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Original Article

Evaluation of the effects of autologous adipose derived mesenchymal stem cells in combination with polyacrylamide hydrogel and nanohydroxyapatite scaffolds on healing in rabbit critical-sized radial bone defect model

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Abstract

Objective- In this study, the bone regeneration ability of polyacrylamide and nanohydroxyapatite scaffolds (PAAH/NHA) and stem cells derived from adipose tissue (ADMSCs) in the healing of critical sized bone defects in rabbit radius was assessed.

Design- Experimental Study

Animals- 12 New Zealand white male rabbits

Procedures- 12 New Zealand white male rabbits were divided into 3 groups. These groups were as follows: Control (bone defect without any treatment), PAAH/NHA (embedding of PAAH/NHA scaffolds in the bone defect) and PAAH/NHA +ADMSCs (embedding of PAAH/NHA scaffolds with stem cells derived from adipose tissue in the bone defect). The rabbits were anesthetized and 15 mm bone defects were created on the radius. According to the group, PAAH/NHA scaffold and PAAH/NHA +ADMSCs were implanted into the defects. The animals were euthanized 12 weeks after surgery and histopathologic studies were carried out.

Results - microscopic examination revealed significant increase in new bone formation in PAAH/NHA + ADMSCs than PAAH/NHA.

Conclusion and Clinical relevance- The results of this study showed that the use of stem cells derived from adipose tissue implanted on PAAH/NHA scaffold can improve osteogenesis.

Keywords- Adipose derived stromal cells, polyacrylamide, nanohydroxyapatite, bone.

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Introduction

Annually, more than 800,000 bone grafts are performed worldwide to manage bone defects.[1] However, the treatment of bone defects is still very difficult in orthopedics. Cell based therapies have been identified using cells derived from the bone marrow. These cells can be differentiated into osteoblasts.[2] However, harvesting of bone marrow can be painful and it is associated with potential donor site morbidity.

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Several studies have shown that adipose tissues are multipotent cells. They comprise a stroma that is simply isolated. Adipose derived stromal cells (ADSCs) are produced after the purification steps and can differentiate into cells of chondrogenic, osteogenic, myogenic and adipogenic lineages.[3,4] Adipose tissue is more available by liposuction and this can be achieved from multiple sites in the body and through a minimally invasive procedure with the use of local anesthesia. Most patients have an adequate fat supply. Moreover, the rates at which stem cells are obtained from adipose tissue are at least 500 fold greater than bone marrow and as such, ADSCs do not decrease with age.[5] Therefore, ADSCs could be an alternative source to stem cells achieved from bone marrow.

The cells require scaffold to guide and help them proliferate and repair. The combination of cell based therapy and tissue engineering is more attractive for

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researchers. Biological tissues and synthetic materials have been used for bone grafts. They are abundant and available. Also, the shape of scaffold can be designed by bone defects and the risk of disease transmission such as blood bore disease can decrease. Numerous materials with diverse characteristics have been inspected including metals, ceramics, polymers and composites. Hydrogel products comprise a group of polymeric materials having hydrophilic structure that makes them capable of holding a large amount of water in their three dimensional networks. The extensive application of these products in a number of industrial and environmental parts is of great importance. Literature on this subject is on the increase, particularly in the scientific areas of research. Recently, the inverse suspension technique has been widely used for polyacrylamide hydrogels (PAAH) due to its easy removal and management of hazardous, residual acrylamide monomer in the polymer.[6] The aim of this study was to use PAAH as one of the scaffold components for cell based therapy with ADSCs.

Calcium phosphate ceramics (CaP), are the most common materials for scaffolds while hydroxyapatite (HA) because of its excellent biocompatibility and bioactivity, is the most often used.[7] In this study, nano-HA (NHA) was used as other scaffold components. Therefore, the rabbit model was used to evaluate bone healing by the PAAH/NHA scaffold loaded with ADSCs.

Material and Methods

Isolation of ADMSCs

All experiments followed the protocols approved by the Animal facility of University of Tehran and were carried out according to the standards of the association for assessment and accreditation of laboratory animal care. Twelve male white New Zealand rabbits weighting 2.5-3 kg were obtained and kept. They were handled for one week prior to surgery and individually housed in cages at 22°C with 12 h light/dark cycles. ADSCs were harvested from the interscapular fat.[8] Fatty tissue, about 10 gr, were dissected and suited in a 50 ml centrifuge tube containing phosphate buffered saline 100 IU/ml penicillin G and 100 µg/ml streptomycin. They were transferred to the cell culture laboratory. Thereafter, under sterile condition, fatty tissues were washed several times with PBS and cut in small pieces for the removal of blood vessels and fibrous connective tissue. For enzymatic digestion, minced fat tissues were placed in tubes containing collagenase type I (Sigma, Aldrich) in a shaking incubator at 37°C with continuous agitation for 60 min. Subsequently, the content was filtered through a 500-µm mesh screen to separate the non-digested fat tissue. The aliquot was added to Dulbecco's Modified Eagle's medium (DMEM) and centrifuged at 400 g for 5 min. The supernatant containing fat droplets was removed. The pellet was suspended in DMEM and centrifuged again. Cells were plated in 25 cm^2 flasks. This initial passage of the primary cells was referred to as passage 0. After 24-48 h of incubation at 37°C in Co_2 incubator, the cultures were washed with PBS and maintained in proliferation medium to P_3. All cells were grown to sub confluence and passaged using the standard methods of trypsinization.[9]

Evaluation of ADMSCs characteristics

The differentiation potential of the isolated cells into osteogenic and adipogenic cell lineages were evaluated. For adipogenesis, passage 3 confluent culture was treated with adipocyte induction media containing 100 nM dexamethasone (Sigma, Aldrich), 50 µmg/ml indomethacin (Sigma, Aldrich) and 3 isobuthyl-1 methylxanthine (1BMX, 0.5 mM). The media were replaced every 3 days. After 14 days, the cultures were rinsed with PBS, and adipocytes differentiation was evaluated using Oil Red O staining. For osteogenesis, passage 3 confluent cultures were treated with osteogenic culture containing 0.05 mmol/L ascorbic acid and 10 mmol/L b- glycerol phosphate and dexamethasone. The media were replaced every 3 days. After 21 days, osteogenic differentiations were evaluated by Alizarin Red.[10, 11]

PAAH/NHA scaffold preparation

Preparation of PAAH/NHA was briefly as follows: hydrolyzed polyacrylamide powder was dissolved in deionized water and sterilized in autoclave. The solution was kept overnight at 25 °C so that the hydrolyzed polyacrylamide chains could obtain their equilibrium conformation. $Cr_{(III)}$ aqueous solution as a crosslink agent with predetermined concentration was filtered and mixed with hydrolyzed polyacrylamide. To accelerate the gelation of hydrolyzed polyacrylamide solution at 37 °C, the prepared hydrogel solution were first preheated at 60°C. Biphasic structure was prepared using optimized hydrogel solution containing 0 and 50 wt% nanohydroxyapatite.[12] The day before transplantation,

scaffold was loaded with the 5×10^5 cells obtained from third subculture. The cells were placed on the surface of scaffold.[13] To observe the cells loaded within scaffold, scanning electron microscopy (SEM) was used.[13]

Surgical procedure

The rabbits were anesthetized with intramuscular injection of 50 mg/kg Ketamine hydrochloride (Alfasan, Woerden, and The Netherlands) and 8 mg/kg Xylazine (Xylazine 2%, Alfasan International BV, Woerden, and The Netherlands). The maintenance of anesthesia was achieved using 1.5% Isoflurane, after endotracheal intubation. Antibiotic (Enrofluxacin 15 mg/kg BW) was administered preoperatively. Pre-operative preparation and surgeries were carried out under aseptic condition. A 2 cm craniomedial incision was made over the radius, soft tissues were dissected, and the bone was exposed by gentle retraction of the muscles. A 15-mm segmental diaphyseal defect was created with a saw.[14, 15] In this study, 5 mm of periosteum was stripped from each side of the remaining distal and proximal fragments. At the end, the defect was irrigated with sterile saline solution, and the scaffold was press fitted into the defect (Fig. 1). The muscles and skin were separately closed over the defect as routine. The animals were divided equally into three groups (N=5 per group). The defects were left empty in the group A (control). In group B, the defects were filled using scaffold without cells. In group C, the defect sites were occupied with scaffold coated with ADSCs. During the postoperative period, the animals were allowed to bear weight on the limbs. After 12 weeks, the animals were anesthetized with intramuscular injection of Ketamine hydrochloride and Xylazine and then sacrificed with intracardiac injection of $MgSO_4$ (magnesium sulfate).

Figure 1. Creating a critical size bone defect in radius of rabbit. Note to fixation of scaffold in the bone defect (Arrow).

Histopathological assessment

The defect sites were removed after 12 weeks and placed in 10% buffered formalin. The samples were decalcified in 6% trichloroacetic acid for 21 days. After washing the specimens with tap water, they were processed as routine at the pathology laboratory. Sections were stained with Haematoxylin and Eosin (H&E) and Goldner's Trichrome, and evaluated under a light microscope. To quantify the presence of bone formation, 5 microscope fields of Goldner's Trichrome stained slides were photographed at 100X magnifications. These sections were selected from both sides of defects, as well as the center. Computer analyzing software (Quickphoto, Germany) was used to assess the percent of new bone formation in microscopic figures. Mean of new bone formation percent of 5 figures were calculated and compared between groups.

Results

Cell differentiation

Based on the results obtained from the Oil Red O staining, lipid droplets were seen in the cells (Fig. 2), indicating adipogenic differentiation of ADSCs. Alizarin Red staining revealed red color in cultured cells (Fig. 3), indicating the osteogenic differentiation of ADSCs.

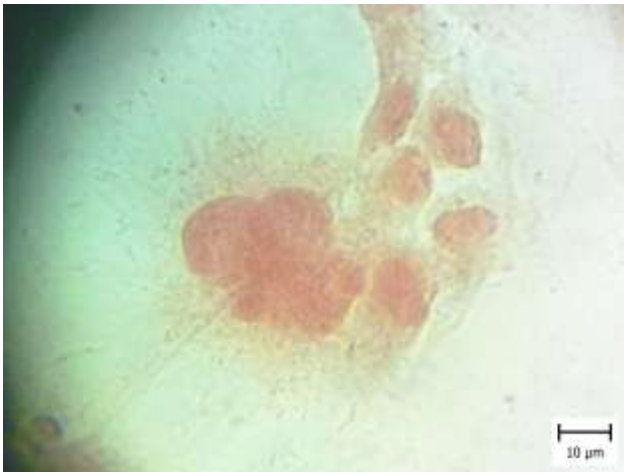

Figure 2. Microphotograph of adipogenic differentiation of cells after 14 days. Note to the red points in the cells indicate fat droplets (Oil Red O, Bar: 10 µm).

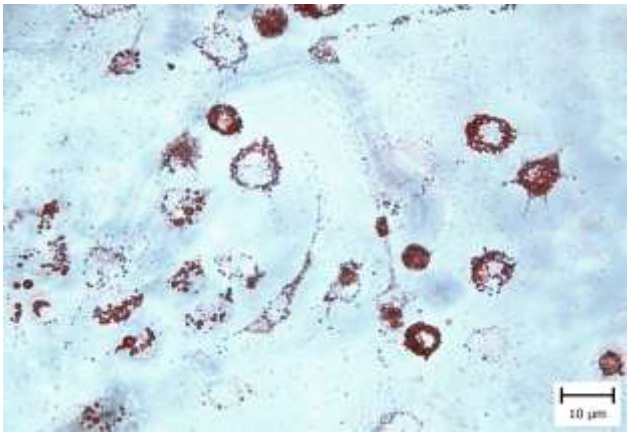

Figure 3. Microphotograph of osteogenic differentiation of cells after 14 days. Note to the red cells indicate mineralization (Alizarin Red, Bar: 10 µm).

SEM results of scaffold

Based on the section prepared from the cell loaded scaffolds, the seeding of cells within the scaffolds and the occupying of its internal spaces were obvious (Fig. 4).

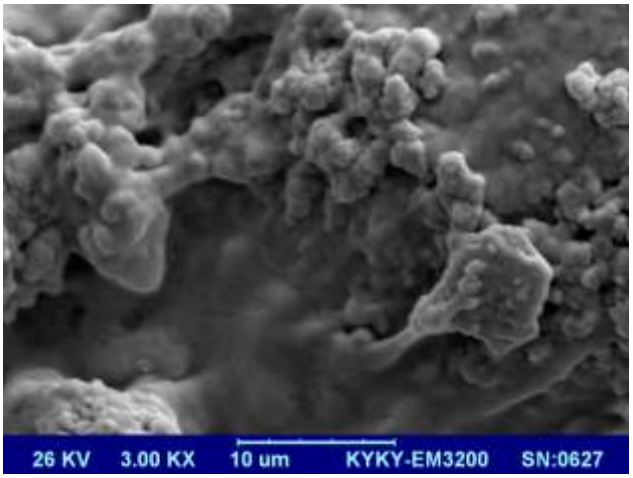

Figure 4. Scanning electron micrographs of PAAH/NHA with ADSCs. Note to attachment of cells (Bar: 10 µm).

Histopathologic evaluation

Microscopic evaluation of the defect site in the group A revealed fibrous stroma accompanied by small foci of new bone formation from both side of defect (Fig. 5). Group B showed well-formed osseous trabecula in both sides of defects within fibrous stroma and soft callus (Fig. 6). The third group showed thicker bone trabecula. In 2 animals, new bone formation completely filled the defect (Fig. 7). New bone trabeculas which were mature were green in color in the Goldner's Trichrome staining (Figure 8). The percentages of newly bone formation which were assessed by computer analyzing software were 27.28, 46 and 75.54% in A, B and C groups respectively (Table 1). The highest rate of ossification were seen in group C and the lowest rate was related to group A.

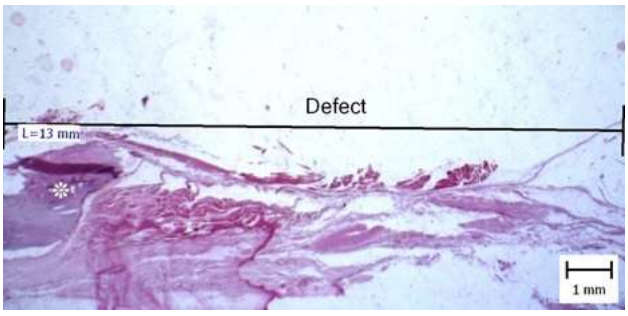

Figure 5. Low magnification of the defect site in Group A. 12 weeks after surgery. The defect was filled by soft callus (H&E, Bar: 1 mm).

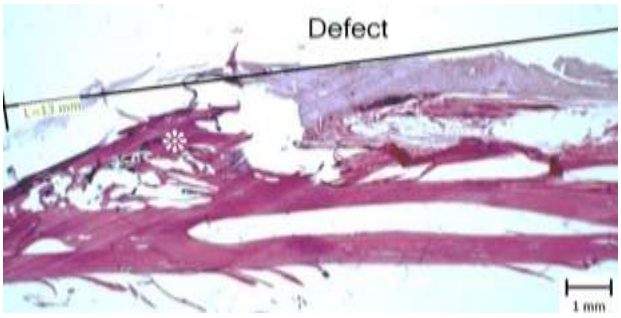

Figure 6. Low magnification of the defect site in Group B. 12 weeks after surgery. The defect was filled by new bone formation (asterisk) (H&E, Bar: 1 mm).

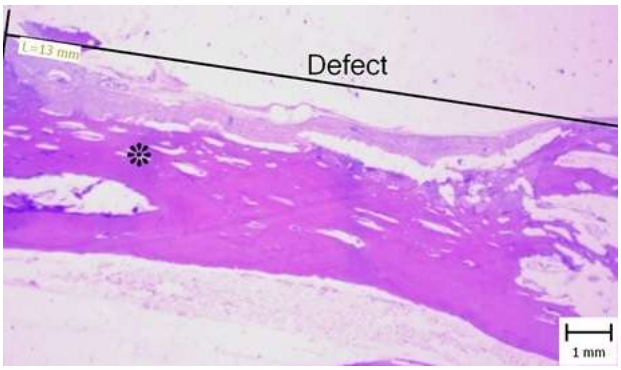

Figure 7. Low magnification of the defect site in Group C. 12 weeks after surgery. New bone formation completely filled the defect (asterisk) (H&E, Bar: 1 mm).

Figure 8. New bone formation in defect site of group C, 12 weeks after surgery (Goldner's Trichrome, Bar: 100 µm).

Table1. Percent of new bone formation in different groups after 12 weeks by analyzing 5 microscopic fields by computer analyzing software.

Groups	New bone formation (%)
A	27.28
B	46
C	75.54

Discussion

In this study, a 15 mm segmental defect on diaphysis of rabbit radius was used. This was accepted as the standard of critical sized bone healing and utilized by several researchers [14, 15]. The supportive role of ulna is imperative in this method and the fixing of injured bone is unnecessary.[16]

In the present study, PAAH were used as scaffold. Koushki et al. (2015) reported that partially hydrolyzed polyacrylamide, as a water soluble polymer, has high potential for the preparation of injectable hydrogel as scaffold for tissue engineering [12]. To the best of author's knowledge, this is the first study to evaluate the effects of PAAH on new bone formation. NHA was also used as a component of scaffold in this study. Among different biomaterials used as bone substitutes, calcium phosphate has excellent biocompatibility and being bioactive, can bind to bone and other tissue. These materials induce an interface mechanism that results in the release of calcium and phosphate ions [7].

Furthermore, ADSCs were used in this study. After cells were obtained from rabbits and passage, differentiation ability was evaluated using two methods, Oil Red O for adipogenic and Alizarin Red for osteogenic potential. The positive results obtained in both staining indicated that these cells were able to differentiate into osteogenic lineage *in vitro*. Several studies on ADSCs have applied these methods to evaluate the differentiation potential of cells.[17-20]

After confirming the potential of ADSCs, they were loaded on PAAH/NHA. The results of SEM revealed loading and adhering of cells with different cytoplasmic process on the scaffold. Accordingly, the microscopic results of group C showed better new bone formation as compared with group A and B. This result is in agreement with the findings of previous studies.[14, 18, 21, 22]

Conclusion

This study demonstrated that PAAH/NHA covered by ADSCs improved the healing of critically sized radial defects in rabbits better than scaffold free of cells. Although further studies are needed to prove this claim.

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Conflicts of interest

None

References

1. Laurencin CT, Ambrosio AM, Borden MD, Cooper JA, Jr. Tissue engineering: orthopedic applications. *Annual Review Biomedical Engineering.* 1999; 1:19-46.
2. Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends in Molecular Medicine* 2001; 7(6):259-64.
3. Morizono K, De Ugarte DA, Zhu M, Zuk P, Elbarbary A, Ashjian P, Benhaim P, Chen IS, Hedrick MH. Multilineage cells from adipose tissue as gene delivery vehicles. *Human Gene Therapy*. 2003;14(1):59-66.
4. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of

multipotent stem cells. *Molecular Biology of the Cell*. 2002; 13(12): 4279-95.

5. Fraser JK, Wulur I, Alfonso Z, Hedrick MH. Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends in Biotechnology*. 2006; 24(4): 150-4.
6. Ahmed EM. Hydrogel: Preparation, characterization, and applications: A review. *Journal of advanced research*. 2015;6(2):105-21.
7. Lichte P, Pape HC, Pufe T, Kobbe P, Fischer H. Scaffolds for bone healing: concepts, materials and evidence. *Injury*. 2011; 42(6): 569-73.
8. Torres FC, Rodrigues CJ, Stocchero IN, Ferreira MC. Stem Cells from the Fat Tissue of Rabbits: An Easy-To-Find Experimental Source. *Aesthetic Plastic Surgery*. 2007; 31(5):574-8.
9. Estes BT, Diekman BO, Gimble JM, Guilak F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nature Protocols.* 2010; 5(7):1294-311.
10. Arrigoni E, Lopa S, Girolamo L, Stanco D, Brini AT. Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. *Cell and Tissue Research.* 2009; 338(3): 401-11.
11. Arrigoni E, de Girolamo L, Di Giancamillo A, Stanco D, Dellavia C, Carnelli D, Campagnol M, Domeneghini C, Brini AT. Adipose-derived stem cells and rabbit bone regeneration: histomorphometric, immunohistochemical and mechanical characterization. *Journal of Orthopaedic Science.* 2013; 18(2):331-9.
12. Koushki N, Katbab AA, Tavasoli H, Jahanbakhsh A, Majidi M, Bonakdar S. A new injectable biphasic hydrogel based on partially hydrolyzed polyacrylamide and nanohydroxyapatite as scaffold for osteochondral regeneration. *RSC Advances.* 2015; 5:9089.
13. Khojasteh A, Behnia H, Sadat Hosseini F, Dehghan MM, Abbasnia P, Mashhadi Abbas F. The effect of PCL-TCP scaffold loaded with mesenchymal stem cells on vertical bone augmentation in dog mandible: A preliminary report. *Journal of Biomedical Materials Research Part B: Applied biomaterials.* 2013; 101(5): 848-54.
14. Hao W, Hu YY, Wei YY, Pang L, Lv R, Bai JP, Xiong Z, Jiang M. Collagen I gel can facilitate homogenous bone formation of adipose-derived stem cells in PLGA-beta-TCP scaffold. *Cells Tissues Organs.* 2008; 187(2):89-102.
15. Hao W, Pang L, Jiang M, Lv R, Xiong Z, Hu Y-Y. Skeletal repair in rabbits using a novel biomimetic composite based on adipose-derived stem cells encapsulated in collagen I gel with PLGA-β-TCP scaffold. *Journal of Orthopaedic Research.* 2010; 28(2): 252-7.
16. Niemeyer P, Szalay K, Luginbühl R, Südkamp NP, Kasten P. Transplantation of human mesenchymal stem cells in a non-autogenous setting for bone regeneration in a rabbit critical-size defect model. *Acta Biomaterialia.* 2010; 6(3):900-8.
17. Zou J, Wang G, Geng D, Zhu X, Gan M, Yang H. A novel cell-based therapy in segmental bone defect: using adipose derived stromal cells. *Journal of Surgical Research*. 2011; 168(1):76-81.
18. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, Quarto N, Contag CH, Wu B, Longaker MT. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. *Nature Biotechnology*. 2004; 22(5): 560-7.
19. Park BH, Zhou L, Jang KY, Park HS, Lim JM, Yoon SJ, Lee SY, Kim JR. Enhancement of tibial regeneration in a rat model by adipose-derived stromal cells in a PLGA scaffold. *Bone.* 2012; 51(3):313-23.
20. Schubert T, Xhema D, Veriter S, Schubert M, Behets C, Delloye C, Gianello P, Dufrane D. The enhanced performance of bone allografts using osteogenic-differentiated adipose-derived mesenchymal stem cells. *Biomaterials*. 2011; 32(34):8880-91.
21. de Girolamo L, Arrigoni E, Stanco D, Lopa S, Di Giancamillo A, Addis A, Borgonovo S, Dellavia C, Domeneghini C, Brini AT. Role of autologous rabbit adipose-derived stem cells in the early phases of the repairing process of critical bone defects. *Journal of Orthopaedic Research*. 2011; 29(1):100-8.
22. Liu G, Zhang Y, Liu B, Sun J, Li W, Cui L. Bone regeneration in a canine cranial model using allogeneic adipose derived stem cells and coral scaffold. *Biomaterials.* 2013;34(11):2655-64.

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چکیده

ارزیابی تاثیرات سلول‌های مزانشیمی جدا شده از بافت چربی خودی همراه با هیدروژل پلی آکریلامید و داربست نانو هیدروکسی آپاتیت در التیام نقیصه استخوان زندزبرین در خرگوش

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هدف- در این مطالعه توانایی استخوان‌سازی داربست هیدروژل پلی‌اکریل آمید و نانوهیدروکسی آپاتیت و سلول‌های بنیادی جدا شده از بافت چربی در التیام نقیصه‌های استخوانی با اندازه‌ی بحرانی در استخوان زند زبرین خرگوش مورد ارزیابی قرار گرفته است.

طرح– مطالعه تجربی در شرایط زنده

حیوانات- ۱۲ سر خرگوش نر نیوزلندی سفید

روش کار- در این مطالعه ۱۲ سر خرگوش نر نیوزلندی سفید در ۳ گروه تقسیم شدند. خرگوش‌ها بیهوش شده و نقیصه‌های ۱۵ میلی‌متری در استخوان زند زبرین خرگوش‌ها ایجاد شد. بسته به گروه مورد مطالعه، داربست به تنهایی یا به همراه سلول‌های بنیادی جدا شده از بافت چربی در محل نقیصه قرار داده شد. این گروه‌ها به ترتیب، گروه اول (گروه کنترل، ایجاد نقیصه بدون هیچ‌گونه درمان)، گروه دوم (ایجاد نقیصه و قرار دادن داربست PAAH/NHA در محل نقیصه استخوانی) و گروه سوم (ایجاد نقیصه و قرار دادن داربست PAAH/NHA به همراه سلول‌های بنیادی جدا شده از بافت چربی در محل نقیصه) میباشند. حیوانات ۱۲ هفته پس از جراحی مورد آسان‌کشی قرار گرفتند و بررسی هیستوپاتولوژی انجام گرفت.

نتایج- : بررسی میکروسکوپی نشان‌دهنده مقادیر بالاتر استخوان تازه تشکیل در گروه‌های دوم و سوم بود.

نتیجه گیری و کاربرد بالینی- . بر اساس یافته‌های این تحقیق استفاده از سلول‌های بنیادی مزانشیمی مشتق از چربی کاشته شده بر روی داربست PAAH/NHA می‌تواند روند استخوان سازی را تحریک نماید.

کلمات کلیدی- سلول های مزانشیمی جدا شده از بافت چربی، پلی آکریلامید، نانو هیدروکسی آپاتیت، استخوان