

Osteogenic Differentiation of Mesenchymal Stem Cells Via Osteoblast-Imprinted Substrate: In Vitro and In Vivo Evaluation in Rat Model

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Abstract

BACKGROUND: Stem cells have great effects in clinical cell-based therapy. Accordingly, controlling the behavior and directing the fate of stem cells cultured in the laboratory is an important issue.

OBJECTIVES: The aim of this study was to evaluate osteogenic properties of adipose derived mesenchymal stem cells (ADSCs) which differentiated toward osteogenic lineage by osteoblast-imprinted substrate.

METHODS: Rat ADSCs seeded on osteoblast-imprinted substrates, alkaline phosphatase (ALP) were measured in cellular supernatant of days 7 and 14. Alizarin Red staining of mineral matrix production in day 17 was performed. In order for in vivo evaluation, after seeding differentiated cells on a simple collagen scaffold, samples were implanted in an 8mm critical size calvarial defect. After 4 weeks defect site was harvested and prepared for histopathological examination.

RESULTS: ALP in both time points was significantly more than in undifferentiated ADSCs. ($p < 0.05$). Alizarin red staining of differentiated cells showed a great production of mineral matrix nodules in cell culture plate. Histopathological investigations revealed greater amount of new bone formation and mostly in the center of defect, indicate osteoinductive effect of differentiated cells in vivo.

CONCLUSIONS: The osteoblast-imprinted substrate can mimic the topography and shape of natural osteoblast, which can mechanically direct ADSCs toward osteogenesis.

KEYWORDS: Adipose derived mesenchymal stem cells (ADSCs), alkaline phosphatase, calvarial defect, cell-imprinted substrate, osteogenic differentiation

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Received: 2019-01-22

Accepted: 2019-05-08

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How to Cite This Article

Gholami, H., Mardjanmehr, S. H., Dehghan, M. M., Bonakdar, S., & Mohajeri, S. F. (2019). Osteogenic differentiation of mesenchymal stem cells via osteoblast-imprinted substrate: In vitro and In vivo evaluation in rat model. *Iranian Journal of Veterinary Medicine*, 13(3), 260-269.

Introduction

Bone tissue has a great capacity of self-regeneration; however, complicated injuries such as fractures, or surgically induced lesions after tumor resection or osteomyelitis are common, in which normal bone healing procedure is disrupted (Zhao et al., 2010). Tissue engineering has recently been developed as a treatment procedure for regeneration of injured or compromised bone tissue. Bone regeneration has involved the use of scaffolds, growth factors and stem cells (Westhrin, Xie, Older, Sikorski, & Strand, 2015). Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into a variety of connective tissue phenotypes such as osteoblasts, chondrocytes and adipocytes (Westhrin et al., 2015).

One of the most intriguing points in regenerative medicine, is how to conduct stem cell differentiation toward a specific favorite lineage. In vivo, suitable differentiation, proliferation and maintenance of potency of stem cells are adjusted by their 3-dimensional microenvironments. Biomaterials can mimic this microenvironment, impressing the in vitro differentiation. As a consequence, tissue regeneration research has been principally focused on finding out how differentiation factors (e.g., chemicals, growth factor, extracellular matrix components, cell morphology, matrix stiffness and mechanical forces) play a part in the stem cell microenvironment (Laurent & Mashinchian, 2015). Among the microenvironmental factors that regulate stem cell fate and function, mechanical factors have been introduced as important determinants. Mechanical factors including rigidity and topology of the extracellular matrix (ECM) can change the fate of stem cells because they can sense physical features of their environment through a mechanism

named mechanotransduction (Macqueen, Sun, & Simmons, 2013). A substrate mimicking surface topography and 3D shape of the original extracellular matrix can be used as a perfect culture substrate for guiding stem cell differentiation. In this regard, several researches have been carried out on stem cell differentiation by a cell-imprinting substrate, in which the footprint of the adult differentiated cells has been used. Further research has studied the differentiation of stem cells toward keratinocytes, tenocytes and chondrocytes, by the cell-imprinting method, which has suggested a new approach to mechanical stem cell differentiation (Mahmoudi et al., 2013) (Kamguyan et al., 2018) (Mashinchian et al., 2014).

The aim of the current study was to evaluate adipose-derived stem cells (ADSCs) differentiation toward osteogenic lineage based on the cell-imprinting method, in vitro and in vivo.

Materials and Methods

Isolation and Culture of Adipose-Derived Stem Cells (ADSCs): Subcutaneous adipose tissue samples were surgically harvested from inguinal region of healthy male 250-gram weight, Wistar rats. Tissue samples were washed 3 times in PBS/penicillin-streptomycin (300 IU/mL) solution. Then samples were cut into 1×1 mm pieces and digested in 0.1 mg/mL collagenase type II (Sigma, United States) for 1h in an incubator. After enzymatic digestion, the solution was filtered through a 150-micrometer filter to remove undigested tissue, followed by neutralization of the enzyme with Dulbecco's modified Eagle's medium (DMEM; Gibco, Scotland) containing 10% FBS, then suspension was centrifuged at 1300 rpm for

5 min and the cell pellets were separated. The solution containing suspended ADSCs was then moved to the culture medium consisting of DMEM supplemented with 10% FBS and penicillin–streptomycin (100 IU/mL, Sigma, United States) in a humidified incubator (37 °C, 5% CO₂). The culture medium was changed every three days until a cell confluence of $\geq 80\%$.

Isolation and Culture of Osteoblast: Two 3 day-old neonatal rats were euthanized and sterilized with 70% ethanol. After removing the skin and brain tissue from the skull using a scalpel and tweezers, any excess tissue and cartilage from around the calvaria were scraped off. Similarly, the skin, muscles and soft tissue around the long bones were scraped away. Epiphyses were cut off and then the bone fragments were placed into a tube containing PBS with antibiotics. Bone samples were washed 3 times and chopped into 1mm pieces. Bone samples were incubated in 0.25% trypsin for 10 min at 37 °C. Trypsin solution was discarded and bone fragments were washed in DMEM. After that, samples were incubated in 0.2% collagenase type II solution for 60 min at 37 °C. The final solution was kept in a 15-ml tube and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cell pellets within culture medium were transferred to 6-well cell culture plates. Plates were incubated at 37 °C 5% CO₂, until the cells became confluent after 5 days (Taylor, Shah, & Orriss, 2014). Osteoblast characterization was performed by alkaline phosphatase measurements and alizarin red staining (Rosa, 2005)(Nikukar et al., 2013).

Fabrication of Cell-Imprinted Substrates: Polydimethylsiloxane (PDMS, SYLGARD 184, RTV, Dow Corning, United States) was employed to fabricate the cell- imprint-

ed substrates. The silicone resin and curing agent were combined with a 10:1 ratio. Confluent osteoblast plates which were fixed by 4% glutaraldehyde solution previously were used as template for imprinting process. The resin-curing agent mixture was poured very gently onto plates and kept at 37 °C for 48 h. The patterned PDMS was peeled off the osteoblasts, followed by several washing steps using 1M NaOH solution to eliminate remained cells and debris. These prepared substrates were fixed in the bottom of cell culture polystyrene plates and after sterilizing procedure, substrates were made ready for stem cell seeding.(Allan et al., 2018) (Bonakdar et al., 2016)

Mesenchymal Stem Cells Seeding on Substrate: ADSCs which were previously isolated and cultured, were transferred onto osteoblast-imprinted substrates, by the ratio of 1×10^4 cells per each 3-cm culture plate. Culture medium for this step was similar to that used in ADSCs culture. Plates were maintained in a humidified incubator (37 °C, 5% CO₂) up to cells covering all of the patterned PDMS surface (14 days).

Characterization of Stem Cells Cultured on PDMS Substrates: Alizarin Red staining and Alkaline Phosphatase (ALP) measurement was performed in order to determine osteogenic properties and compare with undifferentiated ADSCs. The cells in two plates were fixed by 4% glutaraldehyde solution for 24h and washing with PBS, staining with alizarin red stain was done for 45 min, then they were washed with PBS for removing residual stain. Red plaques were seen by an inverted optical microscope (Olympus IX71, Japan) and compared with what was observed in ADSCs.

Osteogenic differentiation was investigated via ALP activity measurements in

cell supernatants at days 7 and 14. Collected samples were stored at -20 °C and tested by an alkaline phosphatase kit (Sigma, United States) according to its standard protocol. The absorbance at a wavelength of 405 nm in a ELISA reader spectrophotometer was measured. (Thibault et al., 2010)

Cell Implantation in rat calvarial defect: Cells were seeded on a commercial collagen scaffold (Nano-Zist-Arayeh, Iran) at the rate of 8×10^5 cells per each 8 cm diameter round scaffold.

The three groups of study (described as differentiated cells by osteoblast-imprinted substrate, ADSC and cell-free scaffold) were implanted in rat calvarial defects for 4 weeks. The sample size ($n = 5$) was selected on the basis of previous studies. (Ekaterina et al., 2012)

Twenty male Wistar rats (2 months old; weight = 300–350 g) were maintained in the animal facility for 2 weeks to become adapted to housing conditions. The rats were anesthetized with intramuscular administration of ketamine (80 mg/kg) and xylazine (10 mg/kg) combination. The surgical site was shaved and scrubbed with iodine. Utilizing sterile instruments and aseptic circumstance, a cranial skin incision was made in an anterior to posterior direction along the midline. Soft tissue, musculature and periosteum were dissected and reflected to expose the calvaria. Central full-thickness critical size defect 8 mm in diameter was created using a saline-cooled trephine drill. The dura mater was not disturbed. Then defect was implanted with the scaffold, the periosteum and skin were reclosed by suture. Post-surgery, the animals were injected with a dose of enrofloxacin subcutaneously. After 4 weeks, the animals were sacrificed by CO₂ inhalation, and the calvarial defect sites with surrounding

bone were harvested for evaluation (Ekaterina et al., 2012) (Narayanan et al., 2013).

Histopathologic Investigation: Samples, including the implantation sites with surrounding bone, were fixed in 10% buffered formaldehyde for 5 days, then transferred into decalcifier solution (10% Ethylenediaminetetraacetic acid, pH=7.2). After complete decalcification (14 days), samples were dehydrated in increasing grade series of ethanol and embedded in paraffin using routine histological techniques. Then the specimens were sectioned at 5 μ m by a rotary microtome and stained with hematoxylin and eosin (H&E). Transmitted light images of the stained sections were taken with a microscope (Nikon Eclips E600) connected to a CCD camera. The sections were analyzed for new bone formation, tissue components, vascularization and inflammatory response (HATT, 2007) (Park, Kim, Moon, & Na, 2009).

Results

Alizarin red staining of cells in day 17 represented a great difference between ADSCs and differentiated cells on osteoblast-imprinted substrate. Obvious red foci of secreted mineral matrix in differentiated cell group indicate remarkable osteogenic differentiation. On the other hand, in ADSCs group, no red stained area was observed (Fig.1).

Statistical analysis of ALP measurement in cellular supernatant of days 7 and 14, revealed significant differences between two groups, in a way that differentiated cells showed greater amount of ALP than ADSCs in both time points. ($p < 0.05$)

In histopathological evaluation, specimens implanted with differentiated cells showed greater bone regeneration compared to those with ADSCs and scaffold alone. This newly formed bone along the defects in group differ-

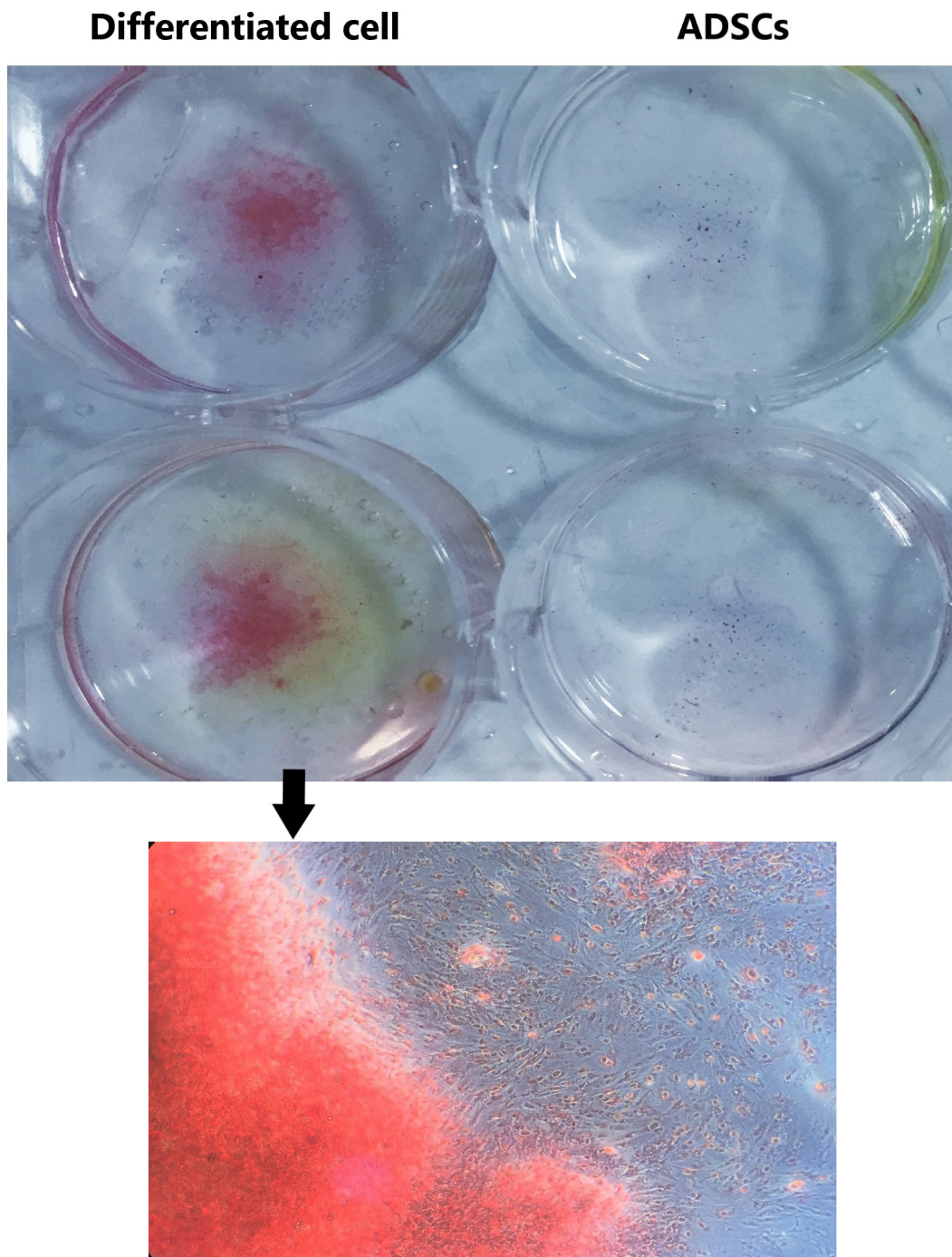


Figure 1. Alizarin red staining of cell culture plates. There is a remarkable red colored area in differentiated cells plates. It represented enormous mineral matrix production as an important characteristic of osteogenic lineage. A photomicrograph of staining cell culture plate, shows the mineralized matrix in a greater magnification.

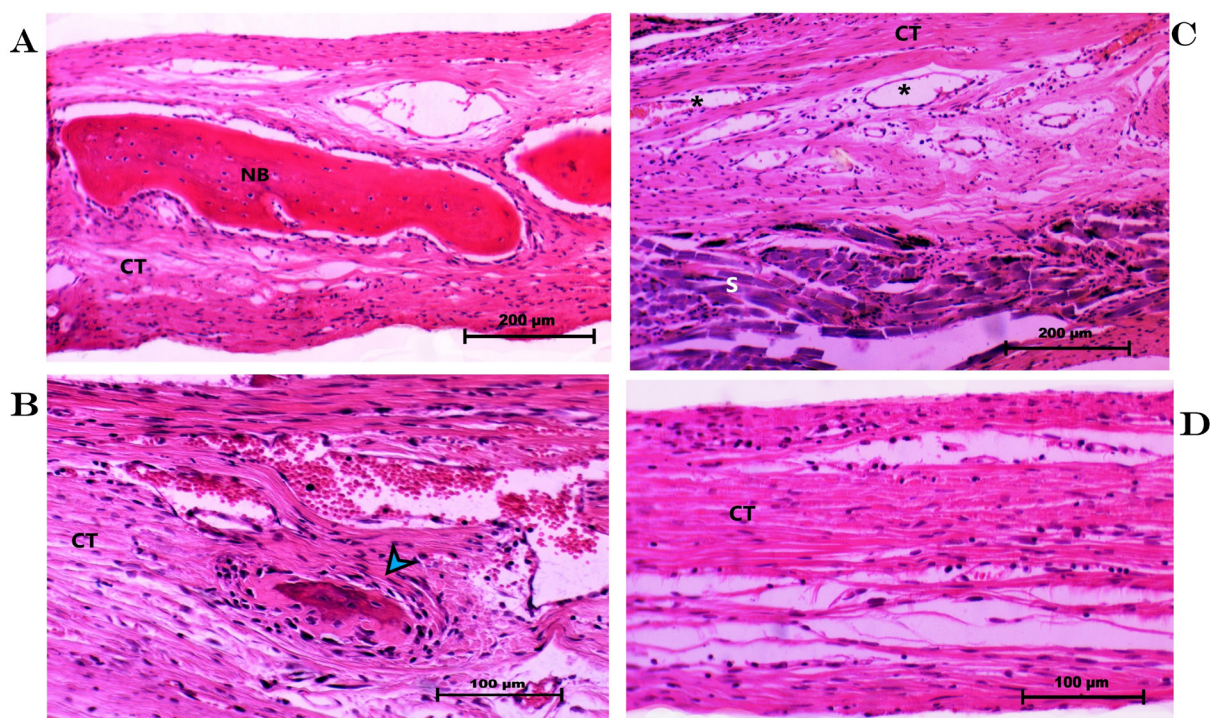


Figure 2. Photomicrographs of calvarial defect area. Pictures A, B and C is related to differentiated group, and D represents part of defect in group ADSCs. Newly formed bone (NB) lamella in the center of defect was seen(A). Within the connective tissue (CT) in differentiated group there is some foci of intramembranous ossification (blue arrowhead in B). Numerous newly formed vessels (*) showed higher rate of angiogenesis in differentiated group(C). Remnants of scaffold showed by a white "S" (C). In ADSCs group except thin to moderate connective tissue, there is not seen new bone lamella or great number of new vessels (D). Hematoxylin & Eosin staining. Bar=200 micrometers (A,C) Bar= 100 micrometers (B,D).

entiated cells was observed both in the center of defect and in the edge of defect, adjacent to old bone. While in groups ADSCs and only scaffold no new bone lamella was seen in the center of defects, just limited bone formation from the edges of defect was observed. Newly formed vessels in differentiated group was more than the other two groups and these structures were distributed all around the defect in spite of ADSCs and scaffold group in which vessels mostly formed near the defect edges. In differentiated cell group, thick connective tissue with active spindle-shaped fibroblasts, numerous new vessels and foci of intramembranous ossification in addition to newly formed bone lamella, connected the edges of defect to each other. On the other hand, in groups ADSCs and scaffold, thin to

moderate connective tissue with less active fibroblast in comparison with differentiated group, without any sign of ossification, connected the defect edges (Fig. 2).

Although differentiated cell group mostly showed moderate mononuclear cell inflammatory response, in some specimens in which the scaffold was not completely degraded, prominent giant cells within the remnant scaffold were seen.

Discussion

Sheep Bone is capable of repairing limited defects through remodeling. Complicated bone lesions (e.g., defects related to severe trauma, tumors, diabetes, osteonecrosis and osteoarthritis), would be helped by cell-based therapy. Tissue engineering

plans have optimized the induction of osteogenic differentiation in order to employ the most effective process of bone regeneration (Waese, Kandel, & Stanford, 2008). Clinical success of bone tissue engineering is greatly dependent on applying suitable cell source for producing functional osteoblasts at the site of repair (Ohgushi, 2014). The biochemical, mechanical and physical factors of cell culture microenvironment can determine stem cell fate (Bae et al., 2010). As established in previous studies, topographical features in cell culture substrate can lead to lineage specifications (Mahmoudi et al., 2013) (Abagnale et al., 2015). Based on this idea, we imprinted osteoblasts nanopattern by PDMS, and used it as ADSCs culture substrate with the aim of enhancing osteogenic differentiation. In a study the success of this method has been determined for osteogenic differentiation in vitro (Kamguyan et al., 2018). Our in vitro findings such as ALP measurement and Alizarin red staining demonstrate a great agreement with their result. In the other study, investigation showed that keratinocyte-imprinted substrate in ADSCs differentiation toward keratinocyte for skin regeneration was highly effective in vitro (Mashinchian et al., 2014). In current study we showed that in addition to in vitro differentiation of ADSCs, differentiated cells as functional osteoblast-like cells can induce osteogenesis in vivo. As the first in vivo study of this method for osteogenic differentiation, the great amount of angiogenesis and marked new bone formation especially in the center of calvarial defect and foci of intramembranous ossification revealed that mimicking osteoblast topographical features in the process of differentiation was not only effective in vitro, but also differentiated cells can act osteoinductively in vivo. It is well

determined that angiogenesis and osteogenesis are coupled procedures throughout bone development and angiogenesis supports osteogenesis. (Ohgushi, 2014). In current study we observed that newly formed bone trabeculae appear in close vicinity of newly formed vessels.

Conclusion

The induction of osteogenic differentiation via osteoblast-imprinted substrate in stem cells could result in new bone formation in 3D microenvironment of repairing bone defect in vivo. The osteoblast-imprinted substrate can mimic the topography and shape of natural osteoblast, which can mechanically direct ADSCs toward osteogenesis. As in this method of differentiation there is no use of chemicals, it would be safer and cheaper to use mechanical differentiation in clinical cell-based therapies.

Conflicts of Interest

The author declared no conflict of interest.

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القای تمایز استئوژنیک در سلول های بنیادی مزانشیمی با استفاده از بستر قالب گیری شده با استئوبلاست: ارزیابی برون تنی و درون تنی در مدل حیوانی رت

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(دریافت مقاله: ۰۲ بهمن ماه ۱۳۹۷، پذیرش نهایی: ۱۸ اردیبهشت ماه ۱۳۹۸)

چکیده

زمینه مطالعه: فناوری های نوین نظیر مهندسی بافت استخوان، با بکارگیری سه جزء مهم: سلول، داربست و ملکول های فعال زیستی، باعث پیشرفت چشمگیری در ترمیم ضایعات استخوانی در سال های اخیر شده است. در همین راستا، بهینه سازی هر یک از این سه جزء، اهمیت بسزایی دارد **هدف:** در این مطالعه، با استفاده از روشی جدید و مبتنی بر تغییرات توپوگرافیک در بسترکشت سلول، تلاش گردید القای تمایز سلول های بنیادی مزانشیمی مشتق از چربی، به رده ی استخوانی صورت پذیرد.

روش کار: در ارزیابی برون تنی این شیوه ی تمایز، میزان فعالیت آنزیم آلکالین فسفاتاز در دو مقطع زمانی ۷ و ۱۴ روزه به روش الایزا، و میزان معدنی شدن ماتریکس پس از ۱۷ روز با استفاده از رنگ آمیزی Alizarin red مورد بررسی قرار گرفت. جهت انجام ارزیابی درون تنی، سلول های کشت داده شده روی داربستی سنتتیک از جنس کلاژن قرار گرفت و در نقیصه ی با اندازه بحرانی مجموعه رت کاشته شد. پس از گذشت ۴ هفته استخوان های مجموعه مورد ارزیابی هیستوپاتولوژی قرار گرفت.

نتایج: در هر دو مقطع زمانی ۷ و ۱۴ روز میزان تولید و فعالیت آنزیم آلکالین فسفاتاز در محیط رویی کشت سلول های گروه تمایزی، به طور معناداری از گروه سلول های غیرتمایزی بیشتر بود ($P < 0.05$). در گروه سلول های تمایزی در اثر تولید فراوان ماتریکس معدنی شده با استفاده از آلیزارین رد پلیت کشت سلول به رنگ قرمز کاملاً مشخصی درآمد. در حالی که پلیت کشت سلول های غیرتمایزی رنگ آلیزارین را به خود نگرفت. مطالعه هیستوپاتولوژی نشان داد که در گروه سلول های تمایزی تیغه های استخوانی به نسبت بیشتری تشکیل یافته و علاوه بر رشد در دو لبه ی نقیصه، به طور مستقل در وسط نقیصه هم ایجاد گردیده بود، که مورد اخیر حاکی از القای ساخت استخوان اختصاصاً توسط سلول های تمایزی می باشد.

نتیجه گیری نهایی: هدایت کنترل شده ی تمایز سلول های بنیادی، با استفاده از تاثیر مکانیکی و به طور خاص با کمک بسترهای قالب گیری شده با سلول های طبیعی، به نحوی که در این مطالعه انجام گرفت، نقش مؤثری در پیشرفت سلول درمانی ایفا خواهد نمود.

واژه های کلیدی:

تمایز استئوژنیک، سلول های بنیادی، قالب گیری سلولی، القای بازسازی بافت استخوانی، هیستوپاتولوژی، آلکالین فسفاتاز