EXPERIMENTAL STUDY

Partial load-bearing rabbit ulnar segmental defects are regenerated with biocompatible grafts with or without bone marrow-derived mesenchymal stem cells

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ABSTRACT

BACKGROUND: The autologous bone grafts still have been used as the gold standard to initiate and facilitate bone healing in cases with bone defects. Because of some disadvantages of autologous bone grafts, the new biocomposite grafts have been researched. The purpose of the present study was to investigate whether the bone marrow-derived mesenchymal stem cells (BM-MSCs) loaded into a biocomposite scaffold enhance bone regeneration.

METHODS: In our study, a 10 mm osteoperiosteal segmental bone defect was created in the middle of the right and left ulnar bones of eight rabbits. The created defects were filled in the right ulnar bones of eight rabbits (Group I) with BM-MSCs loaded onto a biocomposite scaffold (Plexur PTM, Osteotech, Eatontown, NJ, USA) and in the other ulnar bones of the same rabbits (Group II) with only biocomposite graft. Radiographs of each forelimb were taken postoperatively at the end of the 6th week. Then, the rabbits were euthanized pharmacologically for histopathological evaluation.

RESULTS: Were scored using a modified Lane and Sandhu scoring system. All defects healed in both groups. Radiological and histological total scores were slightly better in Group I, but statistical tests did not reveal any significant differences between the two groups at the end of the 6th week radiologically and histologically ($p>0.05$).

CONCLUSION: The results of our study demonstrated that in rabbit ulnar segmental bone defect model was obtained satisfactory regeneration with using biocomposite graft with or without BM-MSCs.

Keywords: Bone regeneration; bone scaffold; bone tissue engineering; mesenchymal stem cells; rabbit; segmental bone defect.

INTRODUCTION

The bone is a special dynamic tissue capable of regeneration and remodeling during a lifetime without causing scar tissue. Many treatment techniques have been tried for the treatment of bone defects no expected secondary healing.[1] Autologous bone grafting is restricted by prolonged healing time, donor site morbidity, prolonged surgery time, limited availability, and increased patient expectations.[2,3] The alternatives to the use of autologous graft are xenogeneic or allogeneic graft, and biomaterials.[4–6] The infectious potential, immunogenicity, and biological inferiority of the xenogeneic or allogeneic bone grafts mandate the investigation and development of a suitable biocomposite bone graft.

Biocomposite materials include ceramic, composite, and polymers.[7–9] It requires osteoinductive and osteoconductive properties to develop an effective bone graft. These materials may serve as a scaffold for deposition and substitution of the bone matrix, and also they promote cell adhesion and differentiation. And then, they disappear gradually replacing them with regenerated tissue.[4,5] Whereas osteoconductive mate-

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rials passively provide mechanical support for vascular and bone ingrowth, osteoinductive materials actively stimulate the formation of new bone by inducing mesenchymal stem cells (MSCs) to differentiate into the osteoblastic phenotype. The problem of the natural materials such as collagen, chitosan, alginate, and silk is the weakness of their mechanical properties.

Implantation of culture-expanded MSCs has been shown to affect tissue regeneration in a variety of animal models.[10–13] Furthermore, the stimulation of differentiation into the osteoblastic phenotype depends on local factors.[11,12,14] Because the adjacent intact radius provides stability, and ulnar segmental bone defect in rabbits is a good option for first phase partial bearing bone models, we chose this bone defect model.[13,15–17]

MSCs based therapies are becoming popular for the treatment of bone defects in recent years.[6–8] Most of the bone tissue engineering models are based on the seeding of the MSCs into three-dimensional scaffolds.[10–13] For this purpose, poly(D,L-lactide-co-glycolide) (PLGA) has been used widely as a biodegradable and biocompatible scaffold material.[11,18,19] A combination of ceramic-polymer materials is usually benefited to augment bone integration of the graft material and the native host tissue. Several studies have demonstrated osteoblast differentiation and matrix mineralization improved on ceramic-polymer scaffolds.[15,18] These biomaterials have well biocompatibility as well as better biomechanical properties without immunological reactions and infective disease transmission.[4,5,18] Several biocomposite grafts offering these properties are commercially available (EasyGraft™ by Degradable Solutions SA, Switzerland, Plexur M™ and P™ by Osteotech, USA, and Cerapatite-Collagen™ by Ceraver Osteal, France). Because hydroxyapatite ceramics have poor elasticity and a substantially high Young's modulus, the use of pure these ceramics for orthopedic purposes is limited. Therefore, in literature is observed to be a tendency to fabricate biomaterials combining the benefits of calcium phosphate ceramics with a level of plasticity/elasticity convenient for surgeons. Plexur P™ (Osteotech, Eatontown, NJ, USA) was approved by Food and Drug Administration for use in filling bony voids of the pelvis and extremities. This graft including osteoconductive property as well as extracellular matrix (ECM) proteins promotes bone healing. In the literature was not encountered any study comparing Plexur P™ with or without BM-MSCs in the segmental bone defect model.

The research question of this study was whether Plexur P™ with or without BM-MSCs could enhance bone regeneration in segmental bone defects. The present study was, therefore, designed to investigate bone regeneration effects Plexur P™ with or without BM-MSCs, using a partial load-bearing rabbit ulnar segmental bone defect model, evaluated using radiographs and histology.

MATERIALS AND METHODS

Animals

A longitudinal controlled study was designed. Test subjects were 14 healthy 10-week-old male New Zealand white rabbits, with a weight of 2.0–2.2 kg. The research protocol for this experiment was approved by the Institutional Animal Care and Use Committee at İstanbul University. This study was performed at the Experimental Animals Laboratory of İstanbul University. All procedures were carried out according to the institutional guidelines for the care and use of laboratory animals. The health status of the animals was monitored throughout the entire study. Three rabbits died due to coccidiosis. Because MSCs could not be reproduced in bone marrow culture, three rabbits also were excluded from our study. The study was completed with eight rabbits.

Biocomposite Material

In our study was used a biocomposite graft material (Plexur P™, Osteotech, Eatontown, NJ, USA) comprising human mineralized bone fibrils, PLGA and PEG. This material also contains ECM proteins, calcium, phosphate, and other trace elements to promote bone healing.

Surgical Procedures

Rabbits were anesthetized with an intramuscular injection of ketamine (50 mg/kg; Alphamine™) and xylazine (5 mg/kg; Rompun™). Iliac crests and both forelimbs were prepared aseptically with povidone-iodine and draped with sterile drapes. The same surgeon performed the all procedures. Ceftriaxone (50 mg/kg; Rocephin™) was administered intramuscularly before 1 h and after 12 h for the surgery for prophylaxis. Meperidine (6 mg/kg, IM; Aldolan™) and as to be 100–300 mg/kg, 30 mg paracetamol (Parol™) put in 15 ml drinking water were given for the post-operative analgesia.

Two surgical procedures were performed for each rabbit. First, a 1 cm skin incision was performed at the superior-anterior iliac crest for bone marrow aspiration. Subcutaneous tissues and muscles were passed and the periosteum was stripped. A 5 ml bone marrow was aspirated from the medullary canal with a heparinized (Nevparin™) 21-gauge injector.

Second, midsagittal skin incisions of 3 cm over both ulnae were made, and fascia and muscles were passed. After the diaphyseal portions of both ulnae were exposed with a soft-tissue cuff, 10 mm osteoperiosteal bone segments were removed using Gigli saw. The wound and the diaphyseal ends of the ulna were thoroughly washed with normal saline. The biocomposite graft material prepared as a 10×4×4 mm rectangular prism was implanted into the left defect area. Furthermore, the biocomposite graft material embedded BM-MSCs were implanted into the right defect area. The deep fascial layer was reapproximated around the defect with 4-0 polyglycolic acid absorbable sutures (Pegesorb™ rapid). The

skin was closed with silk suture. Immobilization was not applied in any rabbits.

The rabbits were sacrificed by an intramuscular injection of xylazine (30 mg/kg; Rompun™) and ketamine (70 mg/kg; Alphamine™) at the end of the 6th week.

BM-MSCs Culture Preparation and Seeding Biocomposite Graft

The aspirated bone marrow was transferred to the laboratory within 50 ml conical tubes with Dulbecco's modified Eagle medium (DMEM-F12; Gibco, UK). Mononuclear cells were isolated from the bone marrow with density gradient centrifugation method at 1500 rpm for 3 min. The separated mononuclear cell layer was collected with a pipette in the airflow cabin and they were transferred to another tube. The cells were washed twice with Ca-Mg free phosphate-buffered solution (CMF-PBS; Gibco, UK), with centrifugation at 400 rpm for 15 min. The cells were seeded in 25 cm^2 tissue culture plastic flasks and cultured at 37°C in a humidified atmosphere with 5% of CO_2. The culture medium was DMEM-F12 (Gibco, UK) media supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis), 100 µg/mL penicillin, and 100 µg/mL streptomycin.

The culture medium was changed twice weekly. The development of BM-MSCs was observed with an inverted microscope (Fig. 1). When 75% of confluence was achieved, the adherent cells were removed with a passage solution contained 0.5% trypsin/ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis). DMEM-F12 (Gibco, UK) solution was used to neutralize the effect of trypsin. After the cells were centrifuged at 3 min for aggregation to the bottom of the tube, the accumulated cells were taken with a pipette and counted with a hemocytometer. The grounds of the 6-well culture plates were covered by 1/1 agar/medium mix. After the biocomposite grafts were placed on the agar/medium mix, the cells were seeded with a pipette into the scaffolds. Finally, 5 ml culture medium was added to each well, and then, the plates were put in the incubator for 3 days for adhesion of the cells to the biocomposite graft.

Histopathological Evaluation

After the rabbits were sacrificed at the end of the 6th week, both ulnar bones were removed as intact. The bones were fixed at 10% formalin room temperature for 72 h. The ulnar bones were then decalcified in 10% EDTA for 2 weeks. Each defect region with healthy tissues was embedded in the paraffin wax. The tissue blocks were sectioned at 4–5 µm thicknesses six slices from the different levels along the long axis and coronal plane of the ulnar defect region using a microtome. Four of these sections were stained with hematoxylin-eosin, and the others were stained with Masson's trichrome. The samples were scanned with Olympus BX61 (Olympus microscopes, USA) digital light microscope providing data transfer to a computer. The best samples were chosen for each staining method. These samples were evaluated with Lane and Sandhu histopathological evaluation system modified by Heiple et al.[20] by two independent histologists.

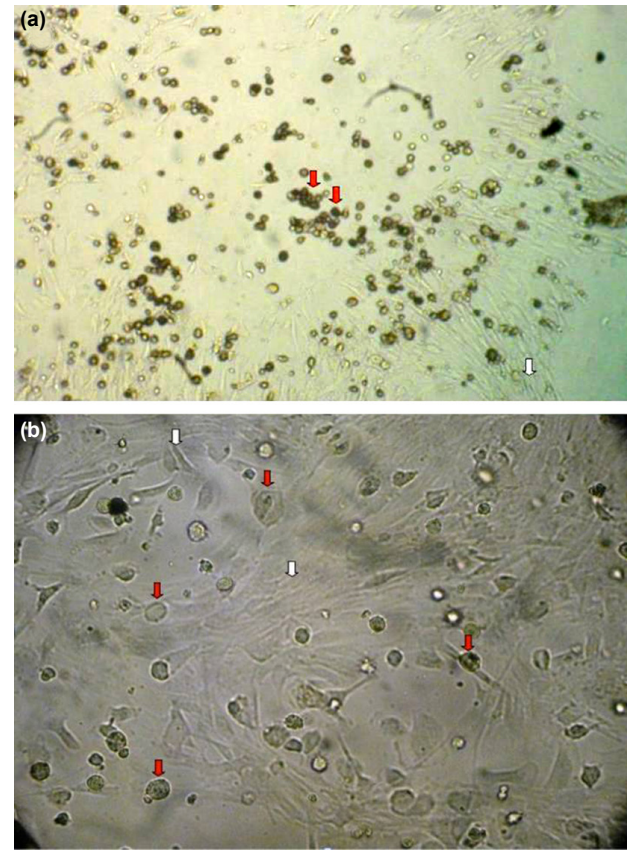

Figure 1. The monolayer primer rabbit bone marrow-derived young cell culture; besides the fibroblast-like cells (white arrow), the cells with wide cytoplasm and round are thought to be multipotent (red arrow) (**a**; ×10), (**b**; ×20).

X-ray Evaluation

Standard radiographs of both ulnar bones were taken at the end of the 6th week and recorded. These images were evaluated with a modified Lane and Sandhu radiologic evaluation system by two independent orthopedists.[21]

Statically Analysis

All statistical calculations were carried out using NCSS (Number Cruncher Statistical System) 2007 and PASS 2008 Statistical Software (Utah, A.B.D). The data were evaluated with descriptive statistical methods (mean and standard deviation). An analysis of independent groups of quantitative data showing no normal distribution was used Mann–Whitney U-test and Kruskal–Wallis test. For crude analysis of independent groups of qualitative data was used Fisher's exact test. Wilcoxon signed-rank test was used for the evaluation of interobserver variability. A 95% confidence interval, significance at $p<0.05$ was accepted.

RESULTS

Histological Evaluation Results

Proximal and distal union, new cancellous and cortical bone formation, and bone marrow formation were better in the study group than the control group (Fig. 2). However, there were no statistically significant differences in terms of histologic union, cancellous, cortical, bone marrow, and total scores in between two groups (Table 1). Total histological scores calculated by both observers were found consistent with each other ($p>0.05$).

X-ray Evaluation Results

The bone formation and proximal and distal union occurred in most rabbits in both groups at the end of the 6th week. In addition, the bone bridge was seen in both groups. However, remodeling was not found in both groups (Fig. 3).

The scores of bone formation, union, remodeling, and total were better in the study group than the control group. However, there were no statistically significant differences in terms of these scores between the two groups (Table 2).

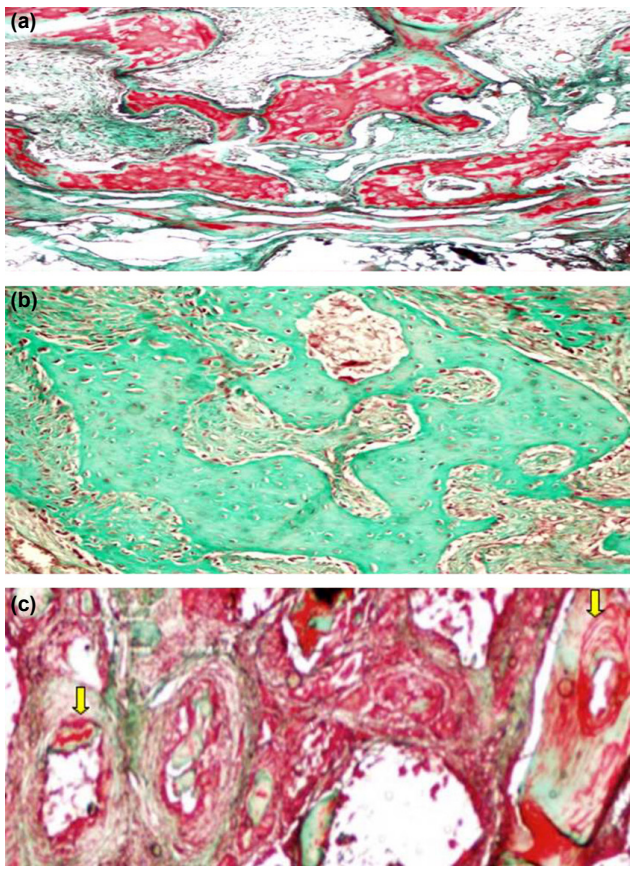

Figure 2. In the study group, the formation of new primary and secondary bone trabecular (**a**; Masson's trichrome × 10). In the control group, the formation of primary and secondary bone trabecular along with more connective tissue is seen (**b**; Masson's trichrome × 10). In the study group, the development of the Haversian systems is seen (**c**; Masson's trichrome × 10).

Table 1. Histological evaluation of the groups

Histological findings	Group I	Group II	p
	Mean±SD	Mean±SD	
Union (proximal and distal evaluated separately)	5.8±1.4	5.6±1.4	0.727
Cancellous bone	3.6±0.5	3.1±0.6	0.112
Cortical bone	2.8±0.7	2.6±0.9	0.654
Marrow	3.6±0.6	3.1±0.6	0.088
Total points	15.8±2.9	14.4±3.4	0.185

Mann-Whitney U test.

Total radiological scores calculated by both observers were found consistent with each other ($p>0.05$).

DISCUSSION

In this study, a rabbit ulnar segmental defect model was created to compare the bone regeneration effect of Plexur P™ with or without BM-MSCs. Because there was no need for internal or external fixation that can influence the healing process, this model has been used in the previous studies.[15–17] The segmental defect model was created as 1 cm in the middle portion of the ulna at least twice the diameter of diaphysis to produce a nonunion model and prevent spontaneous healing.[17,22]

Biocompatible scaffolds absorbed with hydrolytic or enzymatic ways are used for the cell encapsulation approaches.[10] These grafts create suitable scaffolding for the proliferation

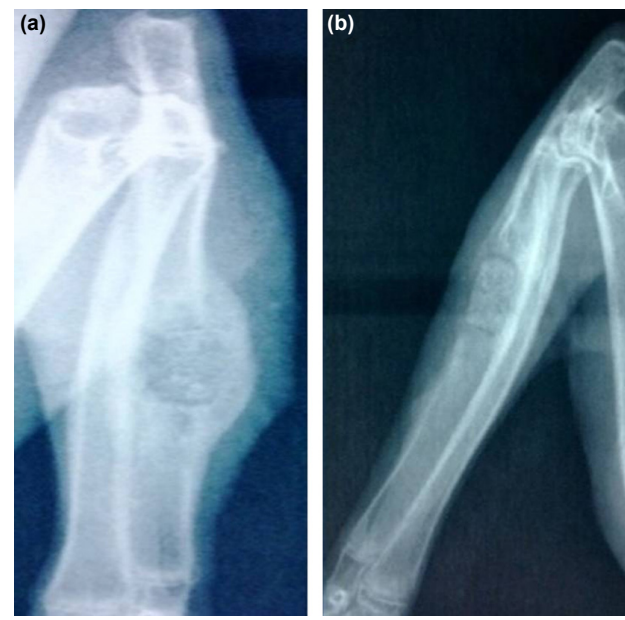

Figure 3. In X-ray views, in the study group, **(a)** graft incorporation and cortical bridge are seen to be more markedly than the control group **(b)**.

Table 2. Radiological evaluation of the groups

Radiological findings	Group I	Group II	p
	Mean±SD	Mean±SD	
Bone formation	3.9±0.4	3.8±0.7	0.927
Union (proximal and distal evaluated separately)	3.9±0.4	3.7±0.4	0.178
Remodeling	0.6±0.9	0.5±0.9	0.351
Total points	8.4±0.9	8±1.4	0.870

Mann-Whitney U test.

and differentiation of cells and help to provide osteogenesis.[9,23] These approaches aim to mimic the natural repair processes. Many investigators have used PLGA as a biodegradable scaffold.[5,15,19] In our study was used Plexur P™ as a scaffold with suitable features such as biocompatibility and degradation time.[24]

When BM-MSCs are placed in a defect area with seeding into a scaffold, they differentiate into the osteoblast and produce an ECM. Several studies have been shown that mineralized ECM creates a bridge on defect area and supports physiological loads.[10] Advantages of these types of polymeric scaffolds include flexibility, biodegradability, and remodeling. Furthermore, they can be shaped according to the defect area.[5,18]

In the present study chosen biocomposite graft includes human mineralized bone fibrils, PLGA, PEG, minerals, trace elements, and ECM proteins to promote bone healing. These ECM proteins such as collagen, fibronectin, and bone sialoprotein contain the RGD (Arg-Gly-Asp) amino acids which are important in controlling the adhesion and spreading surface of a scaffold of MSCs.[21,25] PEG increases the hydrophobic properties and the mechanical strength of the graft. Furthermore, PEG side chains prevent adsorption of non-specific proteins and stimulate RGD-mediated cell adhesion.[25] In addition, calcium, phosphate, and trace elements increase osteoconductive and mechanical properties of this graft.[9,18]

The multipotent cells can differentiate into various tissues.[6–8,26] These cells are available in many tissues such as bone marrow, periosteum, synovium, and muscles.[12,13,26] Furthermore, MSCs have been isolated from peripheral blood in recent studies.[13] Because properties of donor tissues may affect stem cell behaviors, the tissue must be harvested MCs.[27,28] Therefore, the bone marrow-derived multipotent stem cells were used in our study.

The concentration of MSCs is the other important factor for providing tissue regeneration. To ensure the viability of MSCs and differentiate them from osteogenic cells, enough tissue fluid and blood support should be provided.[29] Moreover, the increased passaging number decreases the potentials of duplication and differentiation of MSCs.[8,26] The minimum MSCs number required for providing the bone regeneration in the cell encapsulation approaches is not obvious in the literature.[30] For these reasons, we made a maximum of four passaging, and in between $1\times10^5/mm^3$–$2\times10^5/mm^3$, MSCs were seeded into biocomposite graft in our study.

The age, gender, and local and systemic diseases of the donor can affect the number and the function of the MSCs.[6,26] Furthermore, not only where MSCs are harvested but also the harvesting method is important.[8,26,27] Because of these reasons, MSCs were obtained with the aspiration method from iliac wings of young, healthy, and male rabbits in the present study. This method is easy and safe according to many methods.[31–33]

Several studies have indicated that bone tissue engineering applications may be alternative to the autologous bone grafts in the treatment of clinically important bone defects.[10,18] The several factors mentioned above may affect the results in these complex processes. Furthermore, there is no standardization related to these treatment methods in the literature.

Hernigou et al.[34] stated that grafting with percutaneous autologous bone marrow was an effective and safe method for the treatment of an atrophic tibial nonunion. Furthermore, there are successful clinical studies related to the cell encapsulation approach.[35–37] However, there is no prospective randomized (Level-I) clinic study with these treatment methods in the literature.

In contrast to our study, in there, a rabbit radial segmental bone defect model, Hao et al.[38] found that enhancing in vivo osteogenesis of adipose-derived stem cells wrapped in collagen gel combined with PLGA-b-TCP was more successful than the use of the biocomposite material alone. Furthermore, Bruder et al.[10] reached the same result in their similar study. Besides, Berkes et al.[24] stated that Plexur P™ was as successful as fibula allograft in the management of tibial plateau fracture for filling bone voids.

We think that the reasons for the similar results between both groups in our study were infection by bone marrow cells of the graft in the control group and the use of a non-critical size acute defect model. Because the harvesting of the ulnar bones at the end of the 6th week was too early to allow a determination of the ultimate strength of the grafted ulna, we could not measure the strength of the graft. Furthermore, lack of medullary and cortical remodeling and absence of monitor group was the weakness of our study.

Conclusion

Consequently, our study demonstrated that in a rabbit ulnar segmental bone defect model was obtained satisfactory regeneration using biocomposite graft with or without BM-

MSCs. Plexur P™ as the bone void filler is promising in the treatment of bone defects. We hope that this report will provide new insights into the clinical application of Plexur PTM.

Ethics Committee Approval: This study was approved by the İstanbul University Animal Experiments Local Ethics Committee (Date: 26.11.2008, Decision No: 125).

Peer-review: Internally peer-reviewed.

Authorship Contributions: Concept: S.H.B., G.T.; Design: S.H.B., G.T.; Supervision: S.H.B., M.C.A.; Materials: G.T., A.B., B.T.; Data: G.T., A.B., S.H.B.; Analysis: S.H.B., M.C.A., B.T.; Literature search: A.B., S.H.B., M.C.A.; Writing: S.H.B., B.T.; Critical revision: S.H.B., M.C.A., G.T.

Conflict of Interest: None declared.

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DENEYSEL ÇALIŞMA - *ÖZ*

Kısmi yük taşıyan tavşan ulnar segmental defektlerin biyouyumlu greftler ve/veya kemik iliğinden türetilmiş mezenkimal kök hücreler ile yenilenir

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AMAÇ: Otolog kemik greftleri, kemik defekti olan olgularda kemik iyileşmesini başlatmak ve kolaylaştırmak için altın standart olarak hala kullanılmaktadır. Otolog kemik greftlerinin bazı dezavantajları nedeniyle yeni biyokompozit greftler araştırılmıştır. Bu çalışmanın amacı, bir biyokompozit iskeleye yüklenen kemik iliğinden türetilen mezenkimal kök hücrelerin (BM-MSC'ler) kemik rejenerasyonunu artırıp artırmadığını araştırmaktı.
GEREÇ VE YÖNTEM: Çalışmamızda sekiz adet tavşanın sağ ve sol ulnar kemiğin orta kısmında 10 mm'lik osteoperiosteal segmental kemik defekti oluşturuldu. Tavşanların sağ ulnar defektleri (Grup I) bir biyokompozit iskeleye (Plexur PTM, Osteotech, Eatontown, NJ, ABD) yüklenmiş BM-MSC'ler ile tedavi edilirken, aynı tavşanların sol ulnar kemiklerine (grup II) sadece biyokompozit greft ile tedavi uygulandı. Altıncı haftanın sonunda ameliyat sonrası olarak her bir ön ayağın radyografileri çekildi. Daha sonra tavşanlara histopatolojik değerlendirme için farmakolojik olarak ötenazi yapıldı. Sonuçlar, modifiye Lane ve Sandhu puanlama sistemi kullanılarak puanlandı.
BULGULAR: Her iki grupta da tüm defektler iyileşti. Radyolojik ve histolojik toplam skorlar grup I'de daha iyiydi, ancak istatistiksel olarak altıncı haftanın sonunda radyolojik ve histolojik olarak iki grup arasında anlamlı bir farklılık gözlenmedi (p>0.05).
TARTIŞMA: Çalışmamızın sonuçları, tavşan ulnar segmental kemik defekt modelinin, BM-MSC'ler içeren veya içermeyen biyokompozit greft kullanılarak tatmin edici bir rejenerasyon elde edildiğini göstermiştir.
Anahtar sözcükler: Kemik dokusu mühendisliği; kemik iskelesi; kemik rejenerasyonu; mezenkimal kök hücreler (MKH'ler); segmental kemik defekti; tavşan.

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