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# Formation of repaired hyaline cartilage using PDGF-treated chondrocyte/PCL construct in rabbit knee articular cartilage defect

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**ABSTRACT**

*Articular cartilage is a specialized type of connective tissue which is populated exclusively with chondrocytes [1, 2]. The injured cartilage has a restricted capacity for self repair due to lack of vascularity and scarce number of cells [3], if the damaged cartilage is left untreated, over a period of time progresses to arthritis associated with pain, joint dysfunction and ultimately disability [4, 5]. The results indicates that in comparison to untreated and microdrilling methods in recovery of damaged cartilage, chondrocytes seeded onto PCL scaffolds and pretreated with PDGF has stimulatory effect on formation of hyaline cartilage-like tissue in in vivo condition. Future investigations are needed to evaluate PDGF precise effects on cartilage matrix components after in vivo transplantation.*

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## INTRODUCTION

Articular cartilage is a specialized type of connective tissue which is populated exclusively with chondrocytes [1, 2]. The injured cartilage has a restricted capacity for self repair due to lack of vascularity and scarce number of cells [3], if the damaged cartilage is left untreated, over a period of time progresses to arthritis associated with pain, joint dysfunction and ultimately disability [4, 5]. Conventional surgical procedures for treatment of articular cartilage damages includes abrasion, drilling, debridements, microfracture techniques or arthroscopic shaving that result in the formation of fibrocartilage with abnormal biochemical composition and biomechanical properties [6]. Implantation of *in vitro* manipulated chondrocytes into articular cartilage defects was first presented by Brittberg et al. [7]. This procedure which described as aotologous chondrocyte transplantation (ACT) involves cartilage harvesting, chondrocyte isolation, *in vitro* expansion and delivery of cells into defect site which is believed to result in hyaline-like cartilage formation suitable for normal joint functions [8].

For improving the chance of successful ACT, prolonging the *in vitro* redifferentiation capacity of chondrocytes is important [9]. The original ACT involved injection of cultured chondrocytes into the defect site and its covering by periosteal flap [10], which was insufficient due to lack of homogenous distribution of chondrocytes leading to dedifferentiation of cells that affects production of functionally and structurally competent cartilage extracellular

matrix [4, 11]. Further advances in tissue engineering introduced three-dimensional scaffolds which help to trap the cells and maintain chondrocyte differentiated phenotype [10]. Different scaffolding materials have been introduced for cell delivery in cartilage tissue engineering [8]. Polycaprolactic acid (PCL) is a synthetic biodegradable, biocompatible and semi-crystalline polymer which provides three-dimensional environment desirable for chondrocyte proliferation, differentiation, maturation and proteoglycan production [12]. PCL in the form of nanofiber sheets can be contoured to joint surface due to its flexibility, so it seems to use as an excellent candidate for cartilage tissue engineering [13].

Another factor with potency for augmentation in cartilage repair techniques is growth factor. Growth factors have been shown to support the maintenance of chondrocyte redifferentiation capacity and acceleration of cartilage formation and integration [11, 14]. Anabolic growth factors encourage cell migration into an injured site, increasing cell numbers and matrix production [15]. A number of growth factors like TGF-β, FGF, BMP and IGF, have been explored for their effects on cartilage tissue engineering [8].

Platelet derived growth factor (PDGF) has a positive mitogenic and chemotactic effect on mesenchyme derived cells, and its receptor has been identified also on chondrocytes [16]. The main reason for the usage of PDGF as a promoting factor for cartilage repair comes from the healing response in cartilage defects treated with microfracture. In this method, the formed clot in the defect site can provide an environment enriched with growth factors such as PDGF, exerting chemotactic and mitogenic effects [17]. PDGF has a direct effect on chondrocytes proliferation, differentiation and cartilage proteoglycan production and is thus believed to be able of enhancing tissue regeneration and repair [15, 16].

Although there are differences between human and animal tissues, for examining a novel treatment, a suitable animal model can be used as an important tool in enhancement of regenerative medicine. Furthermore, histological assessment of human articular cartilage after ACT is limited because biopsy for obtaining specimen may result in joint injury. In this study, we used rabbit which is a widely used animal model in study of tissue regeneration.

Regarding the importance of PDGF as promoting factor for cartilage healing, we designed this study to evaluate whether PDGF is able to stimulate transplanted constructs for producing a hyaline-like repaired tissue instead of fibrocartilage one and enhance the integration of chondrocyte/PCL complex implanted in the damaged knee articular cartilage in rabbits.

## MATERIALS AND METHODS

**2.1. Animals:** The ethical committee of Tabriz University of Medical Sciences (TUMS) approved all aspects of this study following laboratory practice guidelines. Twelve rabbits were purchased from veterinary faculty (Tabriz, Iran). Rabbits were 8-12 months and free of musculoskeletal disorders. During study, rabbits were caged individually in stainless steel cages and allowed forage and water ad libitum. They remained in their cages except when undergoing surgery or at the time of sacrifice.

**2.2. Growth medium and chemicals:** Growth medium (Ham`s F-12/Dulbecco`s modified Eagle`s medium (50/50) containing 10% fetal calf serum (FCS), 25 µg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 µg/ml amphotericin B, essential amino acids and L-glutamine) was obtained from Seromed (Munich, Germany). Collagenase and Trypsin/EDTA (EC 3.4.21.4) was purchased from Sigma (Munich, Germany). PDGF-BB was purchased from Acris (Hiddenhausen, Germany).

**2.3. Experimental design:** Rabbits were divided randomly into two groups. In group 1, the 4 mm diameter defects formed in left knees were kept empty during study period as untreated control, while in the right knee defect, microdrilling was performed. In group 2, right knee defect in each rabbit, received chondrocyte/PCL construct stimulated with 10 ng/ml PDGF-bb, while chondrocyte/PCL constructs without PDGF-bb stimulation were transplanted into left knee defects.

**2.4. Cartilage harvesting, chondrocyte isolation and expansion:** To obtain cartilage for allograft transplantation, articular cartilage prepared from healthy femoral condyle, femoral head and interchondylar groove of a 21 days old rabbit and processed in the lab under aseptic condition. Briefly, for chondrocyte isolation the cartilage samples were cut into 1–2mm thick slices and incubated by collagenase (0.5%/ Ham's-F12) (Sigma) in a shaking water bath at

37°C. The digested sample was centrifuged at 1000g/5 min and cells plated at $1x10^6$ cells per T75 flask at 37°C/5%$CO_2$. The first medium change was performed after 24 hours, and following medium changes performed three times per week, when reached 70% confluency, chondrocytes were passaged using trypsin/EDTA.

**2.5 Cell seeding into scaffolds:** Before being used, the scaffolds were cut into pieces of 4mm diameter discs using a biopsy punch and then sterilized by ultraviolet (UV) irradiation in polystyrene petri dish for 2 hours. The PCL scaffolds were prewetted in DMEM/F12 medium for 12 h in 37°C. Scaffolds (n=12) were then placed in a 24-well culture plate (1 scaffold per well). The second-passage cells were harvested by trypsinization and condensed to a concentration of $1 \times 10^6$/100µl. Cell suspension of 100 µl was dropped on the top of the scaffold to allow infiltration of the cells into the porous structure, and then 1 ml of DMEM/F12 medium containing 10% FBS was added carefully to each well. 10ng/ml PDGF-bb was added to 6 of chondrocyte/scaffold constructs. The chondrocyte/PCL constructs were incubated at 37°C/5%$CO_2$ for 4 days.

**2.6. Surgical procedure:** The surgical procedure was performed under general anaesthesia using aseptic techniques including limb preparing and draping. Anaesthesia was induced with intramascular injection of 5-10mg/kg ketamine and 5-10 mg/kg xylazine. No preoperative antibiotics were administered. In each rabbit, both lower limbs were shaved and disinfected using povidoine iodine. Both knee joints were opened via a lateral parapatellar skin incision and the patella was dislocated laterally to expose knee joint. A full-thickness cartilage defect (4mm in diameter, 1-1.5mm in depth) was created in the centre of interchondylar groove using a circular stainless steel punch, with care taken to avoid subchondral bone injury as confirmed by complete absence of bleeding (Fig. 1A). In the first group (untreated control), the left knee articular cartilage lesions were left without any intervention during study, while in the right knee (microdrilling), using a sterile lancet, small holes (approximately 0.5mm in diameter) were created into subchondral bone marrow under the cartilage defect to permit blood fills the defect site. For evaluation of PDGF-bb effects on cell proliferation and extra cellular matrix production, a total of $1x10^6$ chondrocytes stimulated with 10 ng/ml PDGF-bb during culture period seeded on PCL constructs, were implanted into defect sites in the right knee of second group, and the same chondrocyte/PCL constructs without stimulation by PDGF-bb were implanted in the left knee of the same group as revealed in figure 2. Using 6-0 absorbable coated vicryl suture material (Ethicon, W9552), chondrocyte/PCL constructs were fixed into lesion sites (figure 1B). The incision was closed with a layer of 4-0 absorbable suture at capsular joint, superficial fascia and skin were sutured with 4-0 nylon materials. The rabbits were allowed to move freely in their cages and followed up for a period of twelve weeks.

**2.7. Post-operative examination of defects**

***Gross examination***

Twelve weeks after the surgery and transplantation of chondrocyte/PCL constructs, animals were euthanized by over-dose injection of ketamine to retrieve articular cartilages. Macroscopic appearances of defect sites were examined and photographed. Visually acceptable repairs were noted as smooth, firm repair tissue that filled the defects.

***Histological evaluation***

The lower end of femurs were excised and fixed in 4% buffered formalin. To examine both sides of regenerative tissues attached to the native cartilage, each grafted area was dissected along the frontal plane. Specimens were decalcified in 10% nitric acid at least for 2 weeks then dehydrated and embedded in paraffin according to routine methods, sectioned and processed for routine hematoxylin-Eosin (H&E) and periodic acid Schiff (PAS) staining methods for evaluation of cartilage specific extra cellular matrix.

The quality of regenerated tissue in the articular cartilage defect in different groups was scored according to the International Cartilage Repair Society (ICRS) scale [18] (table 1). Each section was examined and scored separately by 3 independent researchers. Sections were graded according to: 1) surface continuity; 2) matrix staining with PAS; 3) integration of regenerative tissue with surrounding articular cartilage; 4) chondrocyte morphology; 5) cartilage thickness; 6) subchondral bone structure.

**Table 1: Modified histological scoring scale for evaluation of articular cartilage repair.**

| *Feature* | | *Score* |
|---|---|---|
| **Surface** | Smooth/continuous<br>Discontinuous/irregularities | 3<br>0 |
| **Matrix** | Hyaline<br>Mixture: hyaline/fibrocartilage<br>Fibrocatilage<br>Fibrous tissue | 3<br>2<br>1<br>0 |
| **Cell distribution** | Mixed/columnar-clusters<br>Clusters<br>Individual/organized Individual/disorganized | 3<br>2<br>1<br>0 |
| **Integration with surrounding cartilage** | Two edges<br>One edge<br>Without integration | 2<br>1<br>0 |
| **Cartilage thickness** | 2/3<br>1/3-2/3<br>< 1/3 | 2<br>1<br>0 |
| **Subchondral bone** | Normal<br>Increasing remodelling<br>Bone necrosis/granulation tissue<br>Detached/fracture/callus at base | 3<br>2<br>1<br>0 |

## RESULTS

### 3.1. Macroscopic results

Macroscopic examination of repair tissue, 12 weeks after surgery in untreated control group, showed that in one case, the defect was left as a dimple with a bony floor, in two cases, the defects were partially filled with relatively thin amorphous soft fibrous tissue that protruded into the joint (Figure 2, A) and in the others, defects were completely filled with a white and soft fibrous tissue.

In the second microdrilling group, two defects remained as dimple with exposed subchondral bone which were not filled by any tissue. Capsular joint attachment to the adjacent cartilage tissue also was observed in one defect, and the others were filled with fibrous whitish tissue as in the untreated group (Figure 2, B).

Macroscopic evaluation of the defects treated with chondrocyte/PCL constructs without PDGF-bb treatment revealed formation of a thin and firm cartilage-like tissue overlying the subchondral bone, which was not filled the entire lesion site (Figure 2, C).

Three articular cartilage defects, in which PDGF-bb treated chondrocyte/PCL constructs have been transplanted, were filled completely with cartilage-like (firm and glassy) tissue. The healed tissue appeared the same as surrounding cartilage (Figure 2, D). Others were filled partially with newly formed cartilage-like tissue.

### 3.2. Histological findings

Samples were graded according to 6 different histological and morphological criteria as shown in table 1, for qualification of newly formed cartilage repair tissue. Huge amount of fibrous tissue was evident in microdrilling and control groups (Figs. 3A, B and E); while in groups treated with cell/scaffold constructs, rare fibrocartilage tissue was present and the lesion sites were mostly filled with hyaline cartilage-like tissue (Figs. 3C and D). Surface architecture in transplanted knee lesions, in comparison to untreated control were filled with repaired cartilage tissue and appeared smooth or showed slight irregularities (Figs. 3C and D). In extracellular matrix, territorial and interterritorial matrices were visible in cell-based treated lesions, while in control and microdrilling groups, thick wavy collagen bundles were evident (Figs. 3A, B and F). Articular cartilage lesions filled with PDGF treated cell/scaffold constructs showed more round and columnar arranged chondrocytes compared with control and microdrilling groups (Fig. 3F). Poor merging of newly formed repaired tissue observed in cell/scaffold treated groups and it was similar in both with/without PDGF treated groups. The thickness of repaired cartilage tissue was also compared with the surrounding articular cartilage which was thinner in microdrilling and control groups due to

fibrous tissue formation (Figs. 3A and B). In subchondral bone examination, detachment and thinning was observed in underlying bone trabeculae.

**3.3 Quantitative findings**

The quantitative data were analyzed and compared using spss 16.0 statistical software by one-way ANOVA and post-hoc Tukey test. Evaluation of matrix properties in repaired tissue showed significant difference ($P<0.005$) between lesions treated with cell/scaffold constructs in the presence or absence of PDGF in comparison to untreated control and microdrilling groups (Fig. 4A). As shown in figure 4A, matrix scoring is higher in transplanted knee joints treated in the presence of PDGF compared to defects treated in the absence of it, but the difference is not meaningful. Surface architecture of newly formed tissue, scored higher in lesions treated with constructs in the presence of PDGF in comparison to other groups (untreated control, microdrilling and constructs without PDGF), but this difference was only significant ($P<0.01$) when compared to untreated control (Fig. 4B). Chondrocyte distribution was also scored in repaired tissues according to their columnar or cluster arrangement. Statistical analysis showed significant difference ($P<0.005$) in cell distribution in transplanted knees treated with cell/scaffold constructs with/without PDGF in comparison to untreated control and microdrilling groups (Fig. 4C). Integration of newly formed tissue with the surrounding articular cartilage was also evaluated and no significant difference was observed between different groups (Fig. 4D). Statistical evaluation of the repaired tissue thickness, showed that there is a meaningful difference ($P<0.01$) between lesions treated with constructs in the presence or absence of PDGF and those in untreated and microdrilling groups (Fig. 4E). Subchondral bone lying beneath the articular cartilage lesion sites was also evaluated in different groups. Statistical analysis showed higher scores in cell/scaffold transplanted knee defects with/without PDGF in comparison to untreated and microdrilling groups (Fig. 4F).

## DISCUSSION

In this study we examined if PDGF-bb treatment of chondrocytes seeded onto PCL scaffolds is able to accelerate chondrocyte extracellular matrix synthesis and integration of transplanted construct to the surrounding cartilage tissue. This study resulted in the following findings: 1) transplantation of chondrocyte/scaffold constructs in knee articular lesion site caused the formation of a newly formed hyaline cartilage-like tissue, 2) PDGF (10ng/ml) significantly promotes healing process in comparison to microdrilling and untreated control, 3) formation of fibrous connective tissue was observed in microdrilling and control groups.

Osteoarthritis (OA) and rheumatoarthritis (RA) occur as a result of an imbalance between synthesis and breakdown of ECM, and apoptosis in chondrocytes within articular cartilage that are consequences of mechanical and biological events [19]. Cytokines and growth factors are believed to have a pivotal role in the pathophysiology of OA [20]. Progression of osteoarthritis is regulated by proinflammatory cytokines which are associated with synovium, cartilage and subchondral bone [21]. Pro-inflammatory cytokines interfere with normal metabolic processes in cartilage. IL-1β is one of the main cytokines that has been implicated in the pathogenesis of degenerative joint diseases such as OA and rheumatoid arthritis (RA) [1, 22]. This cytokine induces the releases of matrix degenerative enzymes (i.e. matrix metalloproteinases (MMPs)) and inhibits the synthesis of extracellular matrix proteins in chondrocytes [23] and also induces chondrocyte apoptosis, which leads to further degenerative changes in cartilage [24]. Accordingly, for osteoarthritis treatment, the factors antagonizing these cytokines should be improved. Obtaining sufficient population of normal differentiated chondrocytes for filling the lesion site is a major challenge in cartilage transplantation technique. In this field, several researchers reported on beneficial effects of different growth factors on chondrocytes growth, extracellular protein synthesis and differentiation [25, 11]. In this regard, TGF-β isoforms, FGF, PDGF and IGF-1 are known to stimulate and augment chondrocyte proliferation and collagen synthesis [23].

Platelet derived growth factor (PDGF) is synthesized by diverse cell types, restored in platelet granules primarily and has a fundamental action in wound healing process [17]. Indirect evidence for PDGF application in cartilage lesion repair raised from microfracture technique, in which small holes are created in subchondral bone to permit blood containing a natural cocktail of growth factors, such as PDGF come into defect site, exert therapeutic effects and promote repaired tissue formation [26].

In the present study, we reported formation of newly formed hyaline cartilage-like tissue in defect sites covered with PDGF-treated chondrocytes seeded onto PCL scaffolds. Previous results from our group showed, PDGF-bb alone or in combination with IGF-1 up-regulated cartilage specific extracellular matrix proteins (β-1 integrin and

collagen type II) expression, and also can suppressed expression of proinflammatory cytokine and enzymes such as MMPs, Cox-2 and caspase-3 in monolayer and high density cultivated chondrocytes [27]. Other researchers reported increased chondrocyte proliferation in the presence of PDGF as evaluated by [$^3$H] thymidine uptake [28, 15]. PDGF also can stimulate proteoglycan synthesis in cultivated meniscal chondrocytes as reported by Imler et al. [29]. Treatment of human chondrocytes in monolayer and three-dimensional alginate beads with different growth factors also lead to increase in proliferation and proteoglycan synthesis by chondrocytes, which was significantly higher in PDGF-treated cells [25]. In an *in vivo* evaluation, PDGF- pretreated costal resting zone chondrocytes seeded onto PLG scaffolds were transplanted intramuscularly in nude mice and evaluated after 4 or 8 weeks. This evaluation reported less hyaline cartilage-like tissue formation after 4 weeks, but significant increase in cartilage amount and prevention of chondrocyte hypertrophy in PDGF-pretreated chondrocytes after 8 weeks [16]. It is possible that chondrocyte stimulation by PDGF can result in formation of hyaline cartilage-like tissue compared with surrounding area.

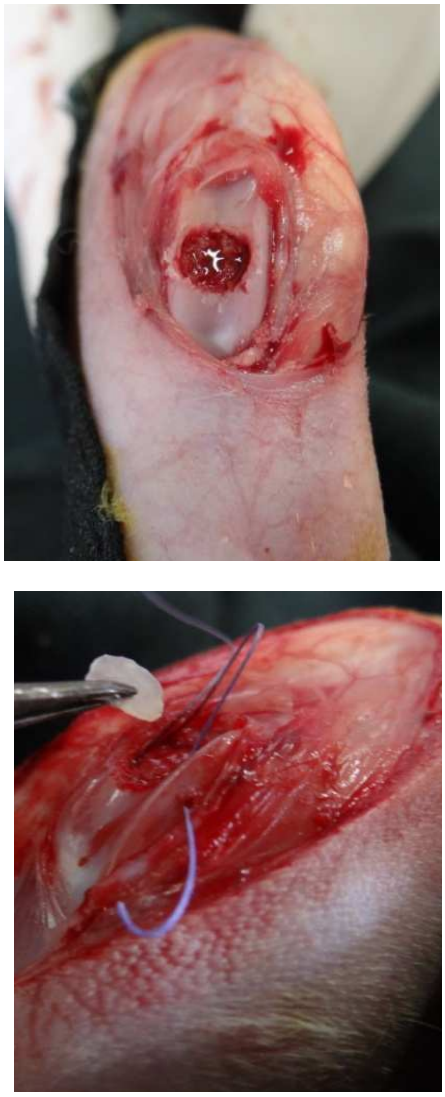

**Figure 1: A: Creation of a lesion with 4mm diameter in articular cartilage. B: Transplantation of chondrocyte/PCL scaffold in cartilage lesion site.**

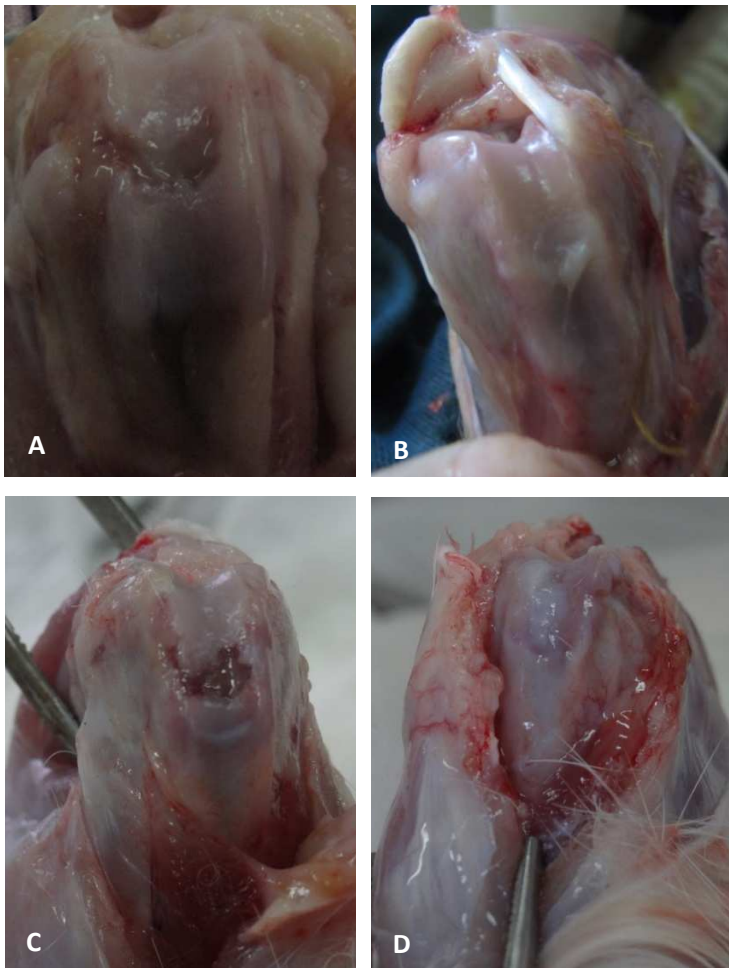


**Figure 2: Post-surgical evaluation of knee joints after 12 weeks. (A) control group, (B) microdrilling group, (C) chondrocyte/scaffold construct transplanted group and (D) PDGF-treated chondrocyte/scaffold transplanted group.**

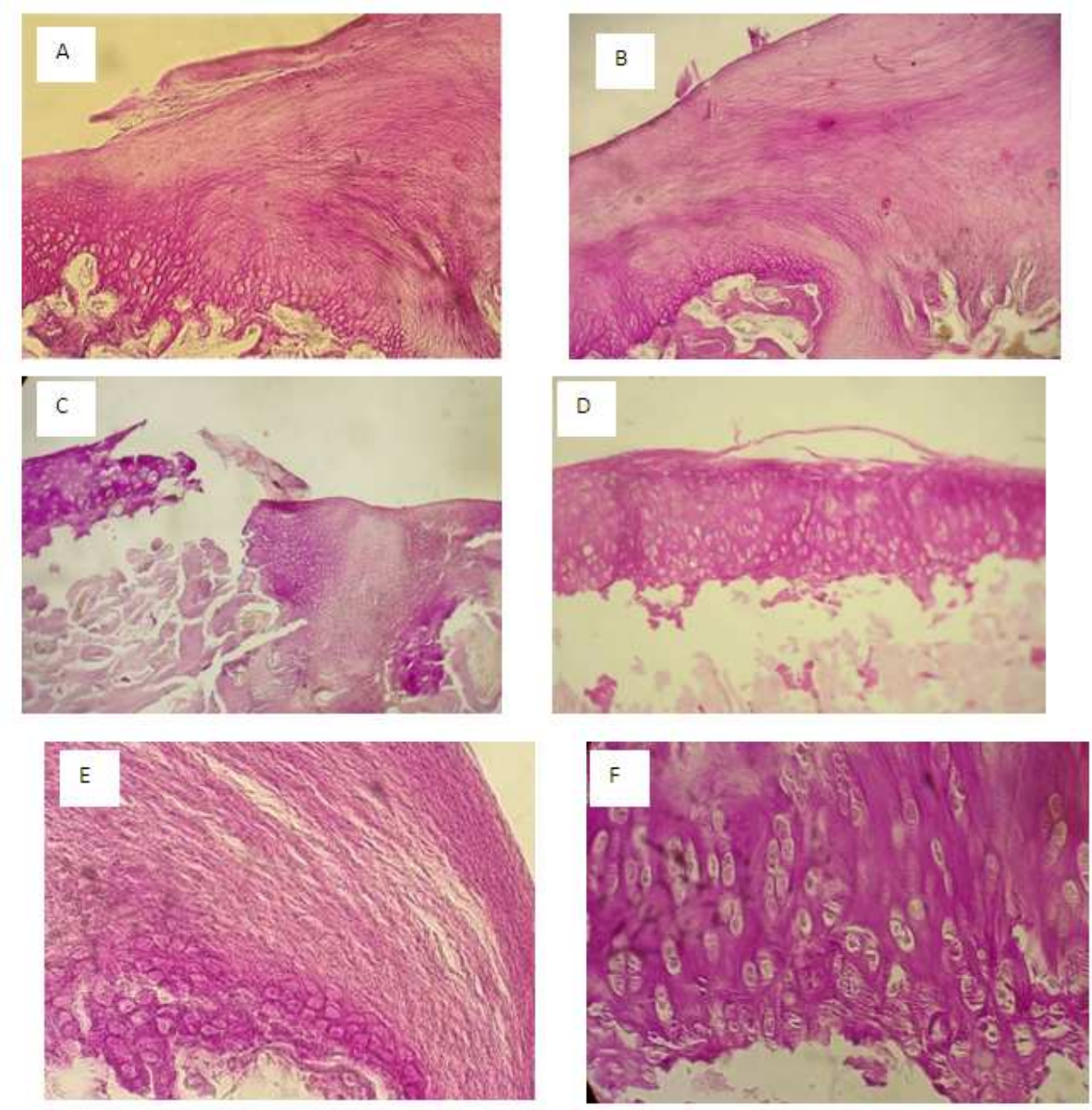


**Figure 3: A) fibrous tissue filled lesion site in untreated control group, chondrocyte clusters are evident at the peripheral tissue (100X). B) Defect site in microdrilling group shows huge amount of fibrous tissue with a thin fibrocartilage formed at the bottom (100X). C) Repaired cartilage tissue formed in cell/scaffold treated defects (100X). D) Hyaline cartilage-like tissue newly formed in defect site (100X). E) Higher magnification of huge amount fibrous tissue formed at lesions treated with microdrilling technique (400X). F) Columnar arranged chondrocytes surrounded by territorial and interterritorial matrices filled the defect site in transplanted knee with PDGF-bb treated chondrocyte/scaffold construct (400X).**

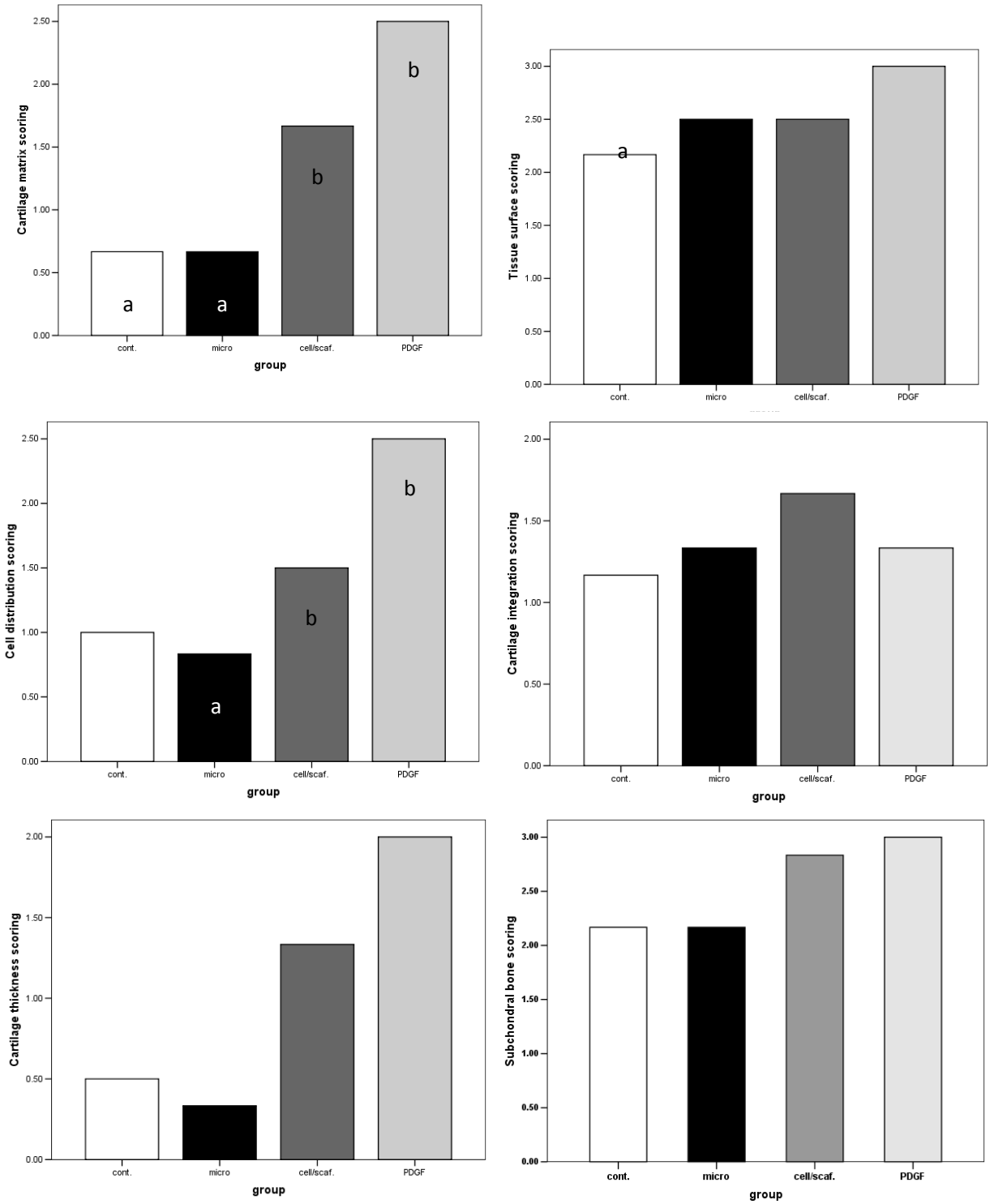


**Figure 4: Quantitative evaluation of repaired tissue properties in different groups.**

*The columns with different letters are significantly (P<0.01) different from each other.*
*The columns with similar letters or without letters are not different from each other.*
*The columns with a letter are not different from columns having no letter.*

## CONCLUSION

The results indicates that in comparison to untreated and microdrilling methods in recovery of damaged cartilage, chondrocytes seeded onto PCL scaffolds and pretreated with PDGF has stimulatory effect on formation of hyaline cartilage-like tissue in *in vivo* condition. Future investigations are needed to evaluate PDGF precise effects on cartilage matrix components after in vivo transplantation.

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