestion process begins at the site of lysosomes fuse with the brush boundary in order to release their substances into the resorption hollow. When osteoclasts anchor to the matrix, actin network provides signals to the nuclei of osteoclasts to initiate the metabolic activation. Activated nuclei coordinate the complicated and temporary cytoplasmic and cell membrane variations necessary for bone breakdown. Osteoclasts create an acidic environment by secreting collagenase and other enzymes and pumping protons. This local acidic environment is formed to dissolve the hydroxyapatite and support the localized digestion of collagen. The cytoplasm neighboring of the resorbing surface is rich in carbonic anhydrase, membrane-bound lysosomes and tartrate-resistant acid phosphatase. While osteoclast activity terminates, macrophages roam into the base of lacunae to phagocytize the organic remnants[1,16,45].

Osteoclast activity is regulated by hormones and local signaling factor. Osteoclasts' receptors sensitive for thyroid hormone and calcitonin, but not for the parathyroid hormone. Osteoblasts are activated with the parathyroid hormone to produce a cytokine called osteoclast stimulating factor. In the light of these, actions of these two

cells are coordinated and both are essential in bone remodeling[1].

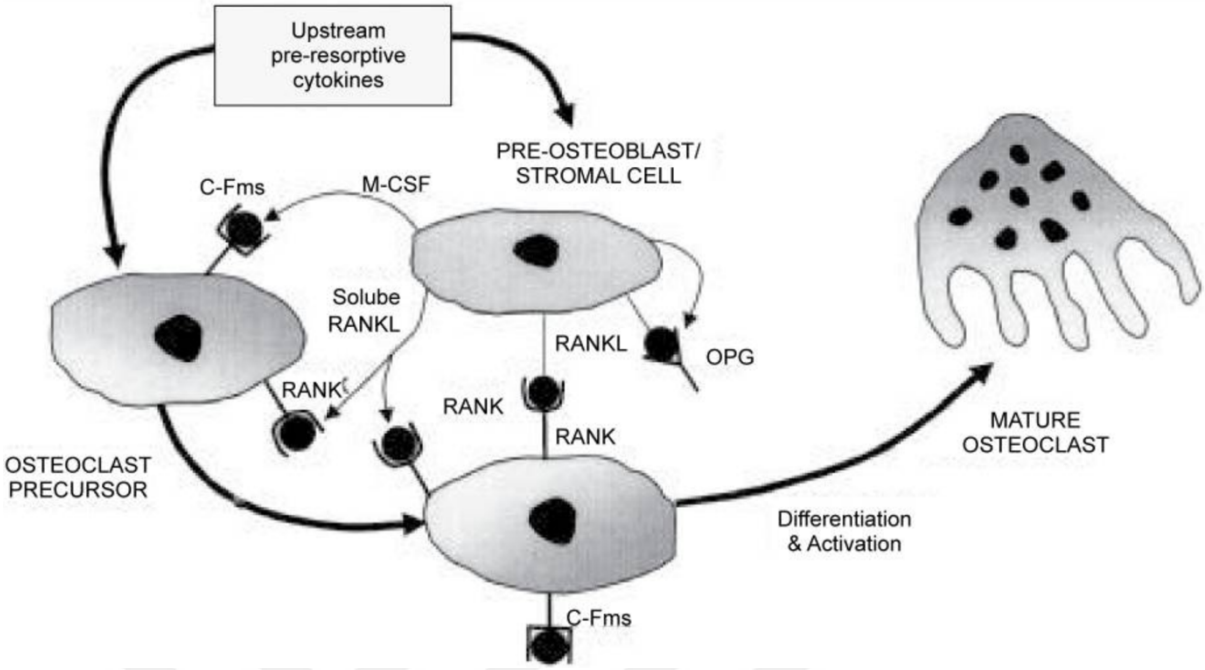

Figure 1 : Osteoclast cycle [46]

2.1.4 Osteogenesis

Osteogenesis, known as the formation of bone tissue, occurs both in the formation of normal skeletal structure in the embryonal period and in the healing of bone fractures in the adult period[47].

Bone formation occurs in two ways; intramembranous and endochondral ossification. Intramembranous ossification occurs with mesenchymal connective tissue and endochondral ossification with cartilage tissue. In both types of ossification, the first bone that appears is the primary bone, which is then replaced by the secondary bone. Reconstruction occurs with the combination of construction and destruction activities during bone growth. Reshaping and bone formation are fast in growing bones, slow in adults and continue for life[1,47].

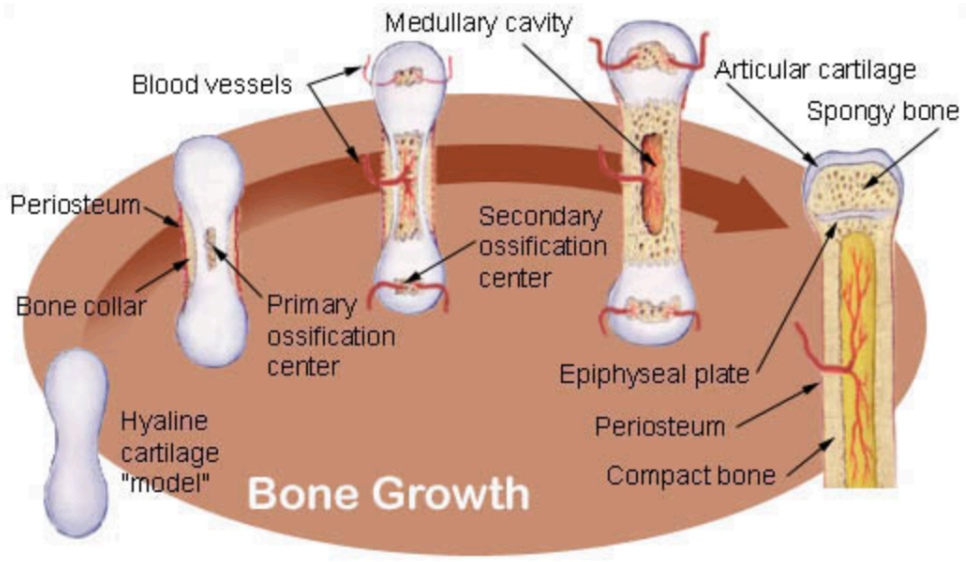

Figure 2 **:** Bone growth[48]

2.1.4.1 Intramembranous Ossification

Intramembranous ossification occurs as a result of the formation of bone tissue directly by mineralization of the matrix secreted by osteoblasts differentiating from mesenchymal stem cells in blood-rich regions of mesenchymal tissue[1].

Ossification center is the starting point for bone formation in which mesenchymal cells differentiate into osteoblasts. Calcification follows the production of osteoid matrix by osteoblasts, which results encapsulation of osteoblasts and differentiate into osteocytes. These developing bone areas form walls that delineate elongated cavities containing capillaries, undifferentiated cells and bone marrow cells. Several such groups arise almost simultaneously at the ossification center and their fusion between the walls gives the bone a spongy appearance. Bone marrow is formed from the connective tissue that remains between the groups at the ossification center with the penetration of growing blood vessels and mesenchymal cells. Bone grows radially at the ossification centers and fuses together to replace the connective tissue[1].

2.1.4.2. Endochondral Ossification

Endochondral ossification is the ossification that occurs when skeletal cartilage models are replaced with bone during fetal period and postnatal life. Endochondral ossification occurs within the newly formed cartilage anlage which is avascular[16,49,50]. The task of the cartilage model not only pioneer the formation of new bone, but also to

prepare the ground for the growth of long bones in the later stages. The growth of long bones depends on the continuity of the growth plate of chondrocytes[51]. The anlage grows both interstitially and appositionally as an outcome of the proliferation of chondrocytes and the accumulation of secreted extracellular matrix, which is composed of proteoglycans and type II collagen with smaller amounts of collagen types IX, X, XI, and XIII[16,21].

Three events ensue at the same development stage in all bones;

1- The mesenchymal stem cells originated from the perichondrium manufacture a layer of osteoblasts nearby the midportion of the cartilaginous shaft to deposit a ribbon of mineralized woven bone on the anlage surface. This is the sign of the conversion of the perichondrium into periosteum. In the middle section of the diaphysis, osteoblasts, the periosteum and the surface layer of bone outline and form the primary ossification center[16].
2- The chondrocytes, wrapped by the periosteal shell of bone, begin to hypertrophy and swell in the anlage shaft center. Chondrocytes begins expansion with an increase in intracellular glycogen and the installation of type X collagen in the neighboring area of the chondrocytes, which undergo soon apoptotic necrosis. Synchronously, the proximate matrix mineralizes, with the aid of matrix vesicles of the chondrocytes. Several matrix mineralization might be occur inside the “holes” of the collagen fibers[16].
3- Periosteal vessels capillary network infiltrate the woven bone via chondroclastic resorption to the primary ossification center. Impending nutrient vessels are developed by pioneer of these capillaries and other primitive mesenchymal cells, incorporating osteoclast progenitor cells[16].

While bone’s cartilaginous core endures resorption continuously, perivascular stem cells’ derived osteoblasts deposit layers of osteoid on the residual longitudinally positioned cross-pieces of mineralized cartilage[16]. Resulting in adhesion of osteoblasts to the calcified cartilage matrix to synthesize continues layers of primary bone which surround the cartilaginous matrix residues[1]. Primary ossification center is formed in the diaphysis of the anlage with this process. Subsequently secondary ossification centers develop at the epiphyses of the cartilage anlage and mature in the same manner. During their expansion and remodeling, bone marrow cavities are formed by both ossification

centers. Cartilage rests in two sides of the secondary ossification center. One of them is articular cartilage, which does not contribute to bone growth in length. The other one is the epiphyseal cartilage also known as epiphyseal plate, which links epiphysis and diaphysis[1].

2.1.5. Bone Healing

Trauma, infection, oncologic or metabolic disease may result in injury to bone tissue. The healing of an injured tissue by creating a tissue that is structurally and functionally unlike the original tissue is called repair. Injured tissues of the organism are repaired by fibrous scar formation. Complete restoration of structure and function is tissue regeneration[47,52]. Bone tissue is able to heal without scar formation. Fracture healing process recaps bone development and may be considered as tissue regeneration. Despite bone tissue's regenerative capacity, this process fails sometimes and fracture zones could heal in unfavorable anatomical locations, perform delay healing or non-unions[53].

2.1.5.1. Indirect Fracture Healing

Indirect fracture healing (secondary healing) involves both intramembranous and endochondral bone healing[54]. This type of healing may be result in non-union or delayed healing under too much load or motion, but it is also enhanced by little weight-bearing and micromotion. Rigidly stable conditions and anatomical reduction are not required with this type of healing[55]. This type of healing usually arises from non-operative fracture treatments and some operative managements in which internal and external fixation is done[56,57].

2.1.5.1.1 The acute inflammatory response

After the trauma occurs, a hematoma is formed by the cells from the blood stream both intramedullary and peripheral including bone marrow cells. Inflammatory response, vital for the healing, is started with the injury. The hematoma coagulates around the fracture sides and within the medulla to form an outline for callus formation. Secretion of proinflammatory molecules is crucial for the regeneration of the tissue[58]. In the first 24 hours, the acute inflammatory response peaks and completes after 7 days[59].

Secretion of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-11, IL-6 and IL-18 is initiated with the proinflammatory response. Inflammatory cells are recruited by these factors and angiogenesis is promoted as well. Peak level of TNF-α concentration has been seen at 24 hours and decreased to baseline at 72 hours post trauma[58,60]. Throughout this period macrophages and some other inflammatory cells express TNF-α to induce secondary inflammatory signals, which act as a chemotactic molecule to recruit important and necessary cells. It has been publicized in vitro that osteogenic differentiation of mesenchymal stem cells are induced by the TNF-α. TNFR1 and TNFR2 are the receptors located on the surface of either osteoblasts and osteoclasts[61,62]. These receptors mediate the differentiation and activation of bone cells. TNFR2 is expressed under the injury circumstances with an exclusive role, although TNFR1 is continuously expressed in bone instead.[62,63] For fracture healing, IL-6 and IL-1 are the most essential ones among the interleukins. IL-1 and TNF-α expressions overlap with a biphasic mode. Macrophages produce IL-1 in the acute inflammation phase and IL-1 induces synthesize of IL-6 in osteoblasts. This expression sequence stimulates the production of the initial cartilaginous callus and angiogenesis at the damaged spot by initiating either of the receptors, IL-1 R I and R II[60,62,64]. Instead, IL-6 is just synthesized during the acute stage and encourages angiogenesis with the vascular endothelial growth factor (VEGF) creation and the differentiation of osteoblasts and osteoclasts[65].

Bone regeneration could occur with the recruitment of specific type of mesenchymal stem cells (MSC) and they multiply and differentiate into osteogenic cells under the expression of some molecules. While utmost data signify that MSCs are derived from adjacent soft tissues and bone marrow, recent data found that the systemic recruitment of circulating MSCs to the injured location may be crucial for an ideal healing response[66,67]. This recruitment's molecular event mediation is still debated. It has been put forward that BMP-2 has a key function in MSCs' recruitment, but records from Bais et al. suggests that this is not the case[68]. Actually, BMP-2 is vital for bone healing[69], but other BMPs for example BMP-7 might play a further more essential role in the recruitment of progenitor cells[68].

2.1.5.1.2 Generation of bony callus

While secondary fracture healing comprises of both endochondral and intramembranous ossification, the construction of cartilaginous callus which subsequent

goes mineralization, resorption and finally replace with the bone is the main feature of this activity[1]. Fibrin-rich granulation tissue formation comes after the development of the primary hematoma. Within the granulation tissue, endochondral formation arises among fracture extremities. These injured sites are stabled with the formation of soft callus by cartilaginous tissue[70]. While soft callus has been matured, hard callus tissue has been generated with the intramembranous ossification at the proximal and distal ends of the rupture subperiosteally. Finally central hard callus is linked the fracture ends to form a semi-rigid structure. This structure would allow weight bearing[54].

Recruitment of MSCs initiate the production of the callus tissues with the molecular cascade involving collagen type I and II matrix fabrication and participate some peptide signaling molecules. In this development, members of transformation growth factor beta (TGF-β) superfamily have been indicated with an important job. TGF-β2, TGF-β3 are involved in endochondral ossification and chondrogenesis. At periosteal sites where intramembranous ossification occurs, BMP-5 and BMP-6 induce cell proliferation[59,71]. BMP-2 is the crucial for the beginning of healing cascade as mentioned above. Tsuji et al. reported that mice with inactivated BMP-2, not capable to arrange callus to repair fractures effectively[69].

2.1.5.1.3 Revascularization at the fracture site

Blood supply to the fractured area is vital for bone restoration. In endochondral healing, apoptosis of chondrocytes and degradation of cartilage as the removal of extracellular matrix and cells allow blood vessel penetrate to the repair site[72,73]. Two molecular pathways, angiopoietin-dependent pathway and vascular endothelial growth factor (VEGF) dependent pathway, regulate the vascularization progression[74]. Angiopoietin 1 and 2 are the main vascular morphogenic proteins, which are responsible for the induction of vascular in-growth from existing vessels from periosteum[75]. Though, the VEGF pathway is deliberated to be the crucial controller of vascular regeneration[73]. The transformation of avascular cartilaginous matrix into vascularized osseous tissue depends on the expression of the VEGF in high levels by hypertrophic chondrocytes and osteoblasts[73].

2.1.5.1.4. Resorption and mineralization of callus

The primary cartilaginous callus should have resorbed and replaced by bonny hard callus[76]. When the fracture callus chondrocytes proliferate, they become hypertrophic and the extracellular matrix becomes calcified. The resorption cascade initiation of the mineralized cartilage is coordinated primarily by macrophage colony stimulating factor (M-CSF), TNF-α, osteoprotegrin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL)[58,77]. During the process RANKL, M-CSF and OPG are suggested to aid to recruit bone cells and osteoclasts to form woven bone[58]. The recruitment of osteogenic potential MSC is promoted by TNF-α, the main role is the initiation of chondrocyte apoptosis. While the hard callus formation advances and the calcified cartilage is interchanged to woven bone, the callus becomes mechanically rigid[54].

2.1.5.1.5. Bone remodeling

Although biomechanical stability is provided by the hard callus, it is not fully restored with the biomechanical abilities of normal bone. In order to achieve the biomechanical properties of normal bone, a second resorptive phase is initiated by IL-1, BMP2 and TNF-α which express highly during the stage. In this point, hard callus is transformed into lamellar bone organization with a central medullar cavity[58,71,72]. The resorption of hard callus by osteoclasts and the deposition of lamellar bone by osteoblasts are performed in harmony. This remodeling process might take years to complete a fully regenerated bone structure[78]. When pressure is applied to the bone's crystalline environment, production of electrical polarity results in bone remodeling[79]. When the loading to the bones occur, one electronegative concave surface and one electropositive convex surface is created. This electrically polarized surfaces undergoes osteoblastic and osteoclastic activity respectively[79].

Successful bone remodeling requests a steady increase in stability and a sufficient blood supply[80]. In situations where neither is succeeded, causing in the development of an atrophic fibrous non-union is observed. If the vascularity is adequate but stability is not, pseudo-arthrosis might occur with the formation of cartilaginous callus[55].

2.1.5.2. Direct bone healing

This type of fracture healing does not occur commonly in nature. This type healing should have stabilized with a correct anatomical positioning of the fracture ends exclusive of any gap formation. This form of stabilization could be acquired with open reduction surgical approach. Direct bone healing appears with direct remodeling of lamellar bone, blood vessels and haversian canals[81]. Gap healing and contact healing are two different natures of primary fracture healing. In both, it is aimed to re-establish correct anatomical organization of lamellar bone structure, function competently[82].

2.1.5.2.1 Contact healing

Fracture fragments should have been restored with the rigid fixation to provide an anatomic restoration in order to achieve significant reduction in interfragmentary tension. Both ending of the bone cortices' must unite to re-establish mechanical continuousness. In order to occur a contact healing, the space among the bone endings must be fewer than 0.01 mm and interfragmentary strain must be less than 2%[82]. Under these circumstances, at the end portion of the osteons close to the crack spot forms cutting cones. Osteoclasts, which lie at the tips of cutting cones, cross the fracture line and generate longitudinal cavities. Subsequently osteoblasts which reside at the edge of the cutting cone synthesize the bone. Osteoblasts play an essential role with the regeneration of the bone tissue simultaneously and restoration of the Haversian system. The restoration of haversian systems allows blood vessels to penetrate through system and carry osteoblastic precursors[83–85].

2.1.5.2.2. Gap Healing

In this healing process, bony union and remodeling of haversian system do not arise simultaneously. This healing type would be achieved with stable conditions and an anatomical reduction, which secure the gap less than 1 mm[85]. Healing in fracture site requires a secondary osteonal restoration in the fracture area where is filled by the lamellar bone positioned vertically to the long axis of the bone[86]. Primary bone is progressively replaced on the each surface of the fracture gap with the lamellar bone, which is synthesized by the osteoblasts. This process takes about three to eight weeks, followed by a secondary remodeling similar to the healing step by contact with cutting

cones. This stage is required in order to achieve the characteristics of the bone both anatomically and biomechanically[82].

2.2. Boron

2.2.1. Introduction

Boron element is one of the essential minerals that shows an important function in some biological processes in plants, animals and also in humans[87]. It is not found in elemental form in nature, but as compounds with other elements named borates[88]. Latest researches have turn into surprise actions for these borates in areas noteworthy to human and animal health[89]. The salient effect of these element is impact on the metabolism of some nutrients, such as vitamin D, calcium, magnesium. In addition, it effects action of some enzymes, formation of steroid hormones[89,90]. Boron is showed as a bioactive food constituent which is beneficial for maintaining health. Deficiency of boron could lead to alter in brain function and bone structure[91].

2.2.2. Boron as Element

Boron element belongs to the family of metalloid. It has an atomic number of 5 and as molecular weight 10.81 g/mol[88,92]. Boron element is not found in nature as elemental form, but it forms compounds with other elements. Since it has strong affinity for the oxygen, resulting in the formation of borates. Boron exists in the form of Boric acid at physiological pH, in addition studies showed that this mineral is cooperating vital role for bone growth, bone health, psychomotor immune actions and embryogenesis[89,91,93]. Boron contains adjacent hydroxyl groups which are in cis configuration and it has a convincing affinity to form complexes with organic molecules. Pyridoxine, riboflavin, polysaccharides, dehydroascorbic acid, pyridine nucleotide are some of the biological substances that boron can interact with. The nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide (NAD+) are ribose containing components, which are active in energy metabolism. These molecules have a strong affinity for boron, results in linking. Therefore, this specific metabolic pathways may be affected by the linkage between boron and these molecules[89,94].

2.2.3.Action in the bone and the body

Boron plays a guiding function in the metabolism of numerous micronutrients. Calcium, magnesium, phosphorous and vitamin D are related micronutrients to the bone metabolism. In the light of these, boron is an important molecule that plays an important function in osteogenesis. The deficiency of the molecule has been shown to adversely influence bone regeneration and development[9–11].

Some studies propose that boron can affect several aspects of vitamin D metabolism. Growth can be influenced by this action. Deprivation of boron in the diet of chickens accompanied by moderate amount of vitamin D in their composition. Microscopic analysis performed in this group showed that calcification of cartilage fragments was delayed and also the density of chondrocytes in the cell proliferation area decreased in boron and cholecalciferol deficiency. In the light of these discoveries, it is proposed that boron can boost cell growth and maturation[91,95]. Another study states that chickens received vitamin D deficient diet but supplemented with boron showed increase in concentration of 1,25-dihydroxycholecalciferol and 25-hydroxycholecalciferol. On the other hand, low levels of boron with the adequate amount of vitamin D results in increasing plasma concentration of 1,25-dihydroxycholecalciferol. These findings suggest a positive correlation between boron and vitamin D[96]. These outcomes suggest that boron might affect half-life or hydroxylation of vitamin D, based on the capability to form complexes with hydroxyl groups into organic compounds. Thus, boron induce bone metabolism[89].

Boron supplementation effects on calcium metabolism, with the way of reducing urinary excretion and increasing ionized calcium concentrations in the plasma. Moreover, calcium supplementation is not enough to prevent bone loss. Studies focused on post- and pre-menopausal women suggested that boron supplementation was able to increase calcium absorption[89,97].

In animal studies, animals which receiving magnesium deficient diet but supplemented with boron showed reduced level of abnormalities triggered by the magnesium deficiency and increased level of magnesium in plasma. In addition, it was reported that post- and pre-menopausal women supplemented with boron had a lower urinary magnesium excretion[89,97].

A study in rats revealed that bone strength was also affected by boron supplementation. The resistance force of vertebrae was tested and found out that boron supplemented group had an increase resistance to the force. Deprived group was showed reduction of bone volume and trabecular thickness[13,14].

In animal studies issued in 2008 and 2009 revealed that, healing of the alveolar socket could be hindered in boron deficient rats. Boron adequate (3 mg/kg/d in the diet) and boron deficient animals (0.07 mg/kg/d in the diet) was compared at seven and fourteen days. Comparison between groups was indicated that boron deficient animals had a noteworthy reduction in osteoblast surface. This result indicate that inadequate boron supplementation could be resulted in impaired bone healing[98,99].

In 2010, a research which was focused on boron affecting mechanism on osteogenesis was published by Hakki et al stated that boron was determined to induce mineralization of osteoblasts by regulating the expression of genes related to tissue mineralization and the action of key hormones such as, testosterone, 17β- estradiol involved in bone metabolism[15].

A report published on 1990 revealed that 3% boric acid solution applied to deep wounds came out with the result of reducing time needed in intensive care by two-thirds[100]. This was the first report about boron on wound healing. In 2000, in-vitro examination using human fibroblast revealed that boric acid solution enhanced wound healing with the effect on extracellular matrix[101]. In addition, specific enzymes found in fibroblasts such as, trypsin-like enzymes, elastase, collagenases, alkaline phosphatase were affected directly by boron. Fibroblasts play a crucial role for the wound healing with the production of collagen and extracellular matrix[102]. Boron eases the key enzyme activities in fibroblasts results in improvement extracellular matrix turnover.

The expression of messenger RNA (mRNA) is regulated by boron. This regulation affects wide range of extracellular proteins and also mineralized tissue proteins such as, collagen type I, bone sialoprotein, osteopontin and osteocalcin. The merged effects of these activities enhance proliferation, osteoblast cell viability and morphology as well as the mineralization of bone cells[15].

3. MATERIALS and METHODS

Our study was approved with the decision report of Yeditepe University Animal Experiments Local Ethics Committee 30.04.2019 dated and 749 numbered . Histopathological evaluations of bone tissues were performed in Istanbul University Institute of Oncology Tumor Pathology and Oncological Cytology Department.

3.1. Animal Care

Twenty-eight thirteen weeks old female rats were used in this study. The animals were intended to be accustomed to the study environment for 10 days. Animals were housed in a cage with a 12-12 hour light / dark cycle, 50% ± 10% humidity and 20 ± PC ambient temperature. The rats were divided into groups by computer-generated stratified randomization to equalize body weight instruments and were scheduled to be assigned to the control and treatment groups. The care and accommodation of the experimental animals were provided at Yeditepe University Experimental Medicine Research Institute.

3.2. Study Design

Twenty-eight rats were used in this study. They were divided into two groups according to their sacrification time. First group (n:14) was sacrificed on the 14th day, and the second group (n:14) was sacrificed on the 28th day. Both groups were divided into two sub-groups as Ia (n:7), Ib (n:7), IIa (n:7) and IIb (n:7). In the surgical protocol, we performed two 5mm diameter critical-sized full thickness defects with trephine burs on each side of the line passing from bregma point on the parietal bone. The defects of the left (L) of the skull were left empty, and 3% boric acid solution was locally applied to the right defects (R) of all groups. In this study, the application of boric acid at a concentration of 3% to the defects was decided based on the work of Blech et al. and Benderdour et al. [100,101]. In addition, systemic dose was decided to use, based on the Nielsen et al. and Gorutovich et al. [98,99]. In the first group, systemic boric acid administration started on the day of the surgery for the group Ia and continued for 14 days until the scarification day. On the other hand Ib group was not administrated any systemic boric acid. Similar protocol was done for the second group. Systemic boric acid administration started on the day of the surgery for the group IIa and continued for 28

days until the sacrification time. On the other hand IIb group was not administrated any systemic boric acid.

The aim of forming the group in this manner is to decrease the number of the animal samples. In local application group, 3% boric acid solution was applied directly to the right defect with the help of Gelatamp®. Ten drops of the solution was embedded to the Gelatamp® sponge and directly transferred to the defect site. This was approximately 0,015 mg. On the other hand, systemic dose of the boric acid was administrated 3mg/kg/day with oral gavage method.

Figure 3: Gavage application

3.3. Study protocol

All procedures were completed in a surgical operating room under sterile environment. All Sprague–Dawley rats were anesthetized with an intraperitoneal injection of ketamine (Ketalar ®, Pfizer, Turkey, 50 mg/mL) and 2% xylazine

(Rompun®, Bayer, Canada, 20 mg/mL, 2 mL/kg). A 2 cm long skin, subcutaneous and periosteal incision was made in the sagittal suture line on the scalp of the rats. Eventually, full-thickness flap was reflected towards the posterior region of the skull in order to expose sagittal bone. Bilateral 5-mm diameter critical sized defects were made with a trephine (Nobel Biocare™, Sweden) used in a low-speed handpiece under constant sterile saline irrigation to prevent thermal damage at parietal bones. During the procedure, bregma point and sagittal suture were recognized as the landmarks. The defect located on the right side was filled with boric acid solution impregnated collagen sponges; the left side defects were left empty and served as the control group. Incision was then sutured with non-resorbable 3/0 silk sutures (Silk; Dogsan; Trabzon, Turkey). All the wound areas were cleaned with antiseptic solution after surgery. After 14 days the first experimental groups and 28 days the second experimental groups were sacrificed using carbon dioxide inhalation. Subsequently, parietal bones were extracted carefully in order not to damage the experimental defected sites.

3.4. Trial groups

We should analyze the groups according to their defects, because in this experiment we aimed to reduce sample size and used adjacent defects at the calvarium of each rat.

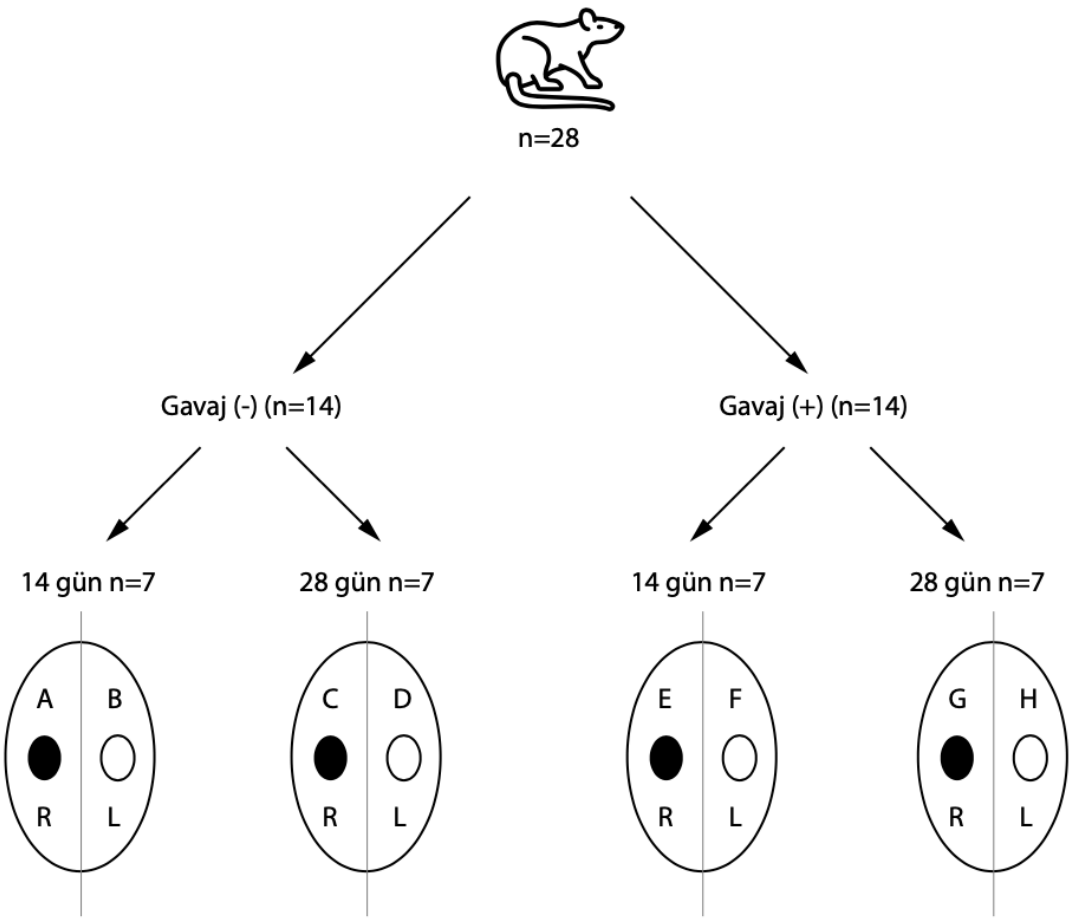

Figure 4: Explanation of the groups

- **Group A (I-b-R)**: Right critical sized defect of the 14 day group without the systemic administration. It was indicating only the effect of local application of boric acid in 14 days period.
- **Group B (I-b-L)**: Left critical sized defect, which was left empty, of the 14 day group without the systemic administration. It was indicating the control group.

- **Group C (II-b-R)**: Right critical sized defect of the 28 day group without the systemic administration. It was indicating only the effect of local application of boric acid in 28 days period.
- **Group D (II-b-L)**: Left critical sized defect, which left empty, of the 28 day group without the systemic administration. It was indicating the control group.

- **Group E (I-a-R)**: Right critical sized defect of the 14 day group with the systemic administration. It was indicating the combination of local application and systemic administration of boric acid.
- **Group F (I-a-L)**: Left critical sized defect, which was left empty, of the 14 day group with the systemic administration. It was indicating only systemic administration of boric acid in 14 days.
- **Group G (II-a-R)**: Right critical sized defect of the 28 day group with the systemic administration. It was indicating the combination of local application and systemic administration of boric acid.
- **Group H (II-a-L)**: Left critical sized defect, which was left empty, of the 28 day group with the systemic administration. It was indicating only systemic administration of boric acid.

- I / II is representing the sacrificed period of the animals. I for 14^{th} day, II for 28^{th} day.
- a/b is representing the systemic gavage during healing period. a stands for systemic boric acid gavage, b stands for no gavage.
- R/L is representing the defects site according to the bregma point. L stands for left defects, which were left empty. R stands for right defects, which were applied local boric acid.

Figure 5: *Operation photo of the critical sized defects*

3.5. Histopathological examination

The bones of the sacrificed experiments were purified from their soft tissues and fixed in a 10% formaldehyde solution for a week. Then, the prepared 50% formic acid was kept in a decalcification solution containing 20% sodium citrate. The samples were decalcified for 45 days, changing the solutions once a week. After decalcification, it was cut and put into the cassette to cover the entire defect site. After routine follow-up, paraffin was embedded in the blocks. 5µ sections from paraffin blocks were removed and placed on the slide and stained with routine Hematoxylin-Eosin after deparaffinization and transparency. Painted sections were examined with Olympus BX60 microscope connected to a computer with a color video camera. All measurements were made by Olympus Image Analysis System 5 for histomorphometric analysis. The images were taken with the camera at x40 magnification and transferred to the computer screen.

We analyzed new bone formation, fibrosis areas in all images which were obtained from the samples at the light microscope with the x40 magnification.

As the terms of fibrosis; Collagens play vital roles in wound healing and fractures regeneration. On the other hand, excessive collagen formation poses a problem, leading to fibrosis in various organs and tissues. In this study, microscopic images were evaluated and connective tissue accumulation seen areas assessed as fibrosis tissue.

As the term of new bone tissue; microscopic images were evaluated and new bone formation seen areas classified as new bone forming areas of the defect site.

3.6. Statistical Method

Data were analyzed on computer using SPSS 25.0 (Statistical Packages of Social Sciences) program. The suitability of the data to normal distribution was evaluated with the Kolmogorov-Smirnov test.. Explanatory statistics are shown as mean ± standard deviation, median, minimum and maximum for continuous variables. Mann-Whitney U test was used to compare data of two independent groups that do not conform to normal distribution. Kruskal-Wallis test was used to compare variables that did not fit the normal distribution between more than two groups, and Mann-Whitney U test was used as pos-hoc test of statistically significant variables. Results were interpreted by Bonferroni correction. If $p < 0.05$, the difference was considered significant.

4. RESULTS

4.1. Statistical Comparisons

Table 1: New bone measurements between groups

New Bone							
Group	N	Mean	St. Deviation	Median	Minimum	Maximum	P Value
A	7	6401,1429	4963,49119	6356,0000	329,00	14332,00	
B	7	5166,5714	3318,96268	5431,0000	1055,00	9869,00	
C	7	6767,2857	6096,70705	3642,0000	1464,00	17224,00	
D	7	5742,2857	5050,02521	3596,0000	1889,00	14847,00	0,502
E	7	6233,1429	7665,97314	2949,0000	1192,00	23018,00	
F	7	3606,0000	4768,06795	2046,0000	610,00	14144,00	
G	7	3813,2857	1658,86083	4404,0000	1622,00	5445,00	
H	7	8133,2857	5046,66790	6752,0000	2299,00	16063,00	

Kruskal- Wallis test.

There was no statistically significant difference between the median values of new bone measurements between groups. p>0,05.

Table 2: Box chart of the groups for new bone measurements

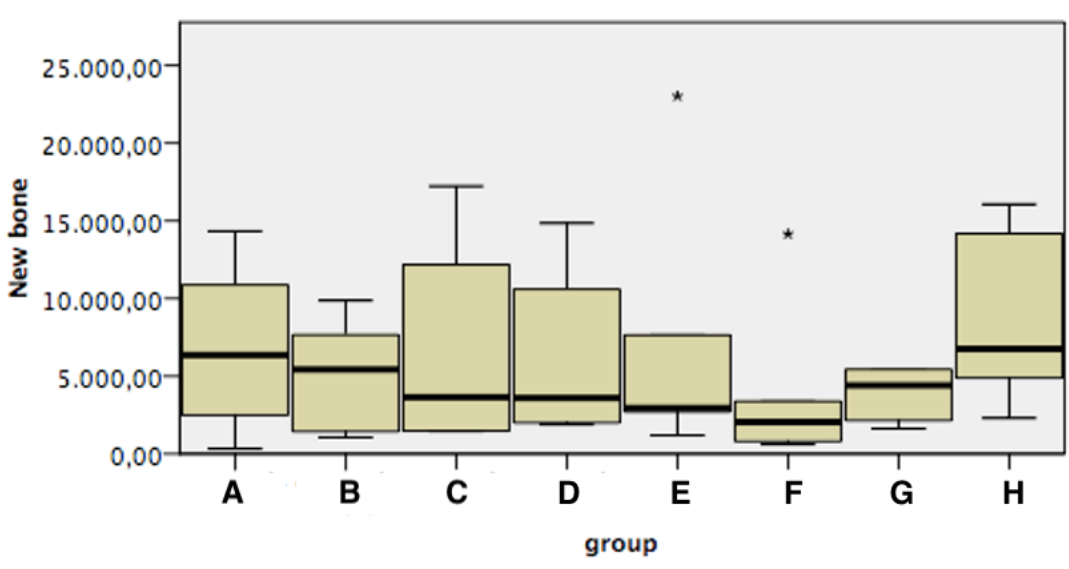

Table 3 : Bar graph of average new bone measurements by groups

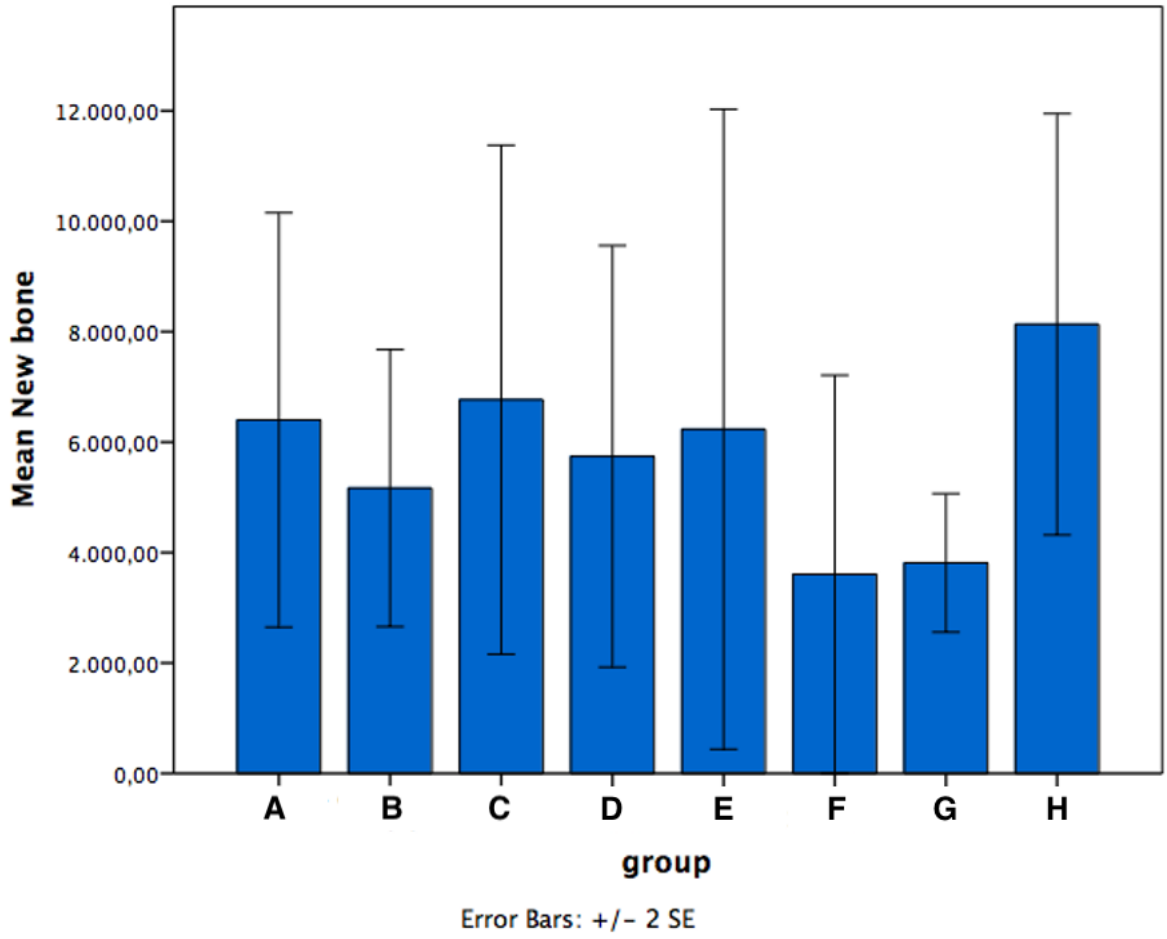

Table 4: Fibrosis measurements between groups.

Fibrosis							
Group	N	Mean	St. Deviation	Median	Minimum	Maximum	P Value
A	7	21695,7143	15920,80448	13752,0000	10907,00	55594,00	
B	7	8117,4286	5871,03682	7776,0000	1878,00	18256,00	
C	7	7883,7143	3924,26310	7449,0000	2809,00	14659,00	
D	7	4628,4286	2719,10352	4200,0000	1760,00	9027,00	0,000*
E	7	20961,1429	11217,22917	21750,0000	7640,00	41091,00	
F	7	6873,1429	3155,79432	7216,0000	3667,00	12163,00	
G	7	6061,2857	2154,93122	5394,0000	4030,00	9670,00	
H	7	2004,7143	1195,59465	2244,0000	250,00	3761,00	

Kruskal- Wallis testi.* p<0,05 statistically significant

A statistically significant difference was found between the median values of fibrosis measurements between the groups. $p<0,05$.

In order to determine between which groups this difference is, the post-hoc test Mann-Whitney u test was performed and p values were interpreted by Bonferroni correction.

According to this;

There are statistically significant differences between H and A groups ($p = 0,000$). The median value of A group is higher than the H group.

There are statistically significant differences between H and E groups ($p = 0,000$). The median value of fibrosis of E group is higher than H group.

There are statistically significant differences between D and A groups ($p = 0.023$). The median value of fibrosis of A group was higher than the D group.

There was a statistically significant difference between D and E groups ($p = 0.023$). The median value of fibrosis of E group is higher than the D group.

Table 5 : Box chart of the groups related to fibrosis measurements

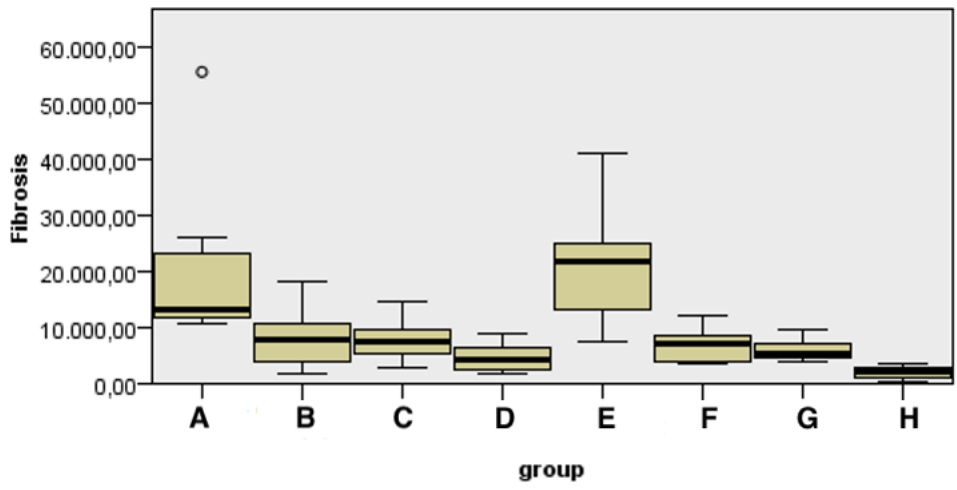

Table 6 : Bar graph of average new bone measurements by groups

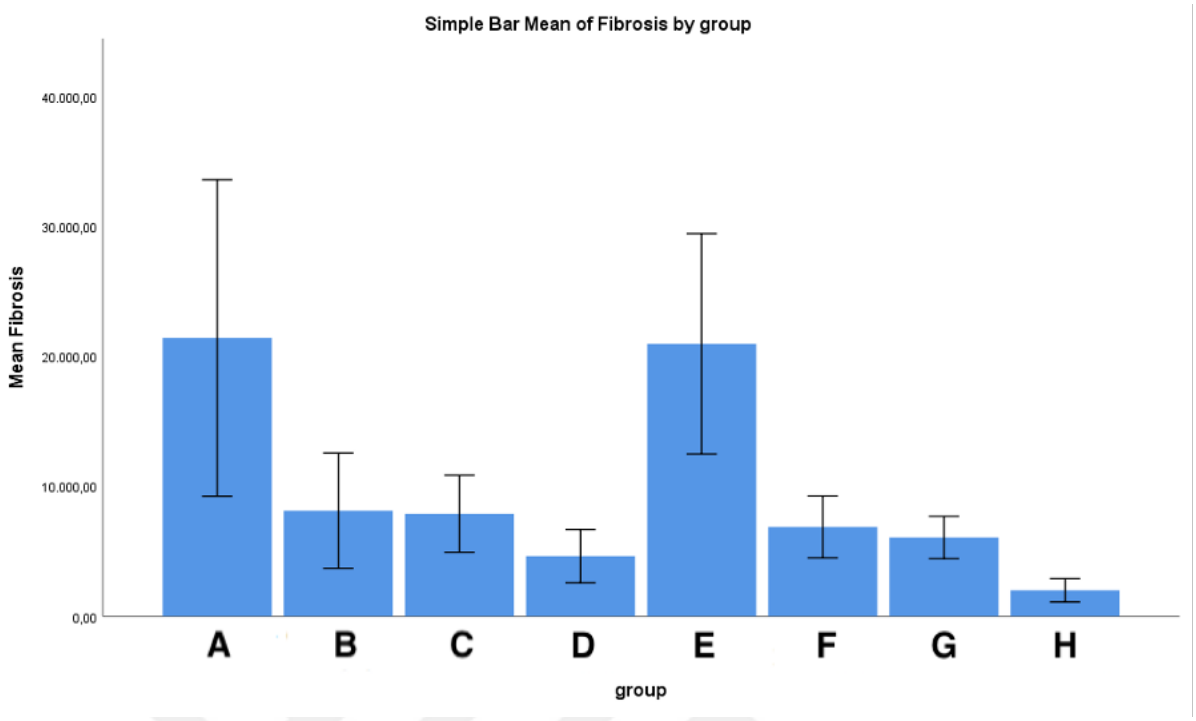

Table 7: Binary comparison between groups A and C

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New Bone	A	7	6453,1429	4890,64612	1848,49048	1,000
	C	7	6767,2857	6096,70705	2304,33867	
Fibrosis	A	7	21695,7143	15920,80448	6017,49847	0,007*
	C	7	7883,7143	3924,26310	1483,23204	

Mann-Whitney U test result

There was no statistically significant difference in the new bone measurement between the 14th and 28th day of the local boric acid application groups. (Group A and group C respectively) ($p > 0.05$).

There was a statistically significant difference in the measurement of fibrosis between the A and C groups ($p < 0.05$). Average value of fibrosis in local boric acid application group on 14^{th} day is more than the 28^{th} day. (Table:7)

Table 8: Binary comparison between groups B and D

	Group	N	Mean	St. Deviation	Std. Error Mean	P Value
New Bone	B	7	5166,5714	3318,96268	1254,44998	0,902
	D	7	5742,2857	5050,02521	1908,73012	
Fibrosis	B	7	8117,4286	5871,03682	2219,04334	0,259
	D	7	4628,4286	2719,10352	1027,72453	

Mann-Whitney U test

There was no statistically significant difference in the new bone measurement between the 14th and 28th day of control groups (Group B and group D respectively) (p> 0.05).

There was no statistically significant difference in the measurement of fibrosis between the 14th and 28th day of control groups (Group B and group D respectively) (p> 0.05). (Table:8)

Table 9: Binary comparison between groups E and G

	group	N	Mean	St. Deviation	Std. Error Mean	P value
New bone	E	7	6233,1429	7665,97314	2897,46550	0,902
	G	7	3813,2857	1658,86083	626,99046	
Fibrosis	E	7	20961,1429	11217,22917	4239,71411	0,002*
	G	7	6061,2857	2154,93122	814,48744	

Mann-Whitney U test (*p<0,05 is statistically significant.)

No statistically significant difference was found in the new bone measurement between the 14th and 28th day of the combined systemic and local application of boric acid groups (Group E and G respectively) (p>0,05).

A statistically significant difference was found in the measurement of fibrosis between the 14th and 28th day of the combined systemic and local application of boric acid groups (Group E and G respectively) (p<0,05).

Fibrosis on 14^{th} day, measurement of combined application group (group E) is higher than Day 28 measurement of the combined application group (group G).(Table 9)

Table 10: Binary comparison between groups F and H

	Group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	F	7	3606,0000	4768,06795	1802,16029	0,026*
	H	7	8133,2857	5046,66790	1907,46117	
Fibrosis	F	7	6873,1429	3155,79432	1192,77814	0,001*
	H	7	2004,7143	1195,59465	451,89230	

Mann-Whitney U test (*p<0,05 is statistically significant.)

A statistically significant difference was found in the new bone measurement between the 14th and 28th day of systemic boric acid administrated groups (Group F and H respectively). p<0,05. The new bone measurements of systemic boric acid administrated groups on Day 28 are higher than on Day 14 (Group H and Group F respectively).

A statistically significant difference was found in the measurement of fibrosis between the 14th and 28th day of systemic boric acid administrated groups (Group F and group H respectively) (p<0,05). Day 14 measurement of systemic empty groups is higher than Day 28 measurement. (Table 10)

Table 11: Binary comparison between groups B and A

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	B	7	5166,5714	3318,96268	1254,44998	0,805
	A	7	6453,1429	4890,64612	1848,49048	
fibrosis	B	7	8117,4286	5871,03682	2219,04334	0,026*
	A	7	21695,7143	15920,80448	6017,49847	

Mann-Whitney U test (*p<0,05 is statistically significant.)

There was no statistically significant difference in new bone measurement between control group and local boric acid application group on the day 14 (Group B and Group A respectively) (p>0,05).

Statistically significant difference was found in fibrosis measurement between control group and local boric acid application group on the day 14 (Group B and Group A respectively) ($p<0,05$). (Table 11)

Table 12: Binary comparison between groups B and E

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	B	7	5166,5714	3318,96268	1254,44998	0,710
	E	7	6233,1429	7665,97314	2897,46550	
Fibrosis	B	7	8117,4286	5871,03682	2219,04334	0,026*
	E	7	20961,1429	11217,22917	4239,71411	

Mann-Whitney U test (*$p<0,05$ is statistically significant.)

There was no statistically significant difference in new bone measurements between control group and combined systemic and local application of boric acid group on the day 14 (Group B and group E respectively) ($p>0,05$).

Statistically significant difference was found in fibrosis measurement between control group and combined systemic and local application of boric acid group on the day 14(Group B and group E respectively) ($p<0,05$). Combined application groups fibrosis measurement is more than control group measurement on the day 14. (Table 12)

Table 13: Binary comparison between groups B and F

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	B	7	5166,5714	3318,96268	1254,44998	0,209
	F	7	3606,0000	4768,06795	1802,16029	
Fibrosis	B	7	8117,4286	5871,03682	2219,04334	0,902
	F	7	6873,1429	3155,79432	1192,77814	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between control group and systemic boric acid application group on the day 14 (Group B and group F respectively) ($p>0,05$).

There was no statistically significant difference in fibrosis measurement between control group and systemic boric acid application group on the day 14 (Group B and group F respectively) (p>0,05). (Table 13)

Table 14: Binary comparison between groups D and C

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	D	7	5742,2857	5050,02521	1908,73012	1,000
	C	7	6767,2857	6096,70705	2304,33867	
Fibrosis	D	7	4628,4286	2719,10352	1027,72453	0,097
	C	7	7883,7143	3924,26310	1483,23204	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between control group and local boric acid application group on the day 28 (Group D and group C respectively) (p>0,05).

There was no statistically significant difference in fibrosis measurement between control group and local boric acid application group on the day 28 (Group D and group C respectively) (p>0,05). (Table 14)

Table 15: Binary comparison between groups D and G

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	D	7	5742,2857	5050,02521	1908,73012	1,000
	G	7	3813,2857	1658,86083	626,99046	
fibrosis	D	7	4628,4286	2719,10352	1027,72453	0,383
	G	7	6061,2857	2154,93122	814,48744	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between control group and combined systemic and local application of boric acid group on the day 28 (Group D and group G respectively) (p>0,05).

There was no statistically significant difference in fibrosis measurement between control group and combined systemic and local application of boric acid group on the day 28 (Group D and group G respectively) (p>0,05). (Table 15)

Table 16: Binary comparison between groups D and H

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	D	7	5742,2857	5050,02521	1908,73012	0,209
	H	7	8133,2857	5046,66790	1907,46117	
Fibrosis	D	7	4628,4286	2719,10352	1027,72453	0,053
	H	7	2004,7143	1195,59465	451,89230	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between control group and systemic boric acid application group on the day 28 (Group D and group H respectively) (p>0,05).

There was no statistically significant difference in the measurement of fibrosis between control group and systemic boric acid application group on the day 28 (Group D and group H respectively) (p>0,05). (Table 16)

Table 17: Binary comparison between groups A and E

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	A	7	6453,1429	4890,64612	1848,49048	0,805
	E	7	6233,1429	7665,97314	2897,46550	
Fibrosis	A	7	21695,7143	15920,80448	6017,49847	0,902
	E	7	20961,1429	11217,22917	4239,71411	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between local boric acid application group and combined systemic and local application of boric acid group on the day 14 (Group A and group E respectively) (p>0,05).

There was no statistically significant difference in fibrosis measurement between local boric acid application group and combined systemic and local application of boric acid group on the day 14 (Group A and group E respectively) (p>0,05). (Table 17)

Table 18: Binary comparison between groups C and G

	group	N	Mean	St. Deviation	Std. Error Mean	P Value
New bone	C	7	6767,2857	6096,70705	2304,33867	0,805
	G	7	3813,2857	1658,86083	626,99046	
fibrosis	C	7	7883,7143	3924,26310	1483,23204	0,383
	G	7	6061,2857	2154,93122	814,48744	

Mann-Whitney U test

There was no statistically significant difference in new bone measurement between local boric acid application group and combined systemic and local application of boric acid group on the day 28 (Group C and group G respectively) (p>0,05).

There was no statistically significant difference in fibrosis measurement between local boric acid application group and combined systemic and local application of boric acid group on the day 28 (Group C and group G respectively) (p>0,05). (Table 18)

Table 19: Binary comparison between groups E and F

	group	N	Mean	Std. Deviation	Std. Error Mean	P value
New bone	E	7	6233,1429	7665,97314	2897,46550	0,383
	F	7	3606,0000	4768,06795	1802,16029	
Fibrosis	E	7	20961,1429	11217,22917	4239,71411	0,007*
	F	7	6873,1429	3155,79432	1192,77814	

Mann-Whitney U test

(*p<0,05 statistically significant)

There was no statistically significant difference in new bone measurement between combined systemic and local application of boric acid group and systemic boric acid administrated group on the day 14. (Group E and group F respectively) (p> 0.05).

A statistically significant difference was found in the measurement of fibrosis between combined systemic and local application of boric acid group and systemic boric acid administrated group on the day 14 (Group E and group F respectively) ($p<0.05$). The average fibrosis value of combined systemic and local application of boric acid group is higher than the systemic boric acid administrated group. (Table 19)

Table 20: Binary comparison between groups G and H

	group	N	Mean	Std. Deviation	Std. Error Mean	P value
New bone	G	7	3813,2857	1658,86083	626,99046	0,038*
	H	7	8133,2857	5046,66790	1907,46117	
Fibrosis	G	7	6061,2857	2154,93122	814,48744	0,001*
	H	7	2004,7143	1195,59465	451,89230	

Mann-Whitney U test

(*p<0,05 statistically significant)

A statistically significant difference was found in the new bone measurement between combined systemic and local application of boric acid group and systemic boric acid administrated group on the day 28 (Group G and group H respectively) ($p <0.05$). The average value of systemic boric acid administrated group is higher than the combined systemic and local application of boric acid group on the day 28 (Group H and group G respectively).

A statistically significant difference was found in measurement of fibrosis between combined systemic and local application of boric acid group and systemic boric acid administrated group on the day 28 (Group G and group H respectively) ($p <0.05$). The average fibrosis value of combined systemic and local application of boric acid group is higher than systemic boric acid administrated group (Group G and group H respectively). (Table 20)

Table 21: Binary comparison between groups A and F

	group	N	Mean	Std. Deviation	Std. Error Mean	P value
New bone	A	7	6453,1429	4890,64612	1848,49048	0,259
	F	7	3606,0000	4768,06795	1802,16029	
Fibrosis	A	7	21412,5714	16102,48461	6086,16711	0,004*
	F	7	6873,1429	3155,79432	1192,77814	

Mann-Whitney U test

(*p<0,05 statistically significant)

There was no statistically significant difference in new bone measurement between local boric acid application group and systemic boric acid administrated group on the day 14 (Group A and Group F respectively) (p> 0.05).

There was a statistically significant difference in the measurement of fibrosis between local boric acid application group and systemic boric acid administrated group on the day 14 (Group A and group F respectively) (p<0.05). The average fibrosis value of the local boric acid application group was higher than systemic boric acid administrated group on the day 14. (Table 21)

Table 22: Binary comparison between groups C and H

	group	N	Mean	Std. Deviation	Std. Error Mean	P value
New bone	C	7	6767,2857	6096,70705	2304,33867	0,535
	H	7	8133,2857	5046,66790	1907,46117	
Fibrosis	C	7	7883,7143	3924,26310	1483,23204	0,001*
	H	7	2004,7143	1195,59465	451,89230	

Mann-Whitney U test

(*p<0,05 statistically significant)

There was no statistically significant difference in new bone measurement between local boric acid application group and systemic boric acid administrated group on the day 28 (Group C and Group H respectively) (p> 0.05).

There was a statistically significant difference in the measurement of fibrosis between local boric acid application group and systemic boric acid administrated group on the day 28 (Group C and group H respectively) ($p<0.05$). The average fibrosis value of the local boric acid application group was higher than systemic boric acid administrated group on the day 28. (Table 22)

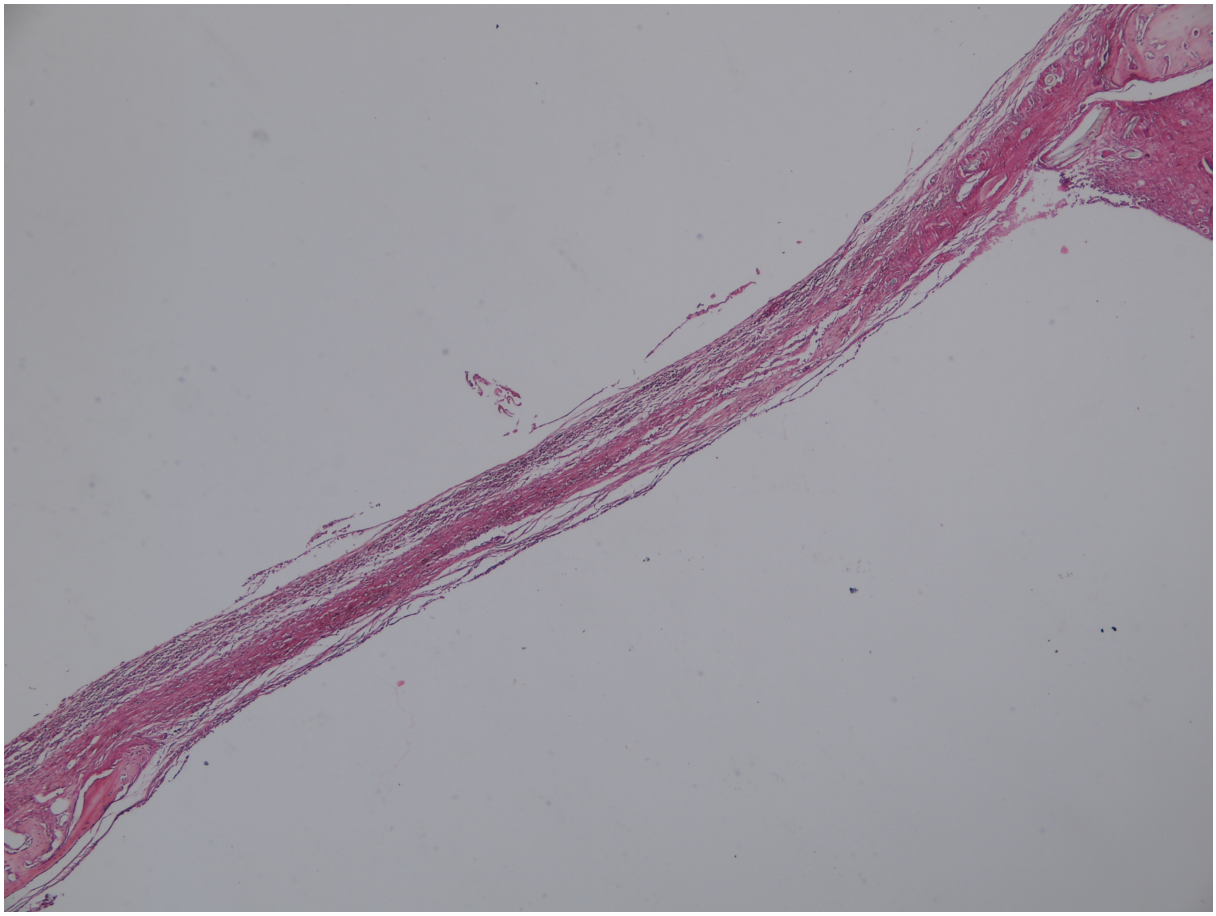

Figure 6: Photo from histological section of group A, showing fibrosis area occurred at the defect site.

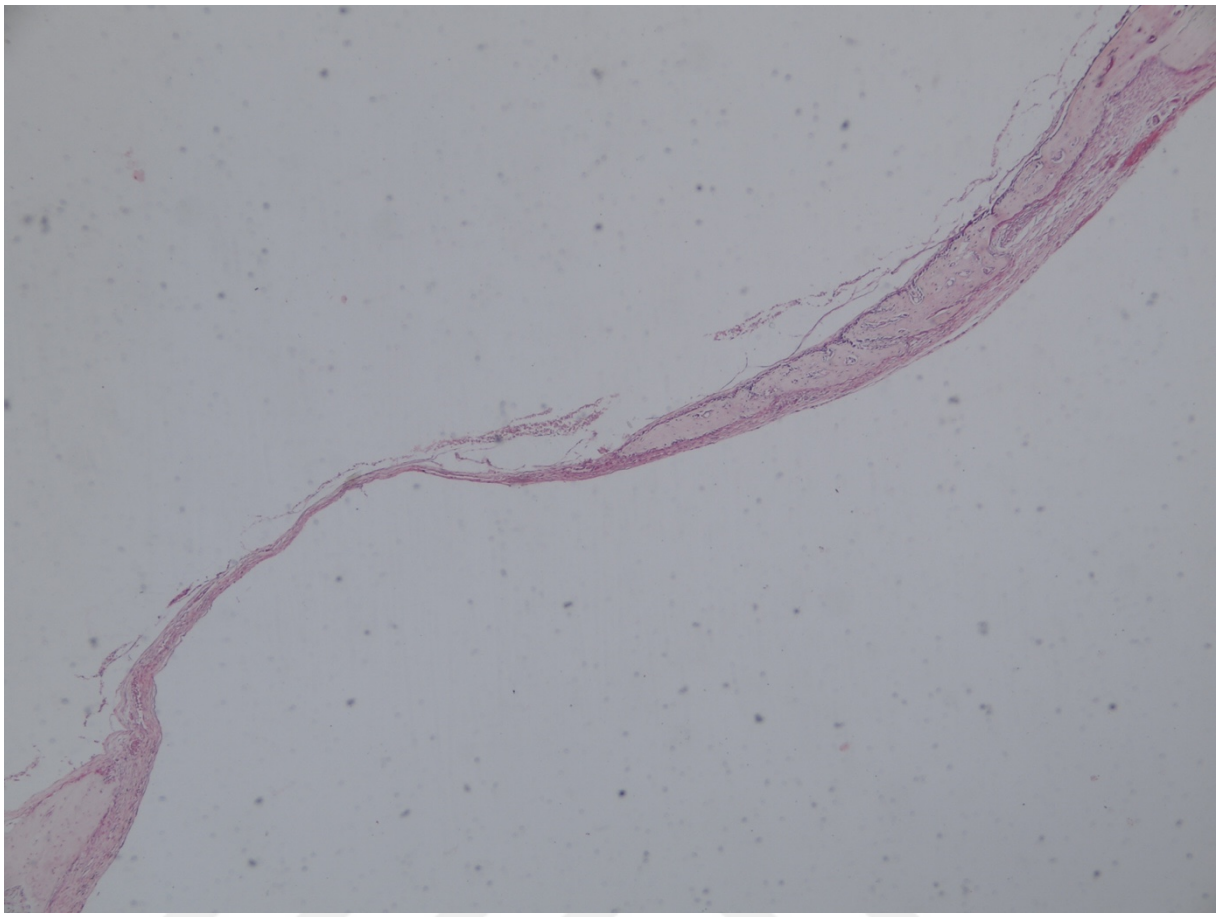

Figure 7 : Photo from histological section of group B, demonstrating new bone formation area at the defect site. (Magnification x40, H&E)

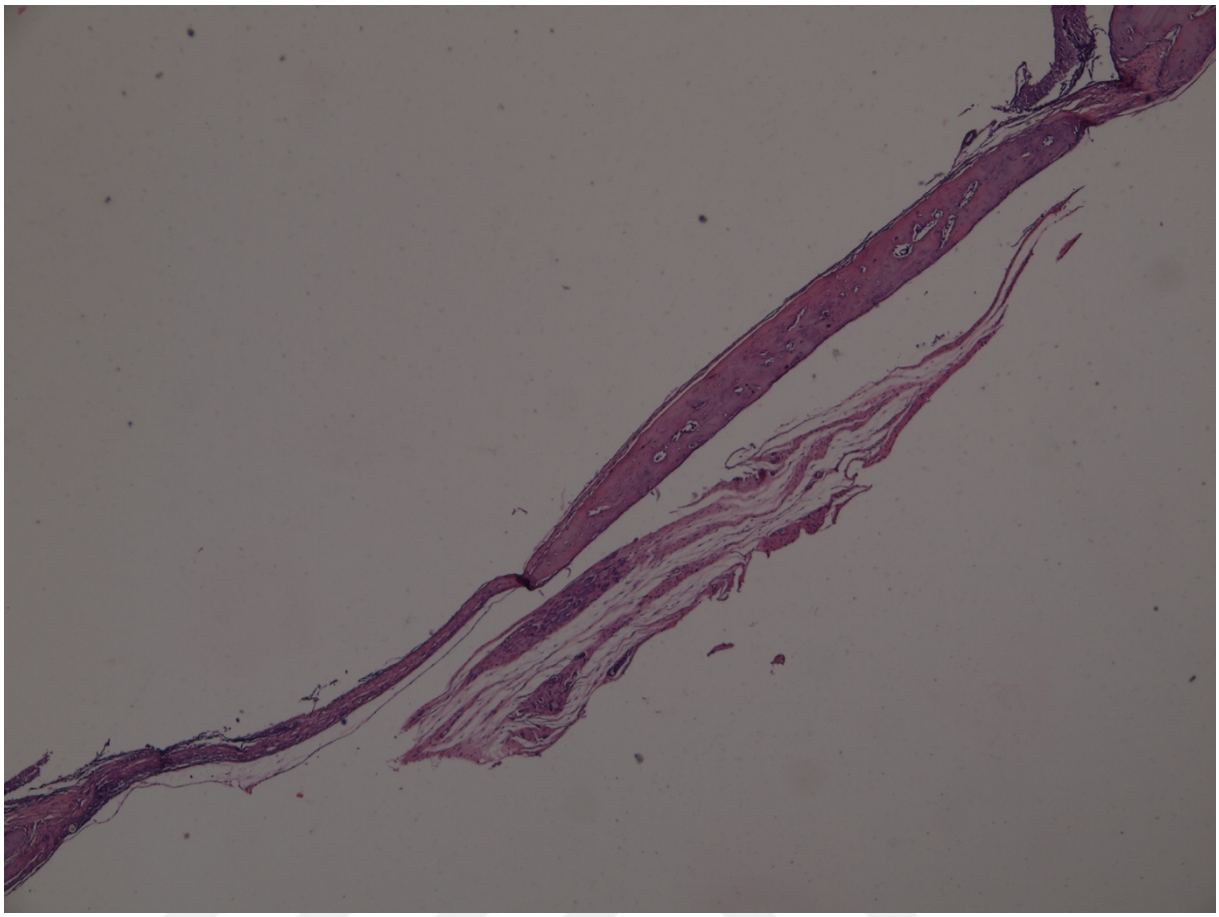

Figure 8: Photo from histological section of group H, screening the bone formation area and the fibrosis tissue. (Magnification x40, H&E)

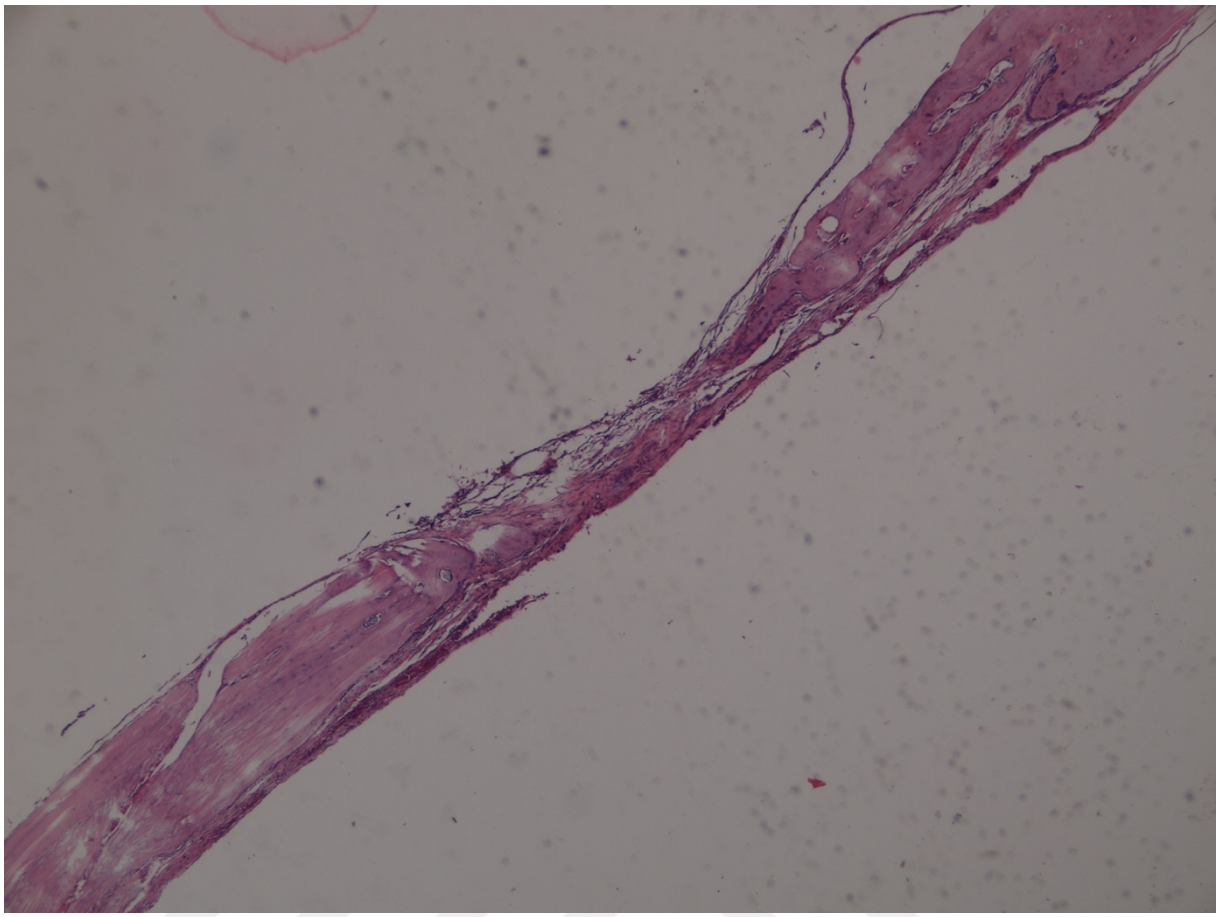

Figure 9: Photo from histological section of group D, revealing the new bone formation. (Magnification x40, H&E)

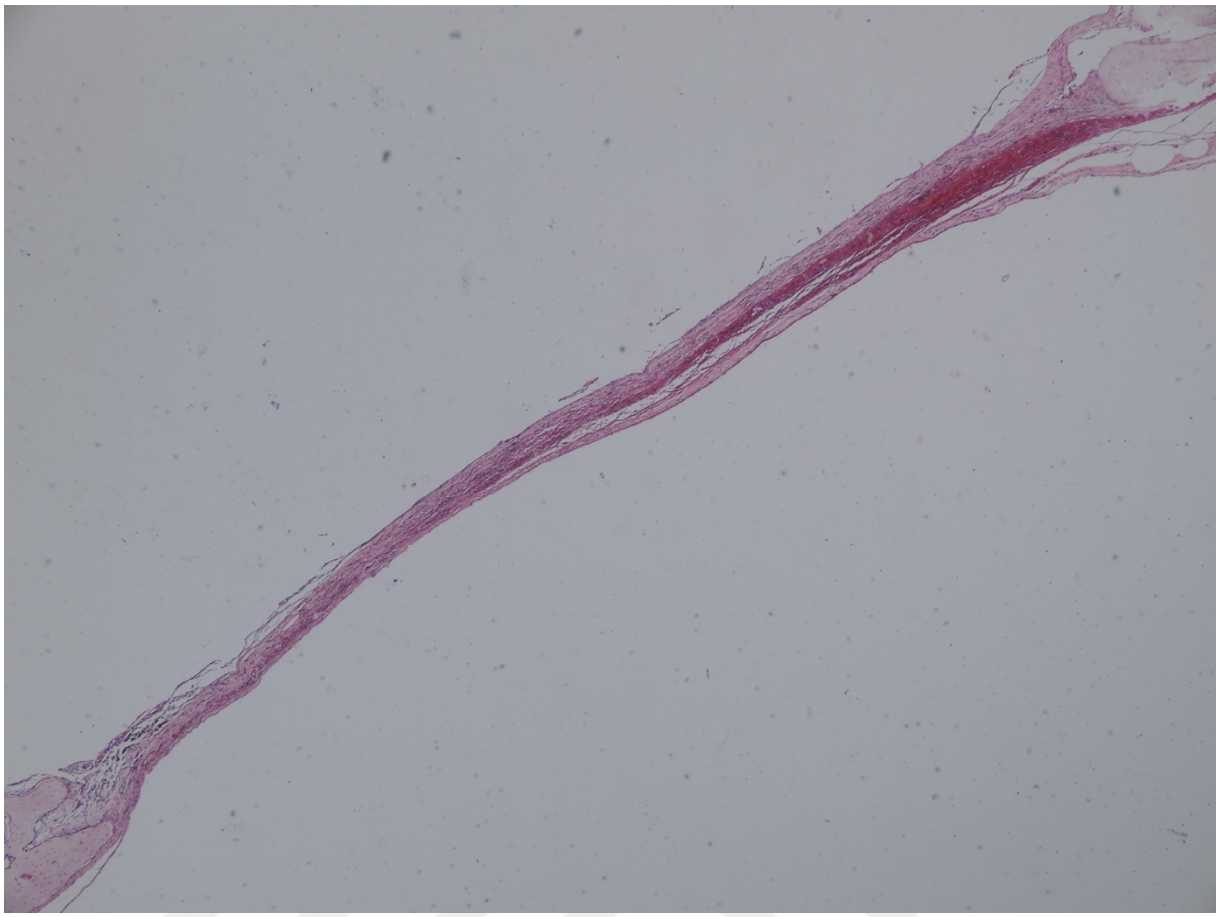

Figure 10: Photo from histological section of group C, showing the fibrosis tissue. (Magnification x40, H&E)

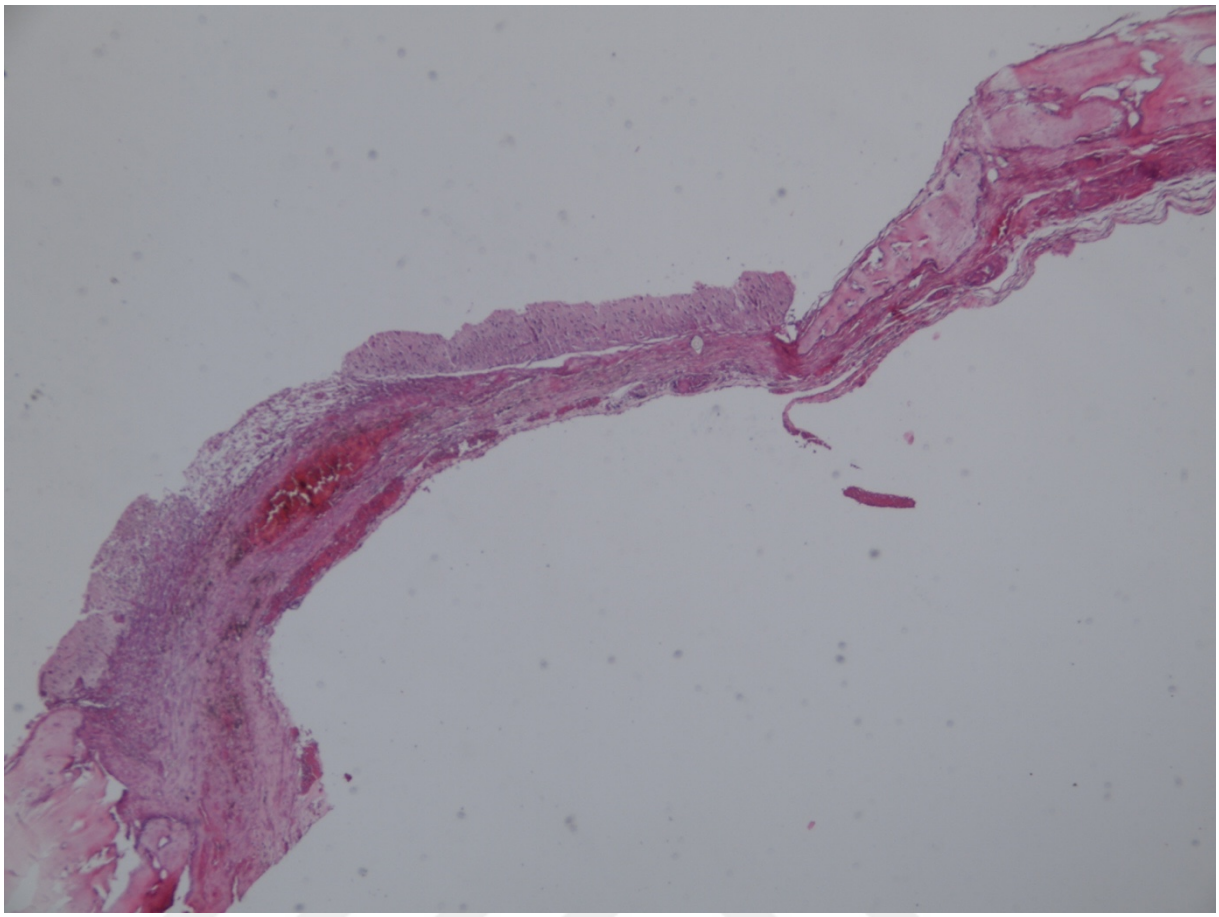

Figure 11: Photo from histological section of group E, demonstrating the connective tissue accumulation as fibrosis tissue and limited amount of new bone formation at the edge of the defect site. (Magnification x40, H&E)

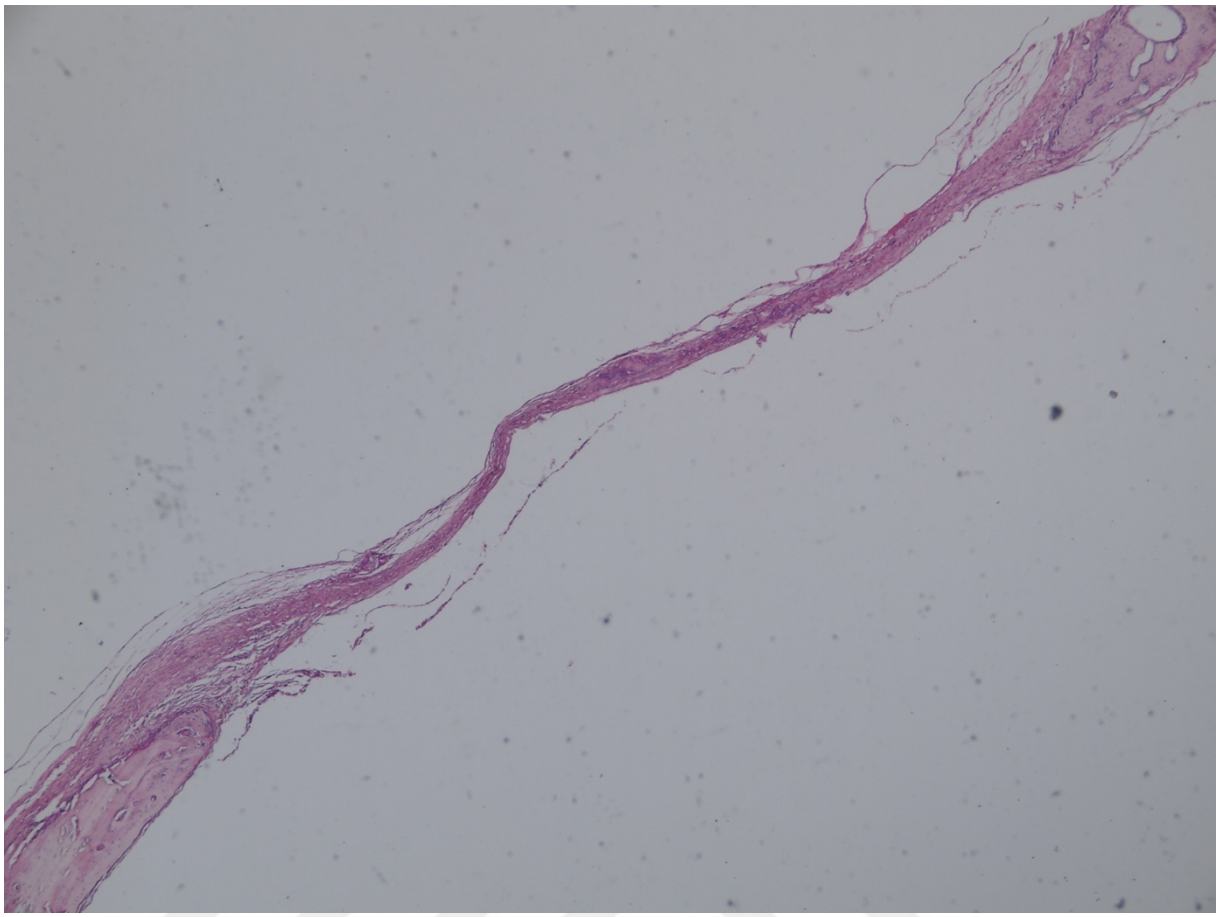

Figure 12: Photo from histological section of group F, revealing that limited amount of new bone formation and fibrosis tissue. (Magnification x40, H&E)

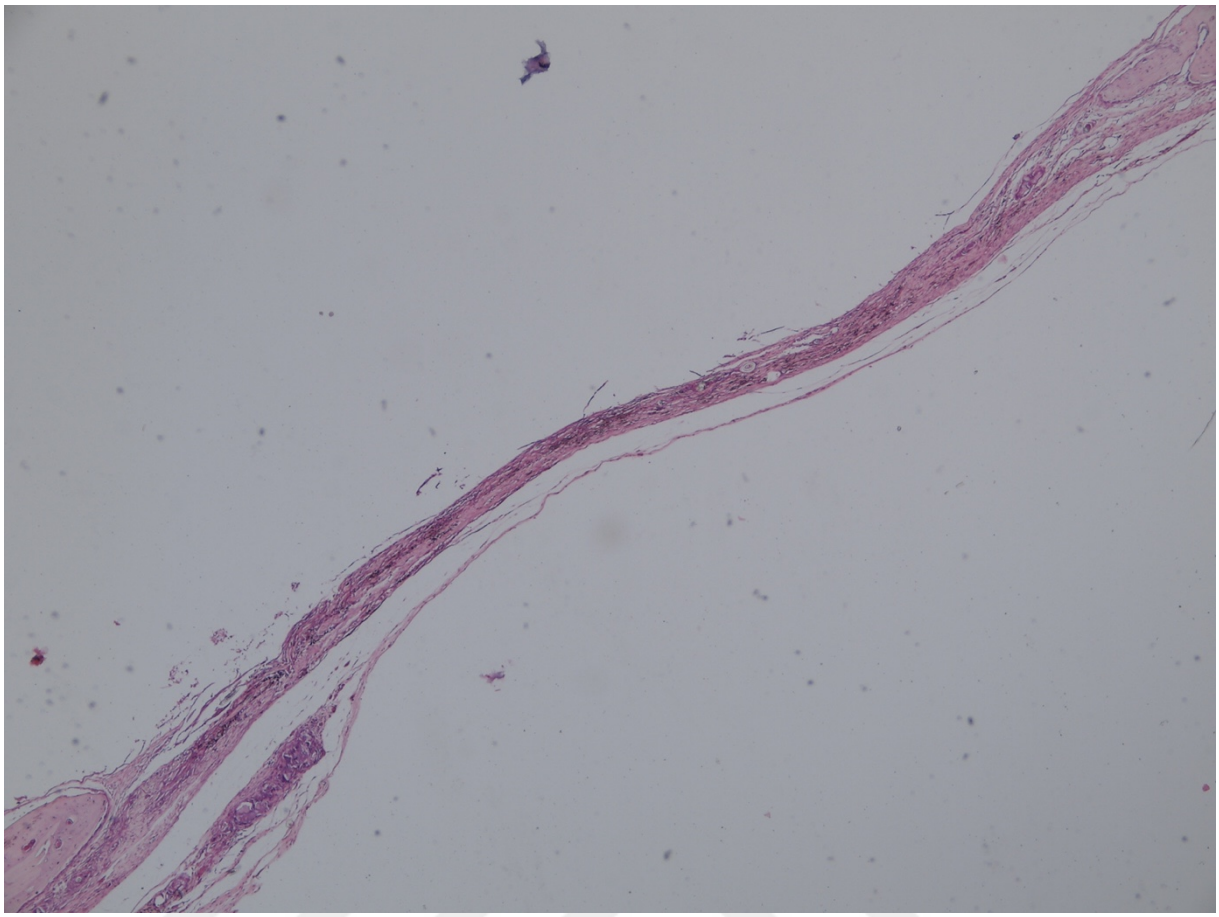

Figure 13: Photo from histological section of group G, showing fibrosis tissue. (Magnification x40, H&E)

5. DISCUSSION

In oral and maxillofacial surgery, the leading causes of bone defects are; tooth extractions, benign or malignant tumors, cystic lesions, congenital diseases and deformities, developmental disorders, infection and trauma. Critical sized defects in the oral and maxillofacial region occur especially due to congenital diseases, injuries, tumor excisions, loss of the structure, function and shape of the bone tissue due to excessive resorption in the alveolar bone after tooth extraction. The treatment of these defects is still controversial, especially for those of critical size[103].

Experimental animal models, which fully and accurately represent regenerative challenges in humans, are in high demand for research on regenerative biomaterials targeting the regeneration of large bone defects[103]. In the literature, there are many biomaterial studies conducted in the tibia, calvaria and mandible of rats, guinea pigs and rabbits in order to increase bone formation in bone defects[8,99,103–109]. These animal experiments are necessary to provide useful scientific information about the most effective and efficient way to treat different clinical conditions[104]. As mentioned above, many types of experimental animals have been used, however, research has focused on rodent models for repeatability, yield and economic reasons. It is often preferred in experiments because rats and mice are low cost, easy to transport and also very similar to humans in terms of genetic, biological and behavioral characteristics. Rodents are also advantageous compared to other experimental animals in terms of repeatability of the experiment, applicability and comparison of a wide variety of materials, and low morbidity and mortality in the experimental process[105]. Since the regeneration rate is high in rodents, there is a special interest in rodents in bone regeneration research. Rats are animals that are easier to work with in terms of size compared to mice[104].

Many areas of the rodent skeleton, such as the femur, spine, mandible, and the calvarium, are suitable sites for bone defect studies[105]. The calvarium is less vascularized and poor in bone marrow than other bones, while the other bones have primary feeding artery, the main feeding vessel of cranium is the middle meningeal artery. The nutrition of the cranium bones is provided by arterioles from the dural arteries. In addition, arteries can enter the skull through muscles such as temporalis but the human skull lacks muscle connectivity compared to the skulls of other mammals. As a result, even small bone defects cannot be repaired by themselves. Regenerative capacity of the calvarium of the

experimental animals is much better than the humans[106]. However, regenerative capacity of the calvaria is lower than the mandible [107]. Calvarial bone healing has many similarities with bone healing in the maxillofacial region. Morphologically and embryologically, the calvaria develops from a membrane precursor and thus resembles membranous bones on the face. Anatomically mandible consists of two cortical bone plates, such as a calvaria, and cancelous bone in between. Physiologically, the cortical bone in the calvarium resembles an atrophic mandible [106]. Given the purpose of the biomaterial or bone regeneration strategy, the rat calvarial defect serves as a fast and highly efficient method in evaluating bone regeneration in vivo. It is a bone defect capable of representing biomaterial, bone regeneration and tissue engineering studies and craniofacial applications in humans without the need for larger animal species [105].

Critical size bone defect study models are the gold standard in evaluating bone regeneration. Critical defects are those that do not heal without intervention or spontaneously during the life of the animal or during the experiment. When left to heal spontaneously in bone defects opened at a critical size, more fibrous connective tissue is formed rather than bone tissue [103,104,110]. Rat calvarial critical sized defect was accepted as 8 mm diameter [106,109]. In cases where fewer animals should be included in the study, two bone defects can be opened in the calvarium. In these circumstances, 5 mm bone defects in circular or square form are opened on the parietal bone[108,111]. Bosch et al.[111], in the defects opened at a diameter of 5 mm, none of them was reported spontaneous bone regeneration except for minor bone formation at the defect boundary, 6-12 months after the experiment. Cooper et al.[109] reported that the 2.3 mm diameter defects opened in the rat's aquarium showed about 35% improvement after 6 weeks. They also reported that the preservation of the dura-meter of the rodent species did not affect calvarial bone healing.

In the light of the animal ethics, rats, the smallest animal species which are useful for the study were used for the experiment. Their usage is also advantageous in the bone studies. Calvarium was selected as the experimental location, because of its similarity to the human jaw bones in both biological and physiological terms. In addition to these, two bone defects can be opened in a single operation site and consequently the number of the experimental animal group size is halved. It was decided to create two 5 mm diameter critical sized bone defects symmetrical to the sagittal suture on the parietal bone in the rat calvarium.

In the 19th century, boron was used to preserve and to prolong the palatability of the food such as meat and dairy products. Boron was contributed a vital role as preventing the food crisis during both world wars. Boron was considered non-toxic, because there were no deaths reported in relation to boron. On the other hand, some researchers were questioning the usage of boron for preserving the products. In the beginning of the 20th century, Wiley[112] reported that, boron might be toxic for human health and specified the doses which might be harmful for humans. After similar reports about boron's negative effects on human health, it was forbidden to be used as a food preservative. Subsequently, in middle of the 20th century, further research about boron revealed that boron was an essential element for plants' metabolism but not for humans[9]. In early 1980s, Hunt and Nielsen[113] showed that boron deficiency exacerbated gross bone deformities in chicks fed marginal amounts of vitamin D. This finding was the beginning of the fall of dogma, which the boron was not having any nutritional benefits on animals[9].

Boron regulates the metabolism of some micronutrients such as phosphorus, calcium, molybdenum, aluminum. Bone metabolism depends on the magnesium, calcium, vitamin D and phosphorus. In addition, it was suggested that boron optimistically influences the metabolism of these nutrients which play a noteworthy function on bone health[90]. Nielsen and Hunt reported that boron supplementation decreased the level of abnormalities caused by inadequate magnesium intake[12]. Nielsen et al.[114] worked with magnesium deficient rats and reported that boron deprived diet led more severe magnesium deficiency symptoms. Hunt and Nielsen[113] studied on the possible linkage between boron and vitamin D in chicks. Vitamin D-deficient chicks were fed by 1 mg/kg basal diet and 3 mg/kg boron supplementation significantly enhanced the growth by 38% and decreased the level of rickets cases. Boron supplementation increase the growth by 11% in the vitamin D-adequate chicks. In addition to these findings, in another study evaluating the relationship between boron and vitamin D, it was found that vitamin D-deficient but boron-supplied diet increased the 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol concentrations. In contrast, for low levels of boron and proper amounts of vitamin D, there was increase in only the 1,25-dihydroxycholecalciferol plasma concentration. These findings suggested the positive correlation between boron and vitamin D metabolism[96]. Moreover, Hunt and Nielsen suggested that, boron has an effect on vitamin D metabolism through an alteration of the calcium, phosphorus and magnesium[12]. On the other hand, Elliot and Edward designed

comparable study about boron supplementation in chicks. They reported conflicting results from their study that, boron supplementation failed to increase growth rate and food consumption in chicks[115]. Furthermore, another research was made by Nielsen about magnesium metabolism on post-menopausal women and reported that the dietary boron supplement had a significant effect on micromineral metabolism[116]. Moreover, Nielsen suggested in another study that deficient boron adversely affected bone strength and mineral element composition in rats. In addition, boron and fat sources act synergistically to beneficially affect bone formation in rats[117]. Consequently, boron could have effect on the bone metabolism and improve bone microstructure[90].

Recently, there has been lot more research about boron metabolism both in vivo and in vitro.

Enhancing effects of boron on bone strength and microstructure was evaluated using the assessment of the breaking forces, micro CT evaluations in different kind of mammals such as rabbits and rats. Bone strength was evaluated referring to bone density values, mineral content and breaking forces on the rabbits. The rabbits were fed by high energy diet and trial groups were given additional boron supplementation. The maximum force to break the femur and the bone mineral composition were both higher in the additional boron receiving group[13]. Naghii et al.[118] reported the bone mechanical properties with the supplementation of calcium and boron in rats. They concluded that boron supplementation increased biomechanical properties of bones compared with the control groups[118]. Another research was published by Nielsen on rats about interaction between dietary oil types and dietary boron. It was concluded that boron and lipid source which contains high in α-linolenic acid act synergistically to affect bone formation in a positive way in rats[117].

An in-vitro study about boric acid's protective effect on DNA damage and wound healing on human epithelial cells was published by Tepedelen et al.[119] . The result of the study indicated that boric acid application induced the reduced wound size by promoting cell proliferation and increased healing capacity. In addition, boric acid might reduce cytotoxic chemotherapy-induced oxidative DNA damage and inflammation in normal human epithelial cells[119]. Another in-vitro study using the MC3T3-E1 cells (osteoblast precursor cell line derived from mouse calvaria) reported that boron displays important roles on bone metabolism. They applied boric acid to cell cultures in different

concentrations in order the evaluate actions and potential toxicity. It was concluded that boron regulates extracellular proteins including collagen, osteocalcin and osteopontin[15]. Ying et al. demonstrated the effect of boron on osteogenic differentiation of human bone marrow stromal cells and reported that boron can increase osteogenic effect by stimulating osteogenic differentiation[14].

M. Gümüsderelioglu et al.[120] conducted an in vitro study with pre-osteoblastic cells differentiation using boron encapsulated bone scaffolds and reported some cellular effectiveness of the boron contained chitosan scaffolds. Mitochondrial activation of the chitosan-boron group was lower at the 3rd day of incubation than the 21st day. Another cellular marker alkaline phosphatase (ALP), which is the early stage marker of the osteogenic differentiation showed no difference on the 7th day of the culture, but on the 14th day of incubation, boron containing groups was presenting significant results on ALP activity. Collagen synthesis is the primary function of differentiated osteoblasts. Type I collagen synthesis showed the highest level on control group on 7th day. Moreover, it was increased in 3 folds in control group on the 14th day while boron-contained scaffold was increased 6 folds[120]. These outcomes could be a proof of short term boric acid application showing any statistically significant results, regardless of the dose and application method.

In this study, it was observed that the rats which were sacrificed on the 14th day, did not show any statistically significant positive effect on bone regeneration in neither local, nor systemic boron applications. The combination of both methods neither showed significant positive change in new bone formation. Local dose selection was made according to Blech et. al. and Benderdour et. al. and systemic dose was decided based on the Nielsen et. al. and Gorutovich et. al. reports [98–101]. Although, it was not observed significant results based on the 14th day measurements, when it was compared with the control group, local application of boron compound was showed up better outcome, combination of local and systemic application was showed up less effective with a small difference but still better effect then the control group. Hakkı et al.[15] reported that higher intensity of boron may decrease cell survival rate at a short time period but later this influence disappears. In the present study, fibrosis measurements' calculation appeared at the highest levels with local application and local and systemic combined application groups on the day 14. It showed up observable decrease in terms of fibrosis on the day 28

for all groups. This consequence presented parallel results with the report of Hakkı et. al.[15] . Ying et al.[14] reported different results on boron treated cell culture groups with varying boron concentrations for different time periods on special marker proteins such as OCN, BMP7, COL1 and ALP. According to their reports, cell proliferation numbers were decreased in all groups with the boron concentrations of 1,10,100 and 1000 ng/ml and the most concentrated boric acid group result was statistically significant. In addition, it was detected that mRNA synthesis of BMP-7 was increased 1.5-folds and 5.5-folds at 100ng/ml boric acid treatment group at 4th and 7th day respectively [14]. The increase between fourth and seventh days could be an explanation of the boric acid effect in early and late stages. When the new bone formation measurements of the groups that were sacrificed in the early period were evaluated, the efficiency order was observed as local application, combined application, control group and systemic group. However, when the evaluation was made in the late period, the efficiency ranking was observed as systemic application, local application, control group and combined application. When the effectiveness of new bone formation was evaluated for the early and late periods, the most effective increase was seen in the systemic application group, followed by the efficiency of local application. A serious decrease was observed in the effectiveness of the combined application in the late period. While the combined application seemed to be more effective in the early period compared to the control group, its effectiveness was not observed in the late period and showed less bone formation than the control group. It can be commented that the effectiveness of systemic application increases over time, on the other hand, the effectiveness of local application occurs faster. While systemic application and local application gave successful results separately in the late period, observation of the effectiveness of the combined application less than the control group can be interpreted as an increase in boric acid concentration.

An in-vivo study was conducted in rabbits on mid-palatal suture expansion experiment with the aid of systemic boron. Uysal et al.[121] reported that mineralized area at the expanding suture site increased significantly and fibrosis area size decreased with the groups of boron applied. Mineralized area of groups with boron intake was significantly higher than the control groups, moreover the changes between boron intake groups of 10 days and 20 days showed increase in 20 days but no statistically significant results were reported[121]. In our study, we obtained statistically significant result between

systemic boron applied groups of 14 day and 28 day. We can construe that systemic boron application effect maximizes in the long term.

Another in-vivo study conducted on rats evaluated the boric acid effect on experimental periodontitis and alveolar bone loss. Experimental periodontitis was demonstrated with the ligature around tooth and control group without ligature. It was reported that, boric acid-treated group with ligature showed enhancing results in osteoclast count, osteoblast activity and bone loss measurements. It was concluded that systemic administration of boric acid inhibited periodontal inflammation and reduced alveolar bone loss in their model[122]. Toker et al.[123] reported that systemic boric acid administration diminishes alveolar bone loss in experimental periodontitis on osteoporotic rat models. Scorei and Rotau[124] reported a study which was on borate molecules acting as an anti-inflammatory agent. Borate compounds, as well as boric acid and other boron sources, might influence macrophage production of inflammatory mediators. Moreover, it can be favorable for the overpowering of cytokine production and inhibit progression of endotoxin-associated diseases. Furthermore, it has been hypothesized that boron interaction with steroid hormones facilitates hydroxylation reactions and acts in some manner to protect steroid hormones from rapid degradation[124]. These effects of boron compounds are the most notable impact on the living organism. Saglam et al.[125] published a study, which was planned as an application of ligature to the tooth in order to demonstrate the periodontitis in rats like the study of Demirer et al.[122] but additionally it was evaluating the RANKL and OPG numbers of the sacrificed tissues[125]. RANKL is a member of the tumor necrosis factor ligand superfamily and its expression is a marked indicator of osteoclastic activity and bone loss. Osteoprotegerin (OPG) is a soluble tumor necrosis factor receptor like molecule which acts as a decoy receptor and prevents the binding of RANKL to RANK and therefore inhibits osteoclastogenesis[126]. Saglam et al.[125] stated that administration of boric acid diminished alveolar bone loss by affecting the RANKL/OPG balance in periodontal disease in rats. In our experiment, we can report that 28-days systemic administration of boric acid reduced the connective tissue accumulation such as fibrosis with statistically significant results. It was demonstrated that systemic administration of boric acid for 28 days came up with the lowest fibrosis value in histomorphology. When the fibrosis formation measurements of the groups that were sacrificed in the early period were evaluated, the efficiency ranking was observed as systemic application, control group, combined

application and local application group. While the same efficiency ranking is observed in the evaluation made in the late period, serious decreases are observed in the formation of fibrosis in the late period. In the related measurements, while much higher fibrosis values were observed in the local boron applied group and the combined application groups compared to the control group in the early period, a serious decrease in the fibrosis values of these groups was observed in the late period. On the other hand, although more fibrosis values were observed in the late period compared to the control group, the difference in value stands out to be much more minimal. The early and late response of systemic boron administration group in terms of connective tissue accumulation is more effective compared to the control group.

The administration of 3% boric acid speeds up particularly deep wound healing and reduces two-thirds of the time necessary to be spent in intensive care[100]. Demirci et al.[127] reported that by promoting cell migration, epithelization, and angiogenesis in burn injuries in rats, the use of boron-containing hydrogel promotes more rapid wound healing. The application of boron-containing hydrogel to wounds in diabetic rats has been shown to contribute positively to wound healing[127]. By affecting extracellular matrix synthesis, which increases the release of collagen, proteoglycans and proteins, boron compounds play an important role in the wound repair process[101]. Moreover, Demirci et al.[128] applied boron-containing hydrogel to the wounds in diabetic rats and reported that on the 8th day of wound healing relative to the control group, the application of boron-containing hydrogel made wound areas shrink faster[128]. Konca et al.[129] conducted an in vivo study with rats using oral (systemic) boron, local boron, and combination of both application methods and evaluated the boron effects on wound healing. The study stated that, boron compounds' antioxidant properties indicate acceleration on wound healing macroscopically. In support of this statement, their study findings claim that systemic boron, local boron, and the combination of both applications, showing significantly smaller wound areas on days 7, 9, 11, 13, 17, 19 and 21 than in the control group. Furthermore, wound areas on the day 7, local boron group significantly smaller than both control and combination therapy group. In the same study, it was drawing attention that, boron compounds were not effective on the day 1, 3, 5 and its efficiency started to show up on the day 7 [129]. In the present study, in terms of connective tissue accumulation, systemic application showed up the best effectiveness and fibrosis value decreased both short and long term. On the other hand, local and combined applications showed up better

results on the day 28. These results can be evaluated in parallel with the results of Konca et al.[129] .

Another in vivo study was conducted by Korkmaz et. al.[130] , to investigate the effect of boron on osteochondral defect repair and also on some parameters of antioxidants and oxidants in both cartilage tissue and blood. In this study, hyaluronic acid and boron compound were applied directly to the intraarticular area, hyaluronic acid and boron compound were compared in the repair of osteochondral defects, and it was proposed that boron was not as successful as hyaluronic acid , but its antioxidant property was superior to hyaluronic acid. In this study, hyaluronic acid and boron compound were applied once a week for four weeks period [130]. On the other hand, in the present study, boron compound was applied locally only on the day of surgery to the defect site. Local application doses should be discussed deeply, because there were no consensus over the local application of the boron compounds directly to the tissues. We applied 3% boric acid solution to the defects locally and this was approximately 15 micrograms of boric acid. This amount of the application might be an inadequate dosage for the cellular efficiency in different time periods.

6. CONCLUSION and ACKNOWLEDGMENT

1- Systemic boric acid application was detected to be the most effective in terms of fibrosis on the 14th and 28th day which is evaluated as both long term and short term effect in the present study.

2- Combined (systemic + local) boric acid applications were not as effective as the systemic application only. The reason behind this result should be the limited local effectiveness of the boric acid in short term or insufficiency of the dose applied to the defects. Combined effect had more contribution to fibrosis decay than sole local application on the 14th and 28th day. This could be a result of the significance of systemic application to lower the connective tissue accumulation and act as an anti-inflammatory drug. Moreover, it can also be explained by the insufficient local dose.

3- Local boric acid application on the 14th day was observed to be ineffective in terms of fibrosis. It also diminished the effectiveness of the systemic application. Connective tissue accumulation displayed the highest value on the 14th day for both combined and local applications. On the 28th day, the combined application diminished the systemic effect of the boric acid on fibrosis.

4- In terms of new bone amount, the best results of bone regeneration was observed on the 28th day systemic boric acid group. the combined application ended up with the lowest result of generated new bone amount. These results were not statistically significant but systemic application should have a moderate effect on value of the regeneration area count.

5- In between 14th day sacrificed groups, the best result was observed in the local application group in terms of bone regeneration area value.

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8. APPENDICES

8.1 Ethical Approval

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU

(YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
30.04.2019	749	02.04.2019	Ahmet Hamdi ARSLAN

'Lokal ve Sistemik olarak uygulanan Borik asitin sıçanlarda kemik iyileşmesi üzerine etkilerinin histomorfometrik olarak değerlendirilmesi' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 3 Yıl	Hayvan Türü ve cinsiyeti: Sıçan ♀	Hayvan Sayısı: 28

GÖREVİ	ADI SOYADI
Başkan	Prof. Dr. Bayram YILMAZ
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA
Raportör	Vet. Hekim Engin SÜMER
Üye	Prof. Dr. M. Ece GENÇ
Üye	Prof. Dr. Rukset ATTAR
Üye	Doç. Dr. Soner DOĞAN
Üye	Doç. Dr. Ediz DENİZ
Üye	Prof. Dr. Gamze TORUN KÖSE
Üye	Doç. Dr. Aylin YABA UÇAR
Üye	Hakan GÖKSEL
Üye	Ahmet ŞENKARDEŞLER

CURRICULUM VITAE

Personal Information

Name	Nevzat Sezer	**Surname**	Işıksaçan
Place of Birth		**Date of Birth**	
Nationality	T.C.	**National ID**	
E-mail		**Telephone**	

Education

Derece	Department	The name of the Institution Graduated From	Graduation Year
Phd	Oral and Maxillofacial Surgery	Yeditepe University	2021
University	Faculty of Dentistry	Yeditepe University	2015
High School	-	Cağaoğlu Anatolian High School	2009

Languages	Grades
English	59
German	B1 diplom

All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS etc.),

Work Experience (Sort from present to past)

Position	Institute	Duration (Year-Year)
Dentist	Private Practice	2020- cont.
Dentist	Yunus Emre Private Hospital	2018-2020

Computer Skills

Program	**Level**
IBM SPSS Statistics	basic
Microsoft Office	average

***Excellent, good, average or basic**

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Cabbar F, Burdurlu MC, Işıksaçan NS, Atalay B, Duygu Çapar G. 'What is the survival rate of dental implants in Turkey? A systematic review.' Biomed Res, 29 (3): 485-495, 2018.
Burdurlu MC, Cabbar F, Dagasan VC, Işıksaçan NS, Olgac V, 'The preventive effect of medicinal herbal extract and gelatin sponge on alveolar osteitis: An in vivo micro-computed tomography and immunohistochemical analysis in rats.' Archives of Oral Biology: 122 (2021), 105002.

Articles published in other journals

Cabbar F, Işıksaçan NS, Burdurlu MC, Özçakır Tomruk C. 'Evaluation of Positive Radiological Findings in Patients Using Complete Prosthesis.' 7tepe klinik, 14(1):31-34, 2018.
Burdurlu MC, Cabbar F, Dagasan V, Işıksaçan NS. 'The evaluation of acquired bone volume with onlay block grafting by dental volumetric tomography.' Yeditepe Dental Journal. (15):317-321, 2019

Proceedings presented in international scientific meetings and published in proceedings book.

Burdurlu MC, Cabbar F, Işıksaçan NS, Cinel L. 'Histopathologic misdiagnosis of a maxillary lesion: The role of surgeon.' International oral maxillofacial surgeons and 12th Oral and Maxillofacial surgery society joint congress, 9-13 May, Antalya, Turkey 2018. (Oral Presentation)
Işıksaçan NS, Cabbar F, Burdurlu MC, Atalay B, Duygu Çapar G. 'What is the survival rate of dental implants in Turkey? A systematic review.' 23rd International congress on oral and maxillofacial surgery, 31 March- 3 April, Hong Kong 2017. (Oral Presentation)

Others (Projects / Certificates / Rewards)

Certificate in Laboratory Animal Science (September 2018)