

An animal model study for bone repair with encapsulated differentiated osteoblasts from adipose-derived stem cells in alginate

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ABSTRACT

Objective(s): Adipose derived stem cells (ADSCs) can be engineered to express bone specific markers. The aim of this study is to evaluate repairing tibia in animal model with differentiated osteoblasts from autologous ADSCs in alginate scaffold.

Materials and Methods: In this study, 6 canine's ADSCs were encapsulated in alginate and differentiated into osteoblasts. Alkaline phosphatase assay (ALP) and RT-PCR method were applied to confirm the osteogenic induction. Then, encapsulated differentiated cells (group 1) and cell-free alginate (group 2) implanted in defected part of dog's tibia for 4 and 8 weeks. Regenerated tissues and compressive strength of samples were evaluated by histological and Immunohistochemical (IHC) methods and Tensometer Universal Machine.

Results: Our results showed that ADSCs were differentiated into osteoblasts *in vitro*, and type I collagen and osteocalcin genes expression in differentiated osteoblasts was proved by RT-PCR. In group 2, ossification and thickness of trabecula were low compared to group 1, and in both groups woven bone was observed instead of control group's compact bone. Considering time, we found bone trabeculae regression and ossification reduction after 8 weeks compared with 4 weeks in group 2, but in group 1 bone formation was increased in 8 weeks. Presence of differentiated cells caused significantly more compressive strength in comparison with group 2 (P -value ≤ 0.05).

Conclusion: This research showed that engineering bone from differentiated adipose-derived stem cells, encapsulated in alginate can repair tibia defects.

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Introduction

Autogenous bone grafting as the gold standard method for treating bone defects caused by cancer, trauma, degenerative disease, or congenital abnormalities has disadvantages such as antigens transfer, need for invasive surgery, and donor site morbidity (1-3). Recently, tissue engineering methods have been suggested as novel approaches for defect repair and tissue regeneration (4, 5).

Natural and synthetic materials are designed as scaffolds for bone engineering. Synthetic scaffolds have some drawbacks such as molding difficulty, becoming brittle (6, 7), and degradation into toxic components (8, 9).

Alginate which is extracted from brown algae and

bacteria is a polysaccharide, composed of D-mannuronic and L-gluronic acid residues that could form hydrogel in presence of bivalent cations (10-12).

Among all natural materials, alginate has suitable biocompatibility, high porosity, injectability, stability *in vivo*, and is easily processed into the desired shape (10, 13).

In addition, alginate can be used in cell encapsulation for tissue engineering and promote cell proliferation and differentiation (14). In several studies, alginate usage with mesenchymal stem cells and osteoblasts culturing for repairing bone defects is reported in animal models (15, 16).

Adipose derived stem cells (ADSCs) in compared

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with bone marrow derived stem cells are obtained easily. ADSCs have an excellent capacity to proliferate and differentiate into variety of cell lineages under appropriate inductive conditions (17). They easily become available in relatively large quantities at harvest with slight injuries (18). Researchers reported that ADSCs in monolayer culture mineralized the extracellular matrix and increased the expression of osteocalcin and ALP (19). The aim of this study is to show bone repair in animal models with encapsulated differentiated osteoblasts from adipose-derived stem cells in alginate.

Materials and Methods

Isolation and proliferation of adipose derived stem cells

Six dogs were used in this study with average age and weight of 3 years and 25 kg. This study was conducted in conformity with ethical principles and international animal research. The canines were anesthetized with ketamine 20 mg/kg, Xyline 2 mg/kg IM, and anesthesia continued with halotan and NO₂ gas. Subcutaneous adipose tissue was collected from neck and shoulder and was washed several times with phosphate buffered saline (PBS) (Sigma). Next, they were sliced and digested with 0.05% type1A collagenase (Sigma) for 40 min at 37°C. After enzyme neutralization, released cells from adipose samples were centrifuged at 1400 rpm. Cell pellet was washed three times with medium, and was then cultured in medium containing DMEM (Sigma) - FBS10% (Sigma) - penicillin/streptomycin (Gibco) in 5% CO₂ and 37°C. Also, the medium was replaced every 3 days. Canine adipose-derived stem cells were characterized by flowcytometry previously (20).

Osteogenic differentiation in alginate scaffold

After three passages, ADSCs cells were suspended in alginate 1.2% at cell density of 5×10^6 cells/ml. Mixed cell / alginate was dropped to CaCl₂ 105 mM by syringe as drop. Next, a NaCl 0.9% solution was added (Figure 1). After washing with control medium, osteogenic medium, consisting of DMEM, FBS10%, ascorbic acid (50 µg/ml), beta glycerophosphate (10 mM), and penicillin / streptomycin 1%, was added to the culture flasks. Medium was changed every three days. In order to confirm osteogenic induction, Alkaline phosphatase assay and RT-PCR method were applied. After two weeks, differentiated osteoblasts encapsulated in alginate were transferred to dogs' defective tibia. In the other group, cell-free alginate was implanted and compared with control group (natural bone tissue).

Alkaline phosphatase assay

Intracellular ALP activity was measured by ALP assay kit (Sigma) which uses p-nitrophenyl phosphate

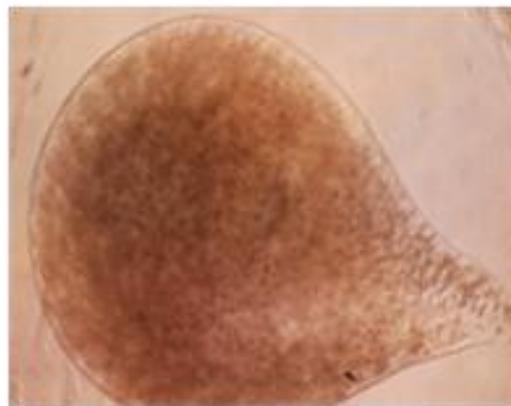


Figure1. Adipose-derived stem cells are encapsulated in alginate hydrogel scaffold

(pNPP) as a phosphatase substrate. Differentiated osteoblasts lysate was prepared using freeze-thaw. 30 µl of the cell lysate was added to a 96 well plate with 50 µl assay buffer and 50 µl pNPP. Then, 20 µl NaOH was added to the wells and the absorbance was read at 405 nm in a micro-plate reader (Wallac Victor3 1420 Multilabel Counter) (21).

Reverse transcription polymerase chain reaction

The expression of bone specific markers (osteocalcin and type I collagen) in differentiated cells was proved with RT-PCR. Total RNA was extracted from the cells using RNx -plus solution (CinnaGen Inc Iran). First strand complementary DNA (cDNA) was prepared using 1 µg of total RNA by oligo(dt)18 as a primer and Revert Aid TM Firststr and cDNA synthesis kit (Fermentas) considering the manufacturer instructions. PCR reaction mixture contained: 2.5 µl cDNA, 1X PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer, and 1 unit Taq DNA Polymerase (Fermentas). Amplification conditions for the PCR were as follow: initial denaturation at 95°C for 5 min, followed by 35 cycles at 93°C, 65°C, and 72°C for 30 sec, 45 sec, and 40 sec, respectively. The PCR products were then analyzed on 1% agarose gel and visualized by ethidium bromide staining and UV trans illuminator.

Cells implantation

All 6 dogs were anesthetized with 20 mg/kg ketamine and 2 mg/kg xylazine IM. After incising the skin and muscles, 4 defects were made on the tibia by trephine bur (Meisinger, Dusseldorf, Germany), and alginate-differentiated cells (group 1) or alginate alone (group 2) implanted separately. After 4 and 8 weeks, the animals were anesthetized and bone samples were taken from repaired and natural bone tissue of tibia (Figure 2).

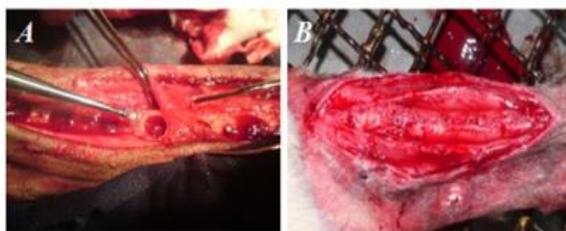


Figure 2. Defects were made on the tibia and alginate-differentiated cells and less-free alginate were implanted separately (A). Repaired defects were remarked by screw (B)

Histology and immunohistochemistry

Biopsy specimens were fixed in formalin, dehydrated by different grade of ethanol, and embedded in paraffin. After sectioning samples in 5 μ thicknesses, staining was performed with Hematoxylin and Eosin. The tissue sections were deparaffinized and treated with trypsin for 30 min for antigen retrieval. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in methanol for 10 min. After washing with PBS, sections were blocked in bovine serum albumin 1.5% for 30 min at room temperature. Monoclonal goat anti human osteopontin antibody was administered (AHP1069 Bio-Rad AbD Sterotec) (1:500 concentration) and allowed to react at 4°C overnight.

Next, antibody conjugated to horse radish peroxidase (DAKO Cytomation, Denmark) was added to the samples for 60 min and finally, after applying diaminobenzidine (sigma), Hematoxylin dye was used to counterstaining (22).

Compressive strength test

Bone samples were embedded in acrylamide (Merck) and tested for compressive strength by Tensometer Universal Machine (Dar Tec) with 5 N/Sec force.

Statistical analysis

Data of compressive strength test were analyzed by ANOVA test and *P*-value <0.05 was considered as statistically significant.

Results

Proliferation of adipose derived stem cells

The adipose derived stem cells were grown with star-shape or fibroblastic appearance with determined



Figure 4. RT-PCR results showed that adipose-derived stem cells of dogs after induction with osteogenic medium expressed bone specific markers such as type I collagen and osteocalcin

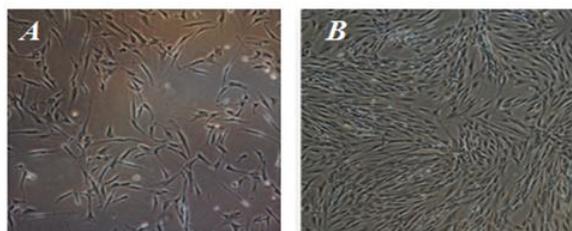


Figure 3. A) Fibroblastic or star like stem cells of monolayer cultured from canine adipose tissue in the primary culture. B) Homogeneous cultivation was obtained from proliferation and increasing number of cells. ×40

nucleus in the primary culture (Figure 3A). In third passage, monolayer cultivation was obtained from proliferation of cells (Figure 3B).

Alkaline phosphatase results

Alkaline phosphatase activity was evaluated in differentiated cells with photometric method at 405 nm with a mean OD of 49±3.6 in comparison with undifferentiated cells with mean 5±1.4.

RT-PCR results

RT-PCR results indicated expression of osteocalcin and type I collagen genes in differentiated adipose-derived stem cells that confirmed differentiation of these cells into osteoblast (Figure 4).

Histological results

After routine staining with hematoxylin–eosin, bone trabeculae formation was observed in all samples. Lacuna with osteoblasts was determined in bone trabeculae. Bone marrow tissue was observed between bone trabeculae. In both groups, woven bone was observed instead of compact bone in control group. Alginate with differentiated cell group showed thicker bone trabeculae compared to cell-less alginate group (Figure 5). Regarding to the time, we found bone trabeculae regression and ossification reduction after 8 weeks compared to 4 week duration in cell-less alginate group. In alginate with differentiated cell group, bone formation was increased in 8 weeks.

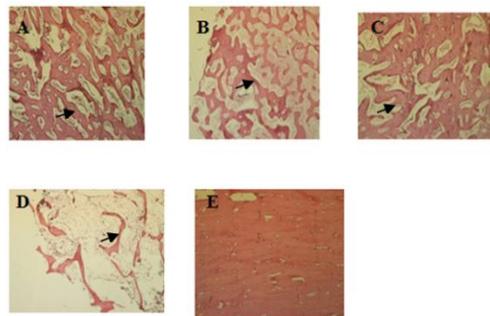


Figure 5. Results of histological study showed bone trabecula formation in repaired sites in different groups A) alginate-differentiated cells, B) cell-less alginate, after 4 weeks, C) alginate-differentiated cells, D) cell-less alginate after 8 weeks and E) control. H&E×60

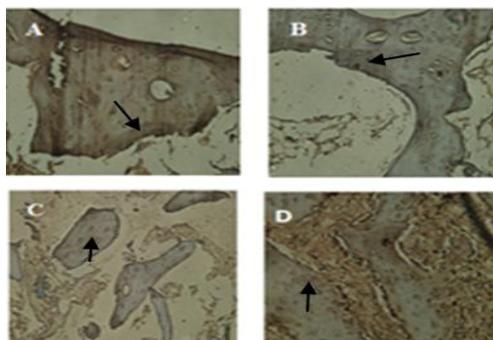


Figure 6. Immunohistochemical results showed osteopontin protein in repaired sites: A) alginate-differentiated cells, 8 weeks, B) alginate-differentiated cells, 4 weeks, C) cell-less alginate, 8 weeks, D) cell-less alginate, 4 weeks. $\times 300$

Immunohistochemistry

Immunohistochemical results showed generated osteopontin in repaired bone matrix in both groups (Figure 6).

Compressive strength test results

At fourth and eighth weeks, compressive strength test results showed that the group of differentiated cells with alginate scaffold is stronger than scaffold alone group and there is a significant difference between two groups (Figure 7) (P -value < 0.05). In the group of differentiated cells with alginate scaffold, strength test results indicated strength increasing after 8 weeks, whereas it was decreased in scaffold alone group.

Discussion

Our study indicated that defects of tibia that have been implanted by encapsulated differentiated osteoblasts from ADSCs in alginate could be repaired after 4 or especially after 8 weeks. The presented bone tissue was acceptable compared with natural bone in strength and histological characters. After 8 weeks, bone trabeculae regression and ossification decrease was found in alginate alone group, but in differentiated cell group, bone formation was increased in 8 weeks. Differentiated cells caused more compressive strength significantly compared to alginate alone group.

Most studies showed that scaffolds are essential for tissue healing and repairing *in vivo* and *in vitro*. Scaffolding has important roles such as a substrate for migration and adhesion of cells, membrane barrier, a delivery vehicle, and also developing new tissue and transplanting it into the body of a living organism (23).

Previous studies indicated that alginate gel is especially attractive for bone and cartilage tissues engineering due to its biocompatibility, biodegradability, and easily injection (24- 25).

Alginate, as an extracellular matter, could provide a suitable ground for cell growth, due to its hydrophilic nature and high amounts of water (26).

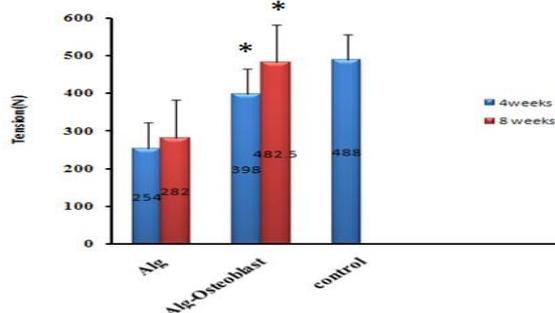


Figure 7. Strength comparison between different groups and times: cell-less alginate group (Alg) and alginate with differentiated osteoblasts from stem cells (Alg-Osteoblast). Strength in group of differentiated cells was significantly different from cell-less alginate group (* P -value ≤ 0.05)

Studies showed that culture of osteoblasts derived from chicken's embryo calvaria in alginate increases osteoblast proliferation and survival during 8 months (27). In accordance with this study, our survey showed that osteoblasts derived from ADSCs in alginate scaffold could proliferate and reconstruct bones *in vivo* during 8 weeks.

In another study, Liao *et al* showed that after 12 weeks, the alginate gel-osteoblasts-bone granule material had changed into bone tissue with few bone granules and some residuary alginate gel in the skull defects in rabbits (15). We showed that alginate - osteoblasts- derived from ADSCs change into bone tissue after 8 weeks. Also, without bone injury for implant, we can repair bone defects with osteoblasts derived from adipose derived stem cells in alginate scaffold.

Abbah *et al* indicated that alginate microbeads can act as a scaffold for mesenchymal stem cell proliferation and osteogenic differentiation (14). Cai *et al* observed that in fourth week after implantation in subcutaneous tissue, bone formation occurred by alginate gel osteoblast derived bone marrow stem cells (10). Our study showed that formed bone after 4 weeks had lower strength and thinner bone trabecula compared to natural bone; in addition, more time is required for obtaining acceptable bone. Also, Lin and colleagues in a study about evaluating alginate gel combined with BMP-2 transfected ADSCs to bone reconstruction, proved that in rats, weak osteogenesis was noted in the epidural region of the defect border in 8 weeks. However, after 16 weeks of treatment, new bone formations throughout the defects were observed to be continued (28). Whereas, our study showed that 8-week duration is an appropriate time for bone repair with osteoblasts derived from ADSCs in alginate scaffold. In our study, compressive strength test after 8 weeks showed that strength of bone formed from osteoblasts derived from ADSCs is significantly higher than cell-free alginate scaffolds. Also, Li *et al* stated same results with differentiated osteoblasts from bone marrow

stem cells on chitosan-alginate hybrid scaffolds after 4 weeks (29).

Conclusion

In summary, this research indicated that engineering bone from differentiated adipose-derived stem cells encapsulated in alginate biodegradable scaffold could repair the defects of tibia with an acceptable compressive strength due to the increased secretion of matrix, and could be used for bone repairing in clinical regeneration if other approaches are unsuitable or impossible.

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Conflict of interest statement

All authors declare that they have no conflict of interest.

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