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RESEARCH ARTICLE

The Contribution of Differentiated Bone Marrow Stromal Stem Cell-Loaded Biomaterial to Treatment in Critical Size Defect Model in Rats [1]

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Summary

Mandibular fractures present a challenge in maxillo-facial surgery due to difficulties in healing and complications. In recent years, advances in bio-engineering as well as stem-cell studies suggest that it may be possible to treat these fractures by stem cell treatment with biomaterials. In the present study, we explored the efficacy of differentiated stem cells placed on biomaterials on fracture treatment and its relation with oxidative stress and apoptosis. A 4 mm circular defect was made on the mandibulae of 20 adults Wistar rats. Hydroxyapatite gel (control, n=5) and bone marrow stromal cells differentiated into osteoblast-seeded hydroxyapatite gel (n=5) were implanted within these defects. We were also used empty cavities (n=5) and cavities filled with only cells (n=5) for negative controls. Animals were sacrificed after a 6-week healing period and samples were examined blindly by histological, immunohistochemical, radiological and morphometric methods. Compared to the control cavities that underwent no procedure or filled with just cells, there were significant (P<0.001) healings in both groups. Hydroxyapatite gel with differentiated stem cells on, however, yielded significantly (P<0.05) better new bone formation and osteoid production decreased fibrous tissue and increased cellular activity. Differentiated stromal cells combined with biomaterial accelerated the treatment in defects of critical volume within a 6-week period of healing, activated and resulted in significant formation of bone of higher quality. Promotion of bone formation by the helps of bioengineering and stromal cells has gained importance in the treatment and reconstruction of fractures.

Keywords: *Bone marrow stromal cell, Healing, Critical size defect, Tissue engineering, Hydroxyapatite gel, NOS, Apoptosis*

Biyomateryal Üzerinde Farklılaşmış Kemik İliği Stromal Kök Hücrelerinin Kritik Hacim Defektli Sıçan Modelinde Uygulanmasının Tedavideki Yeri

Özet

Mandibular kırıklar iyileşmelerindeki zorluklar ve gelişen komplikasyonlar nedeni ile çene cerrahisinde önemli sorunlardır. Son zamanlarda gelişen biyomühendislik uygulamaları aynı zamanda ilerleyen kök hücre çalışmaları bu kırıklarda biyomateryal aracılığı ile kök hücre tedavisinin mümkün olabileceğini göstermektedir. Bu çalışmada biyomateryal üzerindeki farklılaştırılmış kök hücrelerin kırık tedavisinde etkinliği ayrıca oluşan oksidatif stres ve apoptosis ile ilişkisi araştırıldı. Erişkin Wistar sıçanların mandibulalarında 4 mm çapında yuvarlak defekt oluşturuldu. Defekt alanına hidroksi apatit jel ile hidroksi apatit jel üzerinde osteoblasta farklılaştırılmış kemik iliği stromal kök hücreleri yerleştirildi. 6 haftalık iyileşme periyodunu takiben öldürülen deneklerde alınan örnekler histolojik, immunohistokimyasal, radyolojik ve morfometrik yöntemler ile körleme olarak incelendi. Her iki uygulamada da hiçbir işlem yapılmamış veya sadece hücre konmuş kontrol kaviteleri ile karşılaştırıldığında oldukça anlamlı (P<0.001) fark oluşturacak şekilde belirgin bir iyileşme görüldü. Sadece hidroksi apatit jel kullanımı ile kırık kenarlarını kaplayan, yeni kemik oluşumu, osteoid üretimi, fibröz dokunun çokluğu ve hücresel aktivitenin azlığı ile karakterize iyileşme görüldü. Buna karşılık hidroksi apatit jel ile birlikte farklılaşmış kök hücre uygulamasının daha iyi yeni kemik oluşumu ve osteoid üretimi yanısıra fibröz dokuyu azalttığı ve hücresel aktiviteyi arttırdığı bulundu. Farklılaşmış kök hücre uygulamasının biyomateryal katkısı ile birlikte 6 haftalık iyileşme periyodunda olan kritik hacimli defektlerde anlamlı (P<0.05) bir şekilde tedaviyi hızlandırdığı, etkinleştirdiği ve daha kaliteli kemik oluşumunu sağladığı belirlendi. Kemik formasyonunun biyomühendislik aracılığı ve kök hücre yardımı ile etkinleştirilmesi kırık tedavisi ve rekonstrüksiyon uygulamalarında hastalara daha kaliteli bir yaşamı sunması nedeni ile giderek önem kazanmaktadır.

Anahtar sözcükler: *Kemik iliği stromal hücre, İyileşme, Kritik hacim defekti, Doku mühendisliği, Hidroksi apatit jel, NOS, Apoptoz*

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INTRODUCTION

Mandibular fractures are amongst the most commonly problems faced by cranio-facial surgeons. Whether it is elective or emergency, treatment of mandibular fractures is challenging. Infections and complications during ossification affect the efficacy of treatment [1-3] . Critical size defects (CSD) are appealing models to study the manipulations in bone healing. A defect measuring 4 mm in diameter is made on the ramus of the rat mandible does not heal before 16 weeks unless a procedure is carried out [4-6]. Treatment of CSD started with demineralized bone matrix and progressed into treatment with tissue engineering that utilized osteogenic cytokines, including bone morphogenetic proteins [6,7]. More recently, experimental studies with stem cells have been incorporated into these advanced techniques. These cells require a supportive matrix as a suitable milieu and a vehicle in the form of a soluble polymer. This vehicle should be able to give the differentiated cell the necessary support and be compatible with the tissue it is implanted in. By this way, cells that possess characteristics of the environment where the defect is and activate cell migration during wound repair and the biomaterial vehicle will yield wound healing to be more effective and functional [8].

Bone repair and new bone formation require differentiation of stem cells to osteoblasts [9,10]. Growth and differentiation factors affect *in vitro* stem cell differentiation and wound healing [11]. Bone marrow stromal stem cell (BMSC) placed on a biomaterial and implanted in the fracture region could differentiate into osteoblasts and contribute to fracture repair. Biomaterials developed by tissue engineering help by promoting adhesion and migration [12-14]. During healing, cells also show osteopontin (OP), osteocalcin (OC), osteonectin (ON) and alkaline phosphatase/Von kossa (ALP/VK) activities for the production of new matrix. They express osteoblastic markers in the form of nodules stained with alizarin red (AR) [15-17]. As an experimental model, healing in segmental bone defects [18] can be further accelerated by using differentiated BMSC implanted onto osteo-conductive extracellular matrix [19,20].

Synthetic biomaterials such as scaffolds can alter the biocompatibility, surface adhesion, mechanical strength, cell adhesion and separation by the help of tissue engineering. HA ceramics have long been used to enhance the biocompatibility capacity and bioactivity of the host bone tissue [21,22]. Physical and chemical properties of HA increase activation of cell repair and vascularization at the site of fracture. Better repair is achieved by efficient adherence, migration and proliferation between the stem cells and biomaterials. With their extracellular matrix (ECM), biological tissues and organs form a specialized, 3-dimensional structure that produce signals that affect cell behavior [23].

Better repair with osteoblast differentiated BMSCs (odBMSC) in tibial defects has been reported in previous studies [24,25] and implantation of these cells together with biomaterials to the fracture site would play an important role in fracture repair. Therefore, we implanted odBMSC with hydroxyapatite gel (HAG) into a CSD made on rat mandible to achieve a better fracture repair. The effects of this combination on new bone formation, cell activation at the site of fracture and implanted cells were explored. By this way, we aimed to investigate the *in vivo* healing effect of odBMSC on the biomaterials for better treatment of CSD related to oxidative stress and apoptosis.

MATERIAL and METHODS

BMSC Isolation, Culture and Differentiation

Twenty adult male Wistar rats weighing 200±50 g were used in this study, for which approval was given by the Ethics Committee for Animal Experiments of Ege University. BMSCs were harvested from the femurs and tibias as previously described [26,27]. Briefly, bones were aseptically excised from the hindlimbs of the rats following ether euthanasia. The soft tissue was removed, and the proximal end of the femur and distal end of the tibia were clipped off with sterile scissors, a hole was created in the knee joint end of each bone with an 18-gauge needle, and the marrow was flushed from the shaft with control media (CM) 50 ml α-MEM supplemented with 15% FCS (fetal calf serum, St. Louis, MO, USA), 50 µg/ml gentamycin, 100 U/ml penicillin, 100 U/ml streptomycin, 100 U/ml amphotericin B (Sigma, Chemical Co., St. Louis, MO, USA). This solution was resuspended, and the cell suspensions from all bones were combined in a centrifuge tube. The suspension was spun down at 800 rpm for 5 min. Cells were counted by using a haemocytometer (Bürker, Germany). The supernatant was aspirated, and the pellet was resuspended in fresh CM and seeded into twenty four-well plates. This high seeding density was chosen because a large portion of the cells harvested from marrow are nonadherent. The wells were rinsed three times with PBS on the third day of expansion to remove the non-adherent cells [28,29].

Cells were cultivated in twenty four-well plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and incubated in a humidified atmosphere of 95% air, 5%

CO_2 at 37°C (Hera Cell, Kendro Laboratory, Germany). After 3 days, medium containing floating cells which composed of mainly blood cells and fat cells mainly were removed and replaced by fresh osteogenic media (OM) which composed 10 mM Na-β-glycerophosphate, 50 µg/ml L-ascorbic acid and 10^{-8}M dexamethasone (Sigma, Chemical Co., St. Louis, MO, USA). The medium was changed every other day. Cells were microscopically observed under inverted microscope with a phase-contrast attachment and photomicrographs were obtained [30].

ALP/VK and AR Histochemical Staining for Osteoblastic Differentiation

For the ALP/VK histochemistry, the medium was removed, and the cell layers were rinsed with PBS 3 times and fixed in cold 10% neutral buffer formalin (NFB) for 1 h at 4°C. After, the cell layers were washed with deionized water and allowed air dry. Then, the fixed cells were incubated with buffer containing 0.1 mg/ml naphthol AS-MX phosphate disodium salt and 0.6 mg/ml Fast Red TR salt. After 1 h at 37°C, the cell layers were washed with deionized water. Then the cells were stained with 2.5% silver nitrate for 30 min. The cell layers were washed again with deionized water and observed with inverted phase-contrast microscope. Calcium phosphate deposit visualization of the ECM was detected by the VK method in which calcium phosphate deposits were stained brown to black. Using AR histochemistry, the cultured cells were stained for assessing the mineralized matrix. The medium was removed, and the cell layers were rinsed with PBS 3 times and fixed in cold 70% ethanol for 1 h at 4°C. Then, the cell layers were washed with deionized water and allowed to air dry. The fixed cells were stained with 2% AR S solution pH 7.2. After 1 h at 37°C, the cell layers were washed with deionized water and observed with inverted phase-contrast microscope [31].

Biomaterials

The commercial HAG (HAG, BoneMIX®) was supplied by INOVA (INOVA Ltd., Izmir, TURKEY). Bone MIX-HA Gel (HAG), Ca-P nonporous ceramic nanopowders consisting of HA [Ca10(PO_4)6(OH)2] was prepared by aqueous precipitation method. The chemical composition and crystalline structure of the Bone MIX® HAG corresponds to the calcium phosphate component of the natural bone. The fineness of the crystals gives a very large surface area of approximately 175 m^2/gr for dry gel. Particles HA-gel, 50-500 nm in size was selected. Material sample was sterilized by gamma radiation at 25 kGy [32,33].

Surgical Operation

The rats were premedicated with ketamine (90 mg/kg, Pfizer Warner Lambert) + xylazine (8 mg/kg, Alfasan International B.V.) intramuscularly. A linear incision was made through the skin, subcutaneous tissues, and masseter muscle, paralleling the inferior border of the left mandible. The buccal and lingual surfaces of the mandible were exposed with an elevator, and a 4 mm full-thickness defect was created in the body of the mandible, posterior to the root of the incisor. This 1 mm deep ostectomy was performed with a high-speed drill and irrigation and did not interrupt mandibular continuity at the alveolus. The resulting defect was filled with nothing (control=5) , with just cells (control=5), with biomaterial as HAG (control=5) or with cells on biomaterial (control=5). The surgical wounds were closed with 4-0 chromic catgut sutures. The animals were allowed to recover from anesthesia, and then returned to the Department of Laboratory Animal Resources for postoperative care where veterinarians supervised them. The rats were maintained on a diet of ground rat chow and water, to which they had access ad libitum. The mandibulas were radiographed at lateral position by means of a microradiography unit with X-ray film under standardized conditions (20 kV, 5 mA and 1 min) at the end of the experiment. The radiographed films were scanned at 300 dots per inch in a scanner equipped with a transparency adapter and a scanning software (Epson Scan, version 2.61, Seiko Epson Corp) [34].

Immunohistochemistry

Cultures were also assessed immunohistochemically using antibodies against OC (33-5400, Zymed, San Fransisco, ABD), or ON (33-5500, Zymed, San Fransisco, ABD) on selected days as follows. Sections were stained for iNOS (Labvision RB9242, CA, USA) and eNOS (Labvision RB9279, CA, USA). Samples were fixed with 4% paraformaldehyde in PBS (pH 7.4). Endogenous peroxidase was inactivated by incubation with 3% H_2O_2 for 30 min. After incubation with primary antibodies, the sections were incubated with biotinylated secondary antibodies and reacted with peroxidase-conjugated streptavidin using the protocol from a HISTOSTAIN kit (Zymed, San Francisco, USA). Samples were stained with diaminobenzidine (Zymed, San Francisco, USA) to detection of immunoreactivities. The primary antibody was omitted for negative control [30]

In situ Cell Death Detection Assay (TUNEL)

Immunohistochemical detection of cells undergoing DNA fragmentation was carried out using a terminal deoxynucleotidyltransferase-biotin nick end-labelling (TUNEL) method with a commercial in situ apoptosis detection kit (Dead End Colorimetric TUNEL system, Promega G7130). Cells, which were cultured and attached

in twenty four-well plates, fixed with 4% paraformaldehyde in PBS at 4°C for 30 min. After washing with in PBS twice for 3 min, they were incubated with 20 mg/ml proteinase K for 10 min. The cells were rinsed with PBS. Endogenous peroxidase activity was inhibited by 30 min incubation in 3% hydrogen peroxide in methanol at room temperature, and they were then washed several times in PBS. The samples were then incubated with equilibration buffer for 5 min and TdT-enzyme for 60 min at 37°C in a humidified atmosphere. The reactions were stopped by adding 2X SCC solution for 15 min at room temperature. They were then washed with PBS three times for 5 min and incubated with streptavidin–peroxidase for 45 min. Each step was separated by careful washing in PBS. They were then incubated with a solution containing diamino-benzidine (DAB, Sigma Chemical Co., St Louis Missouri, USA) 50 µl for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, Utah, USA) then viewed using an IX71 invert- fluorescence -phase microscope (Olympus, Tokyo, Japan). As negative staining control for TUNEL, TdT was omitted during the tailing of reactions. Staining was examined independently by two of the authors, who had no information about the samples. TUNEL positive cells were counted randomly chosen fields per case and data was given as a percentage. The percentage of apoptotic cells stained brown was determined. Cells in areas with necrosis or poor morphology were not analyzed. A minimum of 200 cells are counted at the same preparation but five different areas. Experiments are repeated at least three different times [35].

Morphometry

Sections from samples were evaluated using a light microscope (Olympus BX40, Tokyo, Japan). New bone formation (BF), osteoid formation (OF), fibrous tissue formation (FF), cellular activity (CA) as healing parameters and immunolabelling intensity was graded independently by two observers blinded to the experimental conditions with a scale starting from weak (+1) to strong (+5) definition [35].

Statistical Analyses

Data are presented as mean ± Standart Deviation and statistical analyses were performed using GraphPad Instat v3.01 software for Windows (GraphPad Software, San Diego, California, USA). The effects of the different media and serum were compared using repeated measures 2-way analysis of variance (ANOVA). ANOVAs were followed by multiple range tests when appropriate. Data are expressed as percentages in statistical analyses. Differences at $P<0.05$ were considered significant [36].

RESULTS

BMSC, under the influence of the OM, differentiated into osteoblasts in a way that included morphometric characteristics. Cells mineralized and expressed osteoblastic markers ALP/VK *(Fig. 1A)*, AR *(Fig. 1B)*, ON *(Fig. 1C)* and OC *(Fig. 1D)*, especially at the center of the colonies.

We examined whether there were any differences in

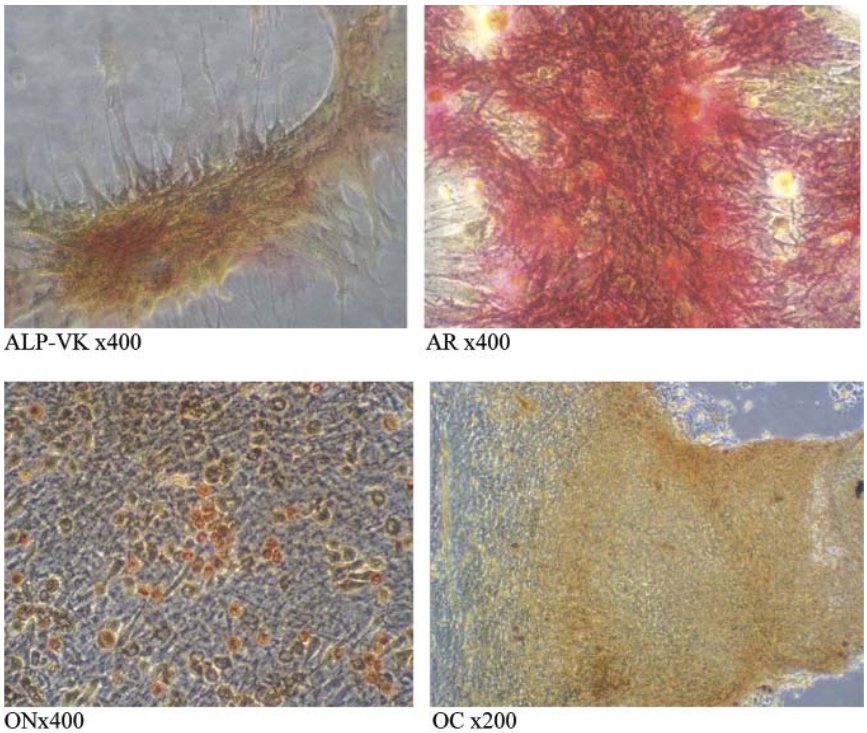


Fig 1. Differentiation of rat BMSCs after 14 days culture with OM. odBMSC showed colony formation and expression of ALP-VK, AR, ON and OC due to mineralization and matrix formation at the centre of the colonies

Şekil 1. Osteojenik medyum (OM) içeren kültür ortamında sıçan kemik iliği stromal kök hücrelerinin (BMSCs) 14 gün sonrası farklılaşması. Osteojenik farklılaşmış kemik iliği stromal kök hücreleri (odBMSCs) koloni formasyonu oluşturup koloni merkezlerinde içerdiği mineralizasyon ve matriks oluşumu nedeni ile alkalen fosfataz,Von kossa (ALP/VK) ve alizerin kırmızısı (AR) ile boyanıp, osteonektin (ON) ve osteokalsin (OC) tanımlaması gösterdi

the outcomes after a 6-week repair stage after odBMSC-seeded HAG or HAG only administration in CSD. Radiographs showed a marked defect in the control group while complete bone repair and closure of defects were observed in the HAG administered group *(Fig. 2)*. On macroscopic images, presence of healing was noted at different levels. We found that only stem cell or differentiated stem cell administrations were not effective and that HAG administration exerted its effects by acting as a space-occupier. Administration of differentiated or undifferentiated cell individually did not yield significant results though, when placed on HAG, they produced significant results. HAG with odBMSC induced more effective repair and ossification occurred in such a way that would close the margins of the defect *(Fig. 3)*.

Macroscopic results were confirmed by histological findings. In HAG administered defects fracture margins could easily be identified and the defects were filled with fibrous tissue. On the other hand, when odBMSC was administered with HAG, the margins of the defect were barely noticeable and covered with very small amount of fibrous tissue. Lymphocytic infiltration was noted in both groups. Morphometric analyses in odBMSC with HAG-administered defects showed that osteoid formation and new mineralized bone formation increased significantly ($P<0.05$), cellular activity was significantly more efficient and development of fibrous tissue decreased significantly ($P<0.05$) *(Fig. 4)*.

iNOS and eNOS activities at the site of defect, which were positive at baseline, decreased significantly in

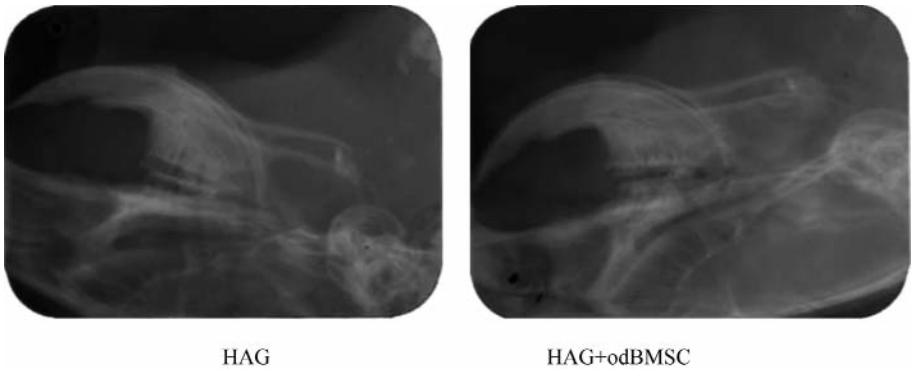


Fig 2. A radiograph of a rat hemi mandible demonstrating the repair process of HAG treated with or without stem cell on CSD

Şekil 2. Kök hücre içeren ve içermeyen hidroksi apatit jel (HAG) ile tedavi edilen kritik hacimli defekt (CSD) tamir sürecini gösteren sıçan sağ yarı çenesinin bir radyografisi

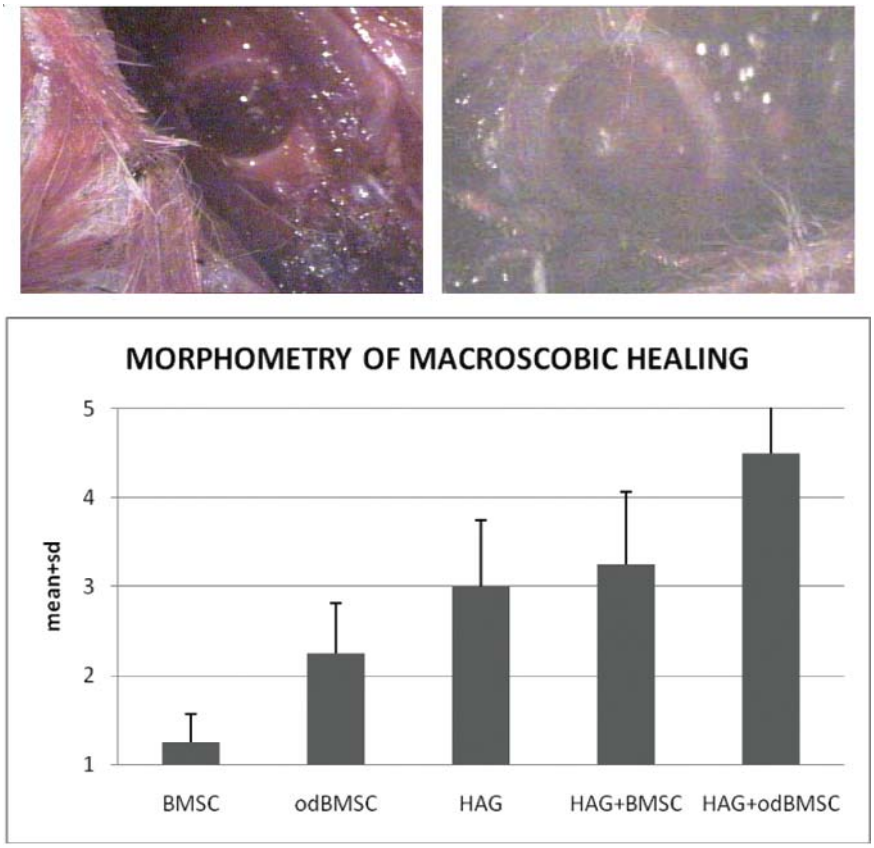


Fig 3. Macroscobic observation of a CSD in rat hemimandible which treated by HAG with or without stem cell. Morphometric analysis showed the best healing with HAG+odBMSC

Şekil 3. Kök hücre içeren ve içermeyen hidroksi apatit jel (HAG) ile tedavi edilen kritik hacimli defekt (CSD) tamir sürecinin makroskobik gözlemleri. Morfometrik analiz en iyi iyileşmenin hidroksi apatit jel (HAG) ile osteojenik farklılaşmış kemik iliği stromal kök hücreleri (odBMSCs) birlikteliği olduğunu gösterdi

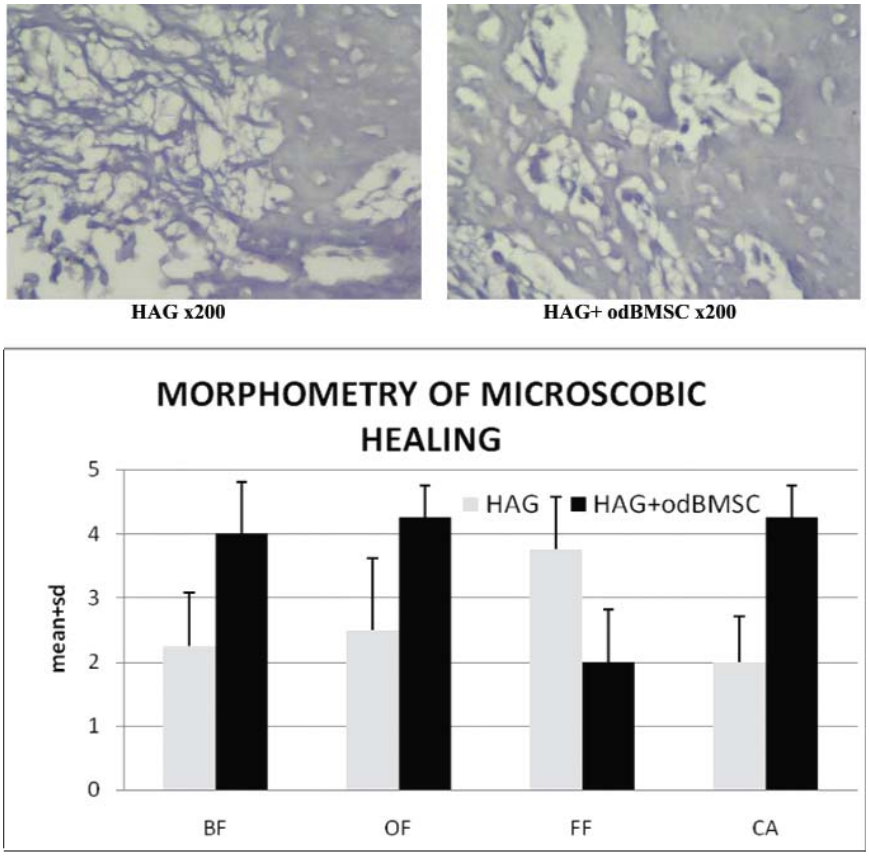


Fig 4. Microscobic histology of a CSD in rat hemimandible which treated by HAG with or without stem cell. Morphometric analysis showed the best healing with HAG+odBMSC. BF: new bone formation, OF: osteoid formation, FF: fibrous tissue formation and CA: cellular activity were used as healing parameters

Şekil 4. Kök hücre içeren ve içermeyen hidroksi apatit jel (HAG) ile tedavi edilen kritik hacimli defekt (CSD) tamir sürecinin mikroskobik histolojisi. BF: yeni kemik oluşumu, OF: Osteoit oluşumu, FF: Fibröz doku oluşumu ve CA: Hücresel aktivite şeklindeki parametreler iyileşme kriteri olarak kullanıldı

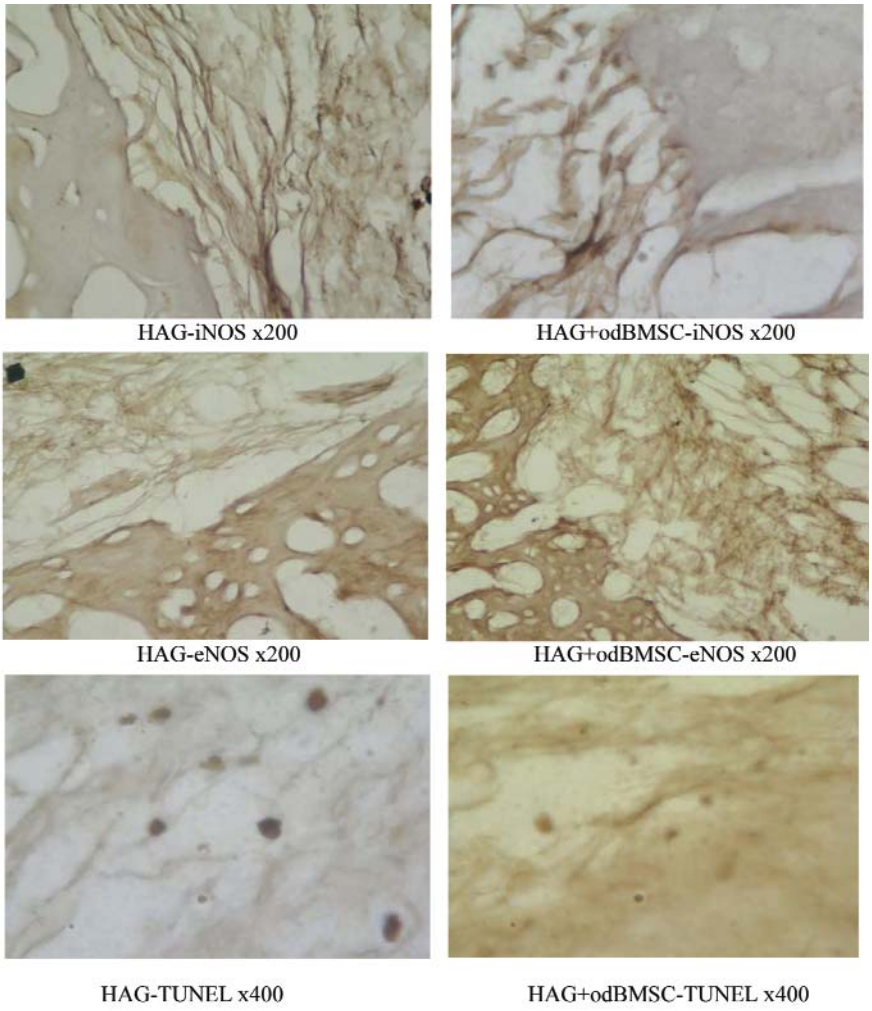
odBMSC with HAG-coated defects (P<0.05) *(Fig. 5)*. The decrease was less pronounced in iNOS. TUNEL staining, similar to NOS immunohistochemistry, was significantly reduced in odBMSC with HAG-filled defects (P<0.05), indicating that fewer cells had gone into apoptosis. There was a positive association between NOS immunoreactivity and increase in the number of TUNEL positive cells *(Fig. 5)*.

DISCUSSION

Stem cell applications developed in the context of tissue engineering in recent years and research on ideal biomaterials have provided important advantages in fracture healing and offered a life of higher quality. In the present study, we aimed to achieve the same advantages offered by tissue engineering in mandibular fractures by using BMSCs differentiated into osteoblasts and placing them in CSD together with HAG. Faster, more efficient and functional membranous bone healing was achieved after a 6-week treatment with odBMSC and HAG.

Even though repair of CSD by various biomaterials and cytokine-like activators was studied previously, there are no data in the literature regarding the use of stem cells. It is possible to achieve more effective ossification by reducing scar formation and fibrous tissue if a matrix environment suitable for the area of defect is created using odBMSC with HAG application, more repair cells are added and host osteoblasts are more effectively activated. Contribution of more cells to wound healing includes reducing toxicity and consequent oxidative phosphorilation. Hence, reduction in the number cells that die due to toxicity helps to increase the osteoblastic function derived from especially from the host tissues. The cytokines and similar osteogenic factors that would activate wound repair are expected to realize a more effective and functional cellular activity and, therefore, bone formation [37,38]. With odBMSC with HAG application, it is believed that cells spread in the area of defect and reduce fibrous tissue and local scar formations and prevent other possible destructive effects. It is predicted that HAG alone prevents soft tissue prolapse into the defect and inflammatory reactions. In the present study, supporting similar predictions, we found the repair effect which indicates cell-biomaterial relation was more important than biomaterial itself.

During reformation of bone fractures, more efficient, rapid and high quality healing has been achieved by new treatment methods developed by tissue engineering. This is, in part, due to the development of ideal biomaterials capable of carrying and conveying the BMSC differentiated into osteoblasts and, in part, to interactions



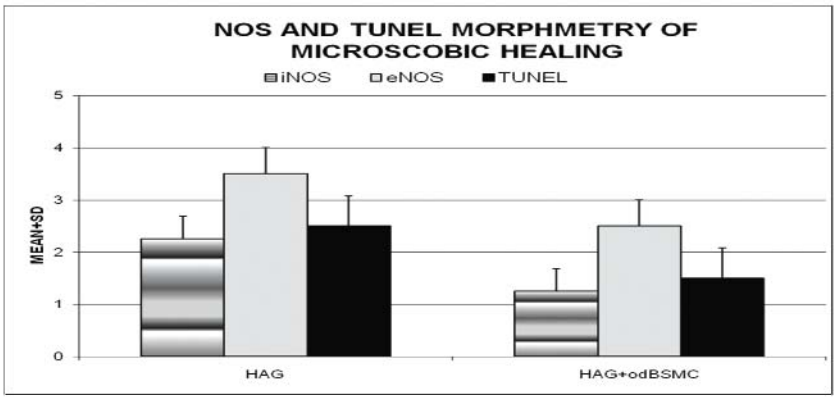


Fig 5. Microscobic analysis of oxidative stress and apoptosis on the histology of a CSD in rat hemimandible which treated by HAG with or without stem cell. Morphometric analysis showed the best healing for these markers was HAG+odBMSC

Şekil 5. Sıçan kök hücre içeren ve içermeyen hidroksi apatit jel (HAG) ile tedavi edilen kritik hacimli defekt (CSD) histolojisi üzerine oksidatif stress ve apoptosisin mikroskobik analizi. Morfometrik analiz en iyi iyileşmenin hidroksi apatit jel (HAG) ile osteojenik farklılaşmış kemik iliği stromal kök hücreleri (odBMSCs) birlikteliği olduğunu gösterdi

between the biomaterial and the osteoblasts. As such, the role of interactions between the BMSC and biomaterials in healing of bone fractures has gained more prominence. Pore sizes and heterogeneous micro structures of bioactive and biocompatible biomaterials such as HA and HAG significantly affect the stem cell behavior. While being a vehicle for these cells, various effects of the vehicle on adhesion, migration, proliferation and differentiation determine the perfection of the biomaterial. Maturity of this interaction will ultimately lead to more active bone formation and, subsequently, more active wound healing [39,40]. In the present study, HAG, thanks to its physical and chemical properties, made osteoblastic differentiation and *in vivo* ossification more efficient by interacting with the cells it carried in a perfect way and, consequently, restored a functional productivity for CSD.

Previous studies showed better biomaterial resorption and new bone formation with administration of BMSC

with a soluble porous ceramic construct into a CSD in a sheep tibia. Tricalcium phosphate (TCP) content of the material decreased more rapidly than HA and, as a result of this resorption, porous structure induced new bone formation. In general, most of the biomaterials are fully resorbed in two years. This is carried out by large osteoclast-like cells and bio-materials are replaced by mineralized lamellar bone [41,42]. Having different composition and microstructure compared to natural bone, synthetic HA may affect matrix production by the stem cells and synthesis of biological factors and adhesion molecules differently. These factors, at the same time, activate ossification by increasing the adhesion of osteoclast precursors [43]. This resorption was not fully understood as a result of insufficient duration of this study but we concluded that HAG affected the stem cells with the aforementioned characteristics and was effective via osteogenic factors such as growth factor, cytokine and Bone Morphogenic Protein (BMP).

Another effect of the biomaterials depends on their surface morphology. With the surface morphology, it is possible to alter the adhesion, proliferation and differentiation of stem cells. Ionic factors affected by these changes act on, for instance, mineralization of inorganic phosphate matrix and cause a more effective osteoblastic differentiation. Moreover, they may promote differentiation by acting on certain transcription factors [44,45] Anada and colleagues showed an increase in ALP, which is an indicator of early stage differentiation [46] and Fialkov and colleagues demonstrated an increase in stained OC as an indicator of late stage differentiation [47]. Matrix production by the porous scaffold is the major determinant of differentiation. This view is supported by the fact that matrix production was more active in the centre of the colonies and differentiation took place more actively here [48,49]. One of the important aims of tissue engineering is to make the surface content and the structure of the biomaterial resemble the bone. Environmental factors such as growth factors are known to be affected by the structure of the biomaterial [50]. In the present study, HAG activated the stem cells through these mechanisms and rendered the repair of CSD to be more effective according to morphometric analyses [51].

The therapeutic potential of stem cells and the ability of the biomaterial to make the cells available within the fracture have recently been noticed by tissue engineers and marked a new era. The clinicians are looking forward to witnessing the contributions of the stem cell-biomaterial pair in fractures that are difficult to heal like CSD. In this study, the efficacy of stem cell coupled with biomaterials was addressed and findings suggested that they would contribute to clinical healing. Moreover, more differentiated BMSC on the fracture area can easily decrease the negative effect of NOS which also cause less apoptotic TUNEL positive cells. We showed that BMSC as stem cell with biomaterial support can produce better and effective fracture healing using oxidative stress and apoptosis mechanism. Using tissue engineering such as HAG as a biomaterial not only promoted the capacity to differentiate to osteoblasts in culture medium but also facilitated more effective healing *in vivo.* By this way, clinically desirable, more efficient re-structuring can be achieved and patients' quality of life can be improved.

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