Partial Resurfacing of the Distal Femoral Cartilage Defect with Stem Cell- Seeded Poly-Vinyl-Alcohol (PVA) Scaffold

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Abstract

Objective- To evaluate the biological compatibility of differentiated stem cells embedded in poly-vinyl-alcohol (PVA) scaffolds for repair of distal femoral cartilage defect.

Design- Experimental in vivo study.

Animals- Twelve adult male New Zealand white rabbits were used which were divided into two groups (I, II) six rabbits each.

Procedures- Mesenchymal stem cells were isolated from humerus bone marrow of group I rabbits and were cultured and differentiated on PVA scaffolds to chondrocytes. Scanning Electron Microscopy (SEM) showed well distribution of the cells inside the scaffold. A 4 mm diameter full thickness cartilage defect was created on central region of bilateral distal femoral joint surface (patellar groove) in all rabbits. In group (I) the defects were covered with autologous differentiated MSCs-seeded scaffolds; whereas the group II rabbits were left without any treatment as control ones. One month and three months after operation, three

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rabbits were sacrificed from each group, randomly. Histopathologic evaluation of defects was performed with H&E and trichrome staining.

Results- The findings showed that in the engineered cartilage with the PVA scaffold, the defects were filled with smooth, shiny white tissue macroscopically at three months after the transplantation. Despite much connective tissue formed in defect area after three months, there was no evidence of chondrocytes in control group, whereas the defects of experimental group were almost completely filled with hyaline cartilage.

Conclusion and Clinical Relevance- The results indicated there is positive possibility for partial resurfacing of cartilage defect using stem cell-seeded PVA scaffolds

Key Words- Stem Cells, Rabbit, Cartilage, Defect, PVA.

Introduction

Joint cartilage allows gliding action of synovial joints. Damage to articular cartilage, either by trauma or disease, can affect joint function, as adult articular cartilage has a limited capacity for repair.[1] A variety of treatments have been attempted for those with focal, symptomatic osteochondral lesions such as abrasion chondroplasty, microfracture, transplantation of osteochondral plugs or the use of cultured autologous chondrocytes.[2] Although each of these treatment strategies has shown some temporary benefit in improving patient symptoms, no studies have yet documented the ability these forms of treatment to delay or halt the relentless progression of the joint towards end stage degeneration. Thus, most patients treated for early focal osteochondral lesions will ultimately require total joint replacement in the future as the disease process advances.[3] As all of these treatments have limitations, recent efforts have focused on developing methods to bioengineer articular cartilage constructs.[4] Over the past decade, tissue engineering approaches have been developed, which combine cells, bioactive molecules and scaffolding materials.[5] Cartilage tissue engineering techniques involving scaffolds made from biodegradable and biocompatible materials hold great promise for the treatment of cartilage defects.[6] Scaffolds prepared from natural materials have been associated with disadvantages such as uncontrolled degradation rates, sterilization difficulty, sourcing issues concerning possible pathogens, viruses and other bioburdens, and safety issues related with breakdown products.[7] The Poly vinyl alcohol (PVA) is a semi-crystalline hydrophilic polymer with good chemical and thermal stability. PVA is highly biocompatible and non-toxic and its hydrogels may be synthesized to mimic the water content of articular cartilage and possess a low coefficient of friction, which is an important characteristic for lubrication of articular joints.[8] To date several cell sources have been investigated as potential candidates for the cell therapy based approach for cartilage tissue engineering, including normal and osteoarthritic (OA) chondrocytes and mesenchymal stem cells (MSC) derived from a variety of tissues.[9,10,11] Subcutaneous adipose tissue and bone marrow represent attractive mesenchymal stem cell sources for tissue engineering because they are abundant and easily accessible with minimal donor site morbidity. Huang et al. suggest that in a culture system with minimal selection, bone marrow shows greater promise than adipose tissue as a cell source for cell-based cartilage tissue engineering.[12]

In the present work, we hypothesized that PVA scaffold was able to facilitate cell viability and chondrogenic differentiation as well as mimicking articular cartilage. In order to prove this hypothesis, the study was designed to evaluate the biological compatibility of differentiated stem cells embedded in poly-vinyl-alcohol (PVA) scaffolds for repair of distal femoral cartilage defect.

Materials and Methods

PVA Scaffold

The biodegradable scaffolds (Stem Cell Technology Corporation, Tehran, Iran) used in this study were composed of PVA, a flat sheet with 0.5 mm thick. The morphology of PVA nonwoven mats were observed using Scanning electron microscope (SEM, Vega©Tescan, Cranberry Twp., PA, USA) at an accelerating voltage of 20 kV. Mechanical properties of the scaffold could hold the suture strength for surgical operation.

Mesenchymal Stem Cell Isolation and Culture Expansion

Twelve adult male New Zealand white rabbits were used which were divided into two groups of experiment and control, six rabbits each. The rabbits of experiment group were anesthetized by an intramuscular injection of Ketamin (35 mg/kg, Alfasan, The Netherlands) and Xylazine (5 mg/kg, Alfasan, the Netherlands) cocktail. Bone marrow was then isolated from the humeral head of each rabbit using an 18 gauge bone marrow needle and 6–7ml of the marrow was fastened to a 10ml syringe containing 1ml heparin (5000 units/ml, I.P.D.I.C., Rasht, Iran). The released cells were collected in a T-75 flask (Coster Co., Cambridge, MA) containing 15ml of medium. The medium was a DMEM (High glucose, Gibco, Scotland) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin [100units/ml], streptomycin [0.1 mg/ml] and amphotericin B [Fungizone, 0.25 g/ml]; all from Gibco). The cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C and the medium was replaced with fresh medium every 3 days. No growth factors were added.

Differentiation of MSCs to Bone and Adipose Cells

Since MSCs have no specific indicator to prove their mesenchymal nature, it is necessary to differentiate obtained cells to at least two other specialized cell lineages. Therefore, MSCs were differentiated to bone and adipose cells in specialized culture mediums. Osteogenesis was induced in a medium of High Glucose DMEM and 10% FBS containing inducers of Dexamethasone (10×10^{-9}), Ascorbic Acid (35 μg/ml) and β glycerol phosphate (10×10^{-3} M). 10^3 to 10^4 cell/cm² in a plate containing mentioned induction medium were cultured in an incubator at 37°C and 5% CO_2 for 15 days. The culture medium was changed every 3 days.
A cell density of 1×10^3 cells/cm² in a plate containing Dexamethasone (10×10^{-9} M), IBMX (5×10^{-3} M) and Indomethasine (10×10^{-6}) was used for induction of differentiation of MSCs to adipocytes. The mentioned medium was incubated at 37°C with 5% CO_2 for 15 days. The culture medium was changed every 3 days.

In Vitro MSC Differentiation and Culture into the Scaffolds

The cells were passaged three times in 4 weeks until they were purified and reached confluence, covering 80% of surface of the flask. Then the cultured cells were released from the substratum using 0.025% trypsin. The autologous MSCs were seeded onto three-dimensional PVA scaffolds by simple dropping of the MSCs suspension and subcultured in conditioned medium of DMEM,

containing FBS (10%), TGFβ1 (10 ng/ml), bFGF (10 ng/ml) and Ascorbic Acid bi-Phosphate (50 ng/ml) as well as Dexamethasone (10-7 M) to induce the differentiation of MSCs in vitro.

MSC Proliferation Assay

MTT assay was applied to show the survival and proliferation of MSCs on PVA scaffold. Mesenchymal stem cells were seeded at low density (7000 cells/disc) and cultured in DMEM containing 10% FBS. After 0,1,2,3,5,7,9,12,15,18,21,28 days of culture, the media were replaced with 200 mL PBS containing 100 mg MTT. The viable cells were allowed to convert the MTT to formazan for 3 h before lysing cells with DMSO in isopropanol. Absorbance of formazan was read on a spectrometer at 570 nm and normalized.

Immunocytochemistry

The sections of MSC seeded-scaffolds were deparaffinized, dehydrated, and incubated at 37º C in collagenase for 15 min in PBS. Then, the samples were reacted with primary antibodies for Type II Collagen (rabbit polyclonal; dilution 1:500) at room temperature over night. Sections were then incubated at room temperature for 1 h with FITC and PE goat anti-rabbit IgG as a secondary antibody. Non-immune controls were prepared with 1% BSA in PBS without primary antibody.

Scanning Electron Microscopy (SEM) Examination

During static culture, the architecture of the scaffold allowed the MSCs to lie in uniform arrays in palisades for three-dimensional culture of the MSCs. The scaffolds were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer For 2 h at 4°C. Then they were dehydrated through increasing concentrations of ethanol. After this, they were dried and coated with gold. The cells were observed with a scanning electronic microscope.

Surgical Procedures

Under the same general anesthesia described above, the knee joint of all rabbits were opened with a lateral parapatellar approach. The patella was dislocated medially and the surface of the femoropatellar groove was exposed. A full-thickness cylindrical cartilage defect of 4 mm diameter was created in the patellar groove of the bilateral knees using a disposable stainless-steel punch (Fig. 1). After irrigating the joint with sterile isotonic saline, the defects in rabbits of experiment group were covered with autologous Differentiated MSCs/3D-PVA scaffolds and sutured with a 6/0 PDSTM II (EthiconTM) suture material as the engineered cartilage group with twelve knees (Fig. 2). In rabbits of control group, the defects were left without any treatment. After suturing the joint capsule and the skin, the animals were returned to their cages and allowed to move freely without joint immobilization. All the animals were operated on according to the guidelines for animal experiments of The Iran Society for Prevention of Cruelty to Animals (ISPCA).

Figure 1. A full-thickness cylindrical cartilage defect of 4 mm diameter was created in the patellar groove of the bilateral knees using a disposable stainless-steel punch.

Figure 2. The defects in experimental group were covered with differentiated MSCs/3D-PVA scaffold.

Histological Examination of Repair Tissue

One month and three months after operation, three rabbits were sacrificed from each group, randomly. Each cartilage defect area was evaluated both macroscopically and histologically with hematoxylin and eosin (H&E) for cell morphology and Masson's trichrome for total collagen. To evaluate cartilage quality, a modified histological score according to Wakitani et al. [13] was used (Table 1).

Table 1. Modified histological score according to Wakitani et al. [13] for the defects.

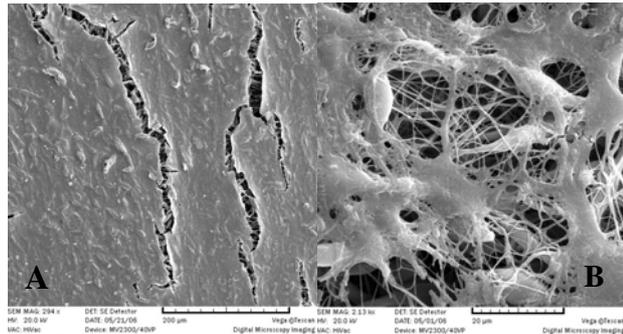| Category		Points	Category		Points
Cartilage formation	Hyaline cartilage	3	**Cartilage thickness**	>2/3	2
	Predominantly hyaline cartilage	2		1/3–2/3	1
	Thin hyaline cartilage	1		<1/3	0
	No cartilage	0	**Binding to articular cartilage**	Both edges integrated	2
Cartilage formation	Normal	3		One edge integrated	1
	Slightly reduced	2		Edges not integrated	0
	Predominantly reduced	1	**Reconstruction of subchondral layer**	Complete	2
	None	0		Incomplete	1
Surface characteristics	Smooth	3		None	0
	Moderate	2			
	Irregular	1	**Maximum of points**		15
	Highly irregular	0			

Results

SEM Examination of MSCs on PVA Scaffold

The morphology of PVA nonwoven mats, with and without cells were observed using Scanning electron microscope (SEM) (Figs. 3 and 4). We observed the scaffold allowed MSCs to infiltrate and lie in a palisade by SEM (Fig 4).


Figure 4. SEM micrographs of MSCs seeded on nanofibrous scaffolds:
A: attachment of anchorage-dependent mesenchymal stem cell on pore structure of nanofibrous scaffold after three days (2130 X); B: The scaffold surface is covered by a layer of attached cells, high magnification view of the cellpolymer constructs after a 3-week differentiation treatment (2940 ×).

Figure 3. SEM micrograph of the PVA nanofibrous structure (700 ×).

Differentiation of MSCs to Bone and Adipose Cells

Under specific culture conditions, the cells obtained in the described manner were able to differentiate into an osteoblastic lineage (Fig. 5A) and also into adipocytes (Fig. 6A).
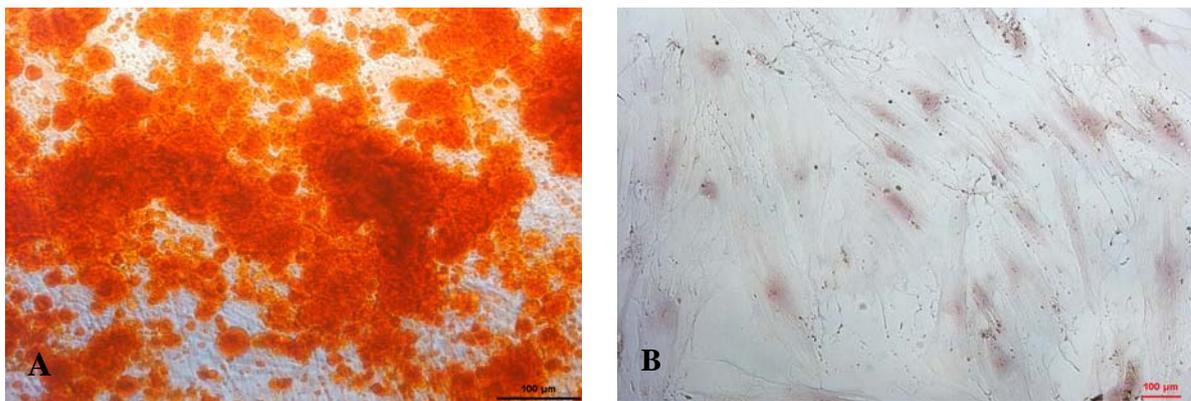
Figure 5. Differentiation of bone marrow MSCs into osteocytes. Alizarin Red staining was used to show differentiation of MSCs. A: The differentiated cells stain red. B: Undifferentiated MSCs are not permeable to Alizarin Red.
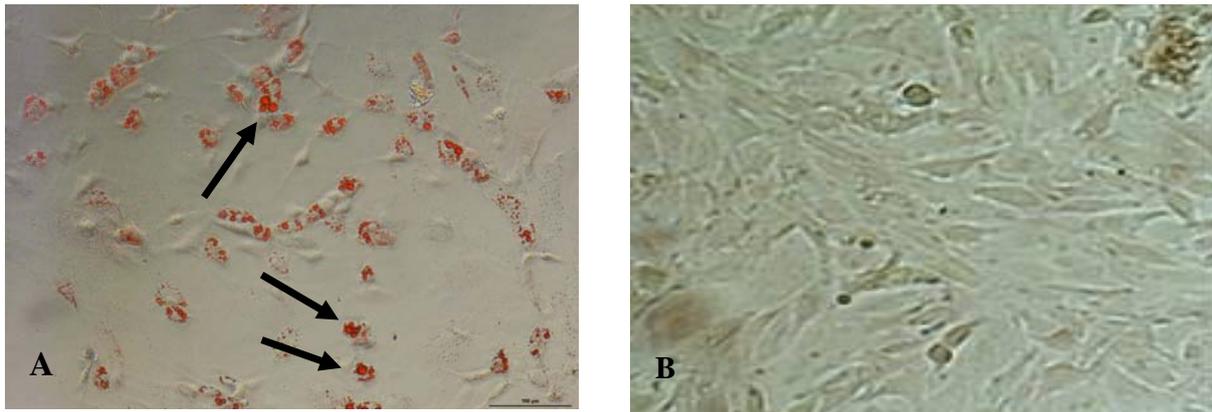
Figure 5. Differentiation of bone marrow MSCs into osteocytes. Alizarin Red staining was used to show differentiation of MSCs. A: The differentiated cells stain red. B: Undifferentiated MSCs are not permeable to Alizarin Red.

MSC Proliferation Assay

The cell number and viability were determined by using MTT assay (Fig. 7).

MSC Adhesion on the Scaffold

The sections of MSC seeded scaffolds after reaction with primary antibodies for Type II Collagen were incubated at room temperature for 1 h with FITC and PE goat anti-rabbit IgG as a secondary antibody. Type II collagen has been shown as red areas Under fluorescent microscope (Fig. 8).

Histological Score of the Repair Tissue

Repair of defects by MSC seeded scaffolds in comparison with control group was evaluated by microscopy at varying time points of 1 and 3 months. Modified score of Wakitani et al. was used to quantify the assessment. There were no significant changes at the site of the defect in one month duration in control group as small amount of fibrous and connective tissue was noted in the defect (Fig .9). There was no evidence of chondrocytes in newly formed tissue which had filled the defect even after 3 months in control group (Fig.10). Control group could not get any score after one month, but one point after 3 months (Fig. 15).
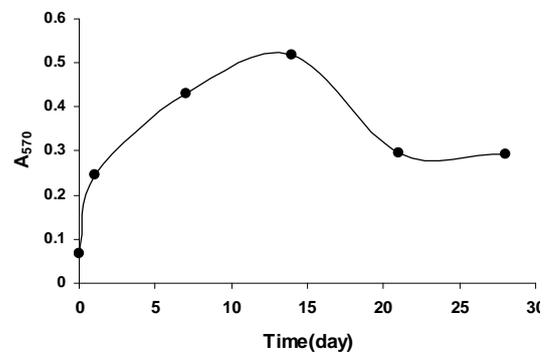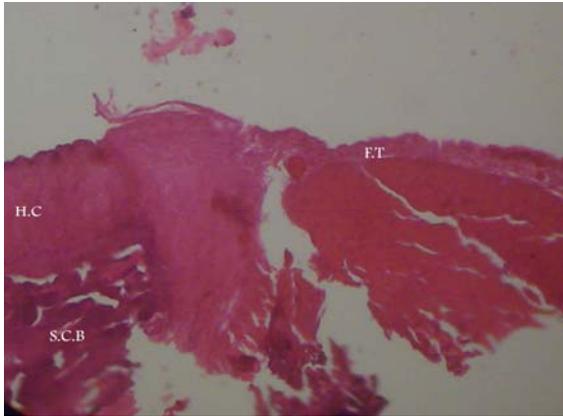

Figure 7. Cell proliferation of rabbit MSCs cultured on PVA scaffold in several days analysed by the MTT test.
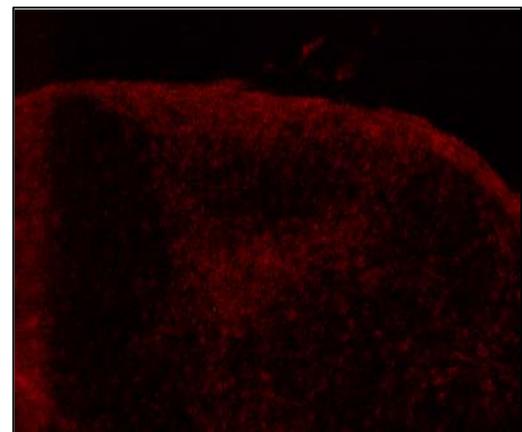

**Figure 8.** Type II collagen staining with PE shown as red areas (×100).

The observation of chondrocytes, collagen fibers and granulation tissue filling the defect were the major changes of attachment of PVA with cells a month after implantation (Fig. 11).


Figure 9. A part of damaged articular surface has been covered with connective tissue (FT) after one month in control group. HC: hyaline cartilage, SCB: subchondral bone. (H&E ×64).
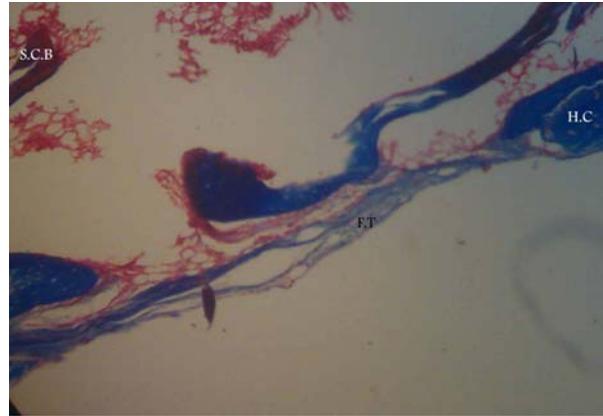
Figure 10. A part of original hyaline cartilage (HC) and subchondral bone (SCB) are seen, with fibrous tissues (FT) filling the defect of control group after three months (Trichrome ×64).
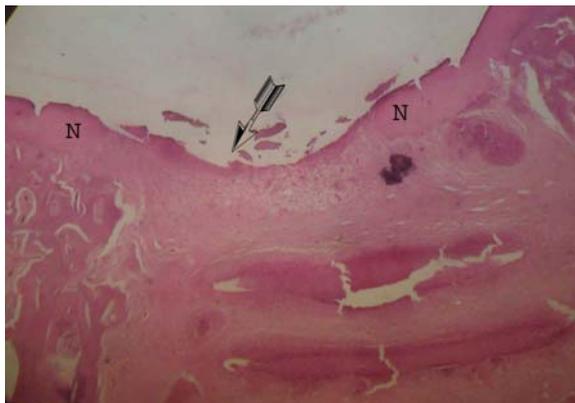

Figure 11. The defect area (arrow) between normal cartilages (N) was shown in experiment group after one month (H&E ×64).
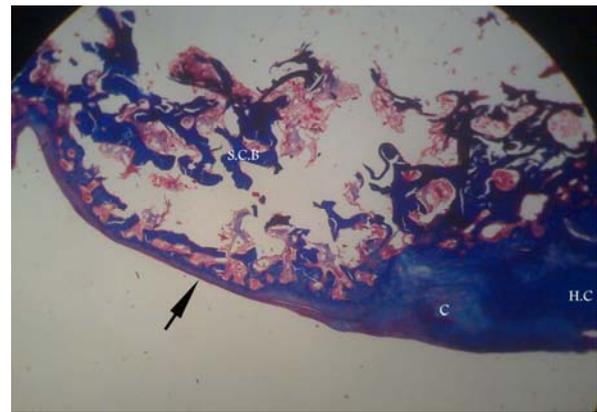

Figure 12. Experiment group after three months shows newly formed hyaline cartilage (arrow) and chondrocytes (C) in defect area (Trichrome ×64).

The presence of complete coverage of the defect with well spread hyaline cartilage and normal chondrocytes were noticed in the samples of implanted PVA with cells after lapsed of 3 months (Fig. 12). Mature chondrocytes, collagen fibers, connective tissue and also blood vessels were seen in experiment group after three months (Figs. 13 and 14). Defects treated with differentiated cell-seeded scaffolds after one month reached 9 points. Differentiated cell-seeded scaffolds with complete binding to original articular cartilage after three months reached on average 13 points (Fig. 15).
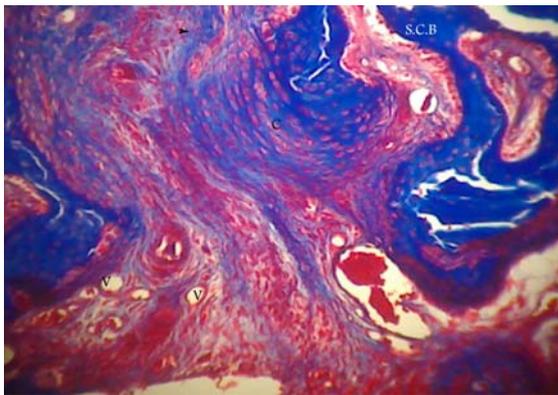
Figure 13. Higher magnification view of experimental group after three months shows collagen fibers (arrow), mature chondrocytes (C) and immature chondrocytes (PC) (Trichrome ×640).
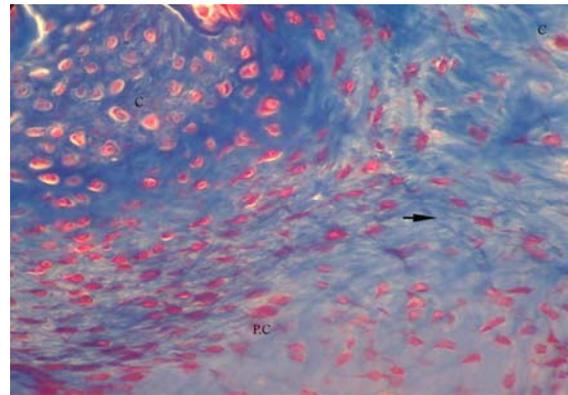
Figure 14. chondrocytes (C), blood vessels (V) and collagen fibers (arrow) are seen (Trichrome ×160).
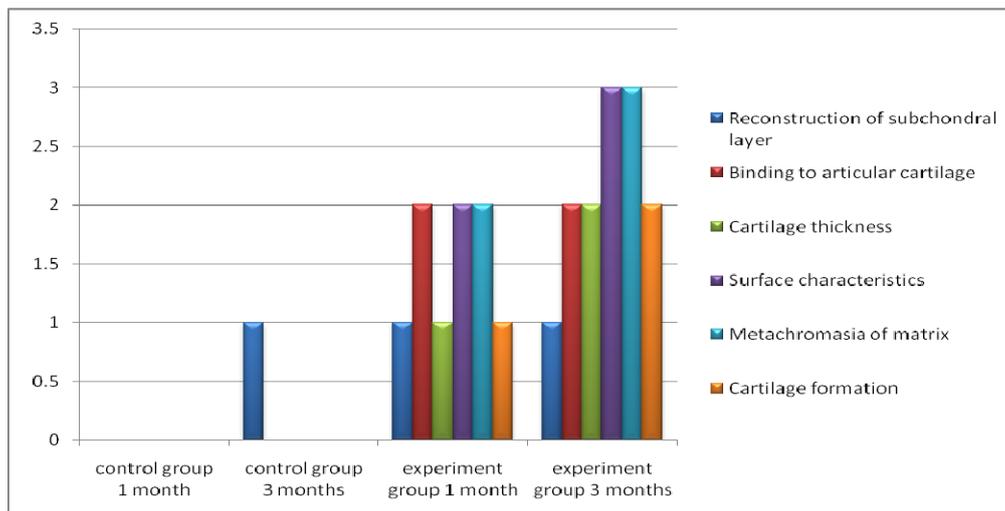
Figure 15. Results of histological scoring according to table 1.

Discussion

The present study showed that the articular cartilage defects were repaired with hyaline cartilage after implantation of differentiated MSCs-seeded PVA scaffold. Our results indicated PVA scaffold was able to provide necessary architectural support and cue for differentiation of MSCs and promote cartilage repair. Previously, PVA has been used successfully as articular layer of glenoid component.[14] Its non-toxicity, non-carcinogenicity and biocompatiblity[15] makes it a good candidate to be used as artificial implants in the body.

The main problem with the use of scaffolds is fixation of them into the defects. In a study by Uematsu et al.[16] poly-lactic-glycolic acid (PLGA) scaffolds were secured by fitting into the defects and applying a fibrin sealant. However, they reported in some cases, the scaffolds became detached from the defect and the percentage success was about 70%. Another study on

regeneration with periosteum fitted using a fibrin sealant reported that the percentage success was about 60%. PVA is a semi-crystalline hydrophilic polymer with good chemical and thermal stability.[8] PVA scaffolds are elastic materials having good mechanical strength to hold sutures.

In this study, the scaffolds were secured by suturing to the surrounding cartilage with an absorbable suture material. Our results showed no evidence of detachment of scaffolds from the defects; therefore, PVA scaffolds may be appropriate candidates to hold the cells in place for successful reconstruction of articular cartilage defects.

The ideal treatment of articular cartilage would replace damaged articular cartilage, restore joint function, and prevent the development of arthritis. Fibrocartilage is unable to withstand the high mechanical loads within a joint and only hyaline cartilage, which has predominantly type II collagen, has the potential for good, long term results. Transplantation of hyaline cartilage has been used for a number of years, but there are few sites where donor articular cartilage can be harvested without damaging the joint. Thus, only small articular defects can be treated with this method.[17] Cartilage tissue engineering techniques with taking small amounts of cells, and increase of their population, hold great promise for the treatment of cartilage defects; however, Successful application of this technology involves overcoming several problems regarding the utilization of the cells.[16] The choice of which cell type, chondrocytes or mesenchymal stem cells, is the first major concern on this line as each has advantages as well as disadvantages. Autologous chondrocyte grafting was developed by Brittberg and colleagues in Gothenburg.[18] In this technique, Cartilage from the margins of the knee joint is harvested by arthroscopy, and the cells are cultured for four weeks, after which they are transplanted into the damaged area. There are, however, several problems which limit efficacy of this technique. The cells may not survive and multiply in culture; the cartilage cells in culture may undergo dedifferentiation to fibroblasts;[19] and the fate of the implanted cells is uncertain.[20] Furthermore, reports on the long term results of regeneration using chondrocytes have shown calcification in the repair tissue, a lack of lateral and basal bonding and extensive fibrillation of the articular surface histologically.[21]

Mesenchymal stem cells (MSCs) are self-renewing progenitor cells that have the potential to differentiate into chondrocytes, osteoblasts, adipocytes, fibroblasts, and other tissue of mesenchymal origin.[22,23] MSC discovery opened new avenues for therapeutic approaches because of their inherent accessibility and repair capacities. Recently, several studies have investigated MSCs in combination with scaffolds for repair of cartilage defects.[4,16,24] Our findings provide supporting evidence that integration of a bony base to chondrocytes seeded into such polymer or constructs is mainly achieved, and this scaffold continues to support tissue elaboration (maintenance of the chondrocyte phenotype).

In our finding using PVA with cells on macroscopic examination, the transplanted tissue was compliant and difficult to be distinguished from native tissue and histomorphologic studies showed total resorption of the PVA scaffold at 12 weeks. A final concept that has come to bear is that cell–polymer constructs may play a role in nonphysiologic places and PVA scaffold may be useful in the design of such a different clinical model. This study brings up the ability to cultivate anatomically shaped tissue constructs aimed at the eventual replacement of the entire articular surface of diarthrodial joints. Furthermore, with the aim of new technologies, it is not unexpected to manufacture the cell-scaffolds compatible with the shape of defect area.

Acknowledgments

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چکیده

پوشش مجدد قسمتی از غضروف انتهای پایینی استخوان ران
با سلول های بنیادی کاشته شده بر روی داربست پلی وینیل الکلی

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هدف- هدف از این مطالعه ارزیابی زیست سازگاری سلول های بنیادی تمایز یافته بر روی داربست های پلی وینیل الکلی برای ترمیم ضایعات غضروف مفصلی انتهای پایینی استخوان ران می باشد.

طرح مطالعه- مطالعه تجربی در محیط بدن.

حیوانات- دوازده سر خرگوش سفید نیوزلندی نر بالغ به دو گروه شش تایی آزمایش و کنترل تقسیم شدند

روش کار- سلول های بنیادی مزانشیمی از مغز استخوان بازوی شش خرگوش گروه آزمایش جدا شده و کشت داده شدند و نهایتاً بر روی داربست های پلی وینیل الکلی به کندروسیت تمایز داده شدند. تصاویر میکروسکوپ الکترونی نگاره نشانگر توزیع یکنواخت سلول ها درون داربست بود. یک ضایعه غضروفی تمام ضخامت به قطر چهار میلیمتر در ناحیه مرکزی شیار کشککی هر دو زانوی تمامی خرگوشها ایجاد گردید. در گروه آزمایش، محل ضایعه با داربست حاوی سلولهای بنیادی مزانشیمی خودی پوشانده شده و توسط بخیه نگهدارنده با نخ پلی دیوکسانون شش صفر در محل خود تثبیت شد. خرگوشهای گروه کنترل بدون هیچ گونه درمانی رها شدند. پس از گذشت یک ماه، سه سر خرگوش از هر گروه بطور تصادفی انتخاب شده و جهت انجام نمونه برداری به روش انسانی معدوم شدند. بقیه خرگوشها سه ماه بعد مورد نمونه برداری قرار گرفتند. ارزیابی هیستوپاتولوژیکی ضایعات با رنگ آمیزی هماتوکسیلین و ائوزین و تری-کروم صورت گرفت.

نتایج- یافته ها نشان داد که در گروه غضروف مهندسی شده با داربست پلی وینیل الکلی سه ماه پس از انتقال، محل ضایعات بطور ماکروسکوپی با بافت صاف، براق و سفیدی پر شده بود. هر چند پس از سه ماه در گروه کنترل، در محل تخریش، مقدار زیادی بافت همبندی تشکیل شده بود، ولی اثری از کندروسیتها دیده نمی شد. در گروه آزمایش پس از سه ماه، محل نقیصه نسبتا با غضروف شفاف پر شده بود.

نتیجه گیری و کاربرد بالینی- بررسی مقاطع هیستوپاتولوژیکی در این مطالعه نشان می دهد که گروه داربست پلی وینیل الکلی با سلول کاشته شده، نسبت به گروه کنترل از میزان التیام بیشتری برخوردار بوده و در نتیجه می-توان اظهار کرد، امکان پوشش مجدد نقیصه غضروفی زانو با استفاده از سلول های بنیادی کاشته شده در داربست های پلی وینیل الکلی وجود دارد.

کلید واژگان- سلول های بنیادی، خرگوش، غضروف، نقیصه، پلی وینیل الکل.