Surface Morphology and Stiffness of Cartilage-Like Tissue Repaired with a Scaffold-Free Tissue Engineered Construct[*]

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Abstract

We have been developing a new tissue engineering technique for cartilage repair using a scaffold-free tissue engineered construct (TEC) bio-synthesized from synovium-derived mesenchymal stem cells (MSCs). A round-shaped chondral defect of 8.5 mm in diameter and 2.0 mm in depth created on the medial condyle of immature (4-month-old) and mature (12-month-old) porcine femur was filled with the TEC. Six months after surgery, a cylindrically shaped specimen of 4 mm in diameter and 4-5 mm in depth was extracted. Stiffness measurements were carried out on the specimen using an AFM after the surface image of the specimen was obtained. The TEC-treated tissues exhibited more irregular surface as compared with normal cartilage, regardless of animal maturity. The stiffness of the superficial layer of the TEC-treated tissue was significantly lower than those of the normal cartilage, indicating 6.8±2.3 mN/m in the immature group and 8.8±2.3 mN/m in the mature group. Histological observation indicated that the defects were repaired with a hyaline cartilage-like tissue with positive Safranine O staining, regardless of animal maturity. However, the superficial layer of the repaired tissues was negatively stained with Safranine O. The present study suggests that the recovery of the superficial layer is delayed in both immature and mature animals although the treatment with TEC enhances the repair of partial chondral defects.

Key words: Cartilage Repair, Mesenchymal Stem Cell, Tissue Engineered Construct (TEC), Scaffold Free, Atomic Force Microscope (AFM), Stiffness, Surface Morphology

1. Introduction

Important biomechanical functions of articular cartilage are load bearing property and lubrication property. Articular cartilage bears numerous numbers of cyclic load applications for a long period while keeping the frictional coefficient at a negligible level. However, once degenerative disease or physical damage occurs in articular cartilage, such functions immediately deteriorate and are not be restored due to the limitation of healing capacity [(1)]. To solve the problem, various autologous chondrocyte-based techniques using

synthetic or animal-derived scaffolds have been developed for the repair of full-thickness articular cartilage [2]-[4]. However, this technique may have limitations including the sacrifice of undamaged cartilage tissue within the same joint. In addition, the scaffolds are frequently used for effective cell delivery although a long-term safety and efficiency of such scaffolds still remain unclear.

To overcome such potential problems as regard with autologous cell transplantation in conjunction with the scaffolds, stem cell-based therapies have been developed for a better cartilage repair. Mesenchymal stem cells (MSCs) isolated from synovial membranes have a strong capability of chondrogenic differentiation [5]. We have been developing a new tissue engineering technique for cartilage repair using a scaffold-free tissue engineered construct (TEC) bio-synthesized from allogenic synovial MSCs as a potential MSCs-based therapeutic method [6]-[9]. The TEC is composed of MSCs with their native extracellular matrix (ECM), we believe that it is free from concern regarding long term immunological effects. Our previous studies using immature and mature porcine models indicated that the TEC successfully repaired chondral defects by producing a hyaline cartilage-like tissue without any immunological reaction [7]-[9]. Moreover, quasi-static compressive properties of the cartilage-like tissue were close to those of normal cartilage regardless of animal maturity [7]-[9]. However, TEC-treated repaired tissue exhibited slightly larger creep deformation in response to static and dynamic loading as compared with normal cartilage, which was probably attributable to a higher permeation of interstitial fluid in the tissues [9]. Our previous study indicated that the superficial layer of repaired cartilage-like tissue from immature porcine femur exhibited a significantly higher permeability as compared with normal cartilage [10]. Moreover, the superficial layer of the tissue was negatively stained with Safranine O, which indicated low maturity of proteoglycan [7]-[10]. Thus, our previous studies implied that the mechanical and biological properties of the superficial layer of the TEC-treated tissues were deteriorated although the properties of entire tissues were comparable to those of normal cartilage. For better understanding and improving of the cartilage repair technique, it is required to precisely investigate the structure and mechanical properties of the superficial layer of the TEC-treated cartilage-like tissues.

The atomic force microscope (AFM) is a great tool to investigate the surface morphology and elastic modulus of biological samples in nano-scale resolution. Recently, a number of studies have used AFM to determine the surface morphology and mechanical properties of the superficial layer of articular cartilage [11]-[17]. Therefore, the objective of the present study was to perform an atomic force microscopic analysis on the surface morphology and stiffness of cartilage-like tissues repaired with the TEC.

2. Materials and Methods

2.1 Isolation of the cells

The cell isolation protocol was according to that previously described [6]. Briefly, synovial membranes obtained from 3-4 months old porcine femurs were rinsed with phosphate buffered saline (PBS), minced meticulously, and digested with 0.1% collagenase IV (Sigma, St. Louis, MO, USA) for 1.5 h at 37 °C. After neutralization of the collagenase with growth medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and penicillin/streptomycin (Gibco BRL, Life Technologies Inc., Rockville, MD, USA), the cells were collected by centrifugation, washed in PBS, resuspended in growth medium, and plated in culture dishes. For expansion, cells were cultured in the growth medium at 37 °C in a humidified atmosphere of 5% CO_2. The medium was replaced once a week. After 15-28 days of primary culture, when the cells reached confluence, they were washed twice in PBS,

harvested by the treatment of trypsin-EDTA (0.25% trypsin and 1 mM EDTA: Gibco BRL, Life Technologies Inc., Rockville, MD, USA), and replated at 1:3 dilution for the first subculture. The above-described procedure was repeated 4-7 times for cell passage in the present study.

2.2 Development of TECs

Obtained cells were plated on culture dishes at a density of 4.0×10^5 cell/cm^2 in growth medium containing 0.2 mM ascorbate-2-phosphate (Asc-2P), and then the cells became confluent within a day. After an additional culture for 7 days, a complex of the cultured cells and the ECM produced from the cells was detached from the substratum by application of shear stress using gentle pipetting. The detached monolayer complex was left in suspension to form a three-dimensional structure by natural contraction. This tissue was termed a scaffold-free three-dimensional TEC (Fig.1).
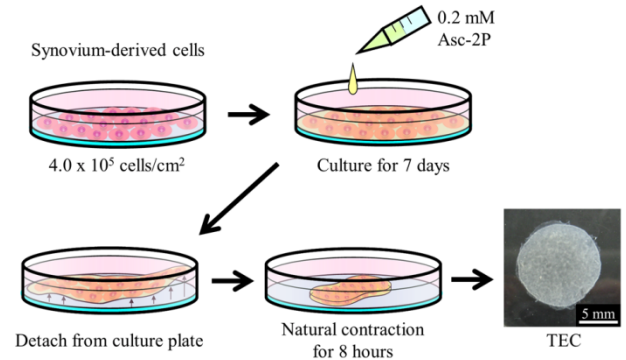

Fig.1 Production method of the TEC.

2.3 Preparation of specimens

A round-shaped chondral defect of 8.5 mm in diameter and 2.0 mm in depth which did not reach the subchondral bone were created on the load bearing area of the medial chondyle of immature (4-month-old) and mature (12-month-old) porcine femurs. A TEC mass of a similar size to the defect was allo-grafted into the defect without any surgical fixation (TEC-treated group).
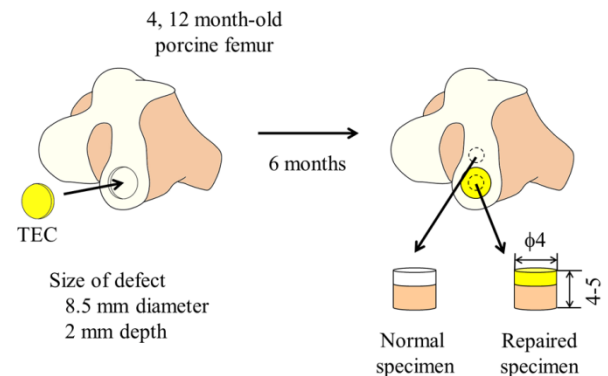

Fig.2 Implantation of the TEC to a porcine chondro defect and extraction of specimens.

For comparison, a chondral defect was created on the medial chondyle of other porcine femurs in the identical manner to the TEC-treated group, but the defect was left empty (TEC-untreated group). All animals were immobilized for 7 days, and allowed to move in cages. Six months after surgery, cylindrically-shaped, repaired tissue-subchondral bone specimens of 4 mm in diameter and 4-5 mm in depth were extracted from the center of the defect. In addition, normal cartilage-subchondral bone specimens of the dimension identical to that of the repaired tissues were also extracted from the load bearing area of the femoral chondyles adjacent to the repaired sites (Fig.2).

2.4 Histological observation

Repaired specimens (n=1 for each group) were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), decalcified with EDTA, and embedded in paraffin. The tissues were longitudinally sectioned to 4 mm thickness in the sagittal plane, and stained with Safranine O.

2.5 AFM analysis

AFM image capture and indentation were carried out using the MultiMode scanning probe microscope (Nanoscope IIIa; Veeco Instruments, Santa Barbara, CA, USA) with silicon nitride probes of a spring constant of 0.06 N/m (DNP-S, Veeco Instruments, Santa Barbara, CA, USA). The specimens were mounted on the sample stage of the AFM and soaked in saline solution at room temperature.

The surface image of the specimen was obtained through a contact mode at a scan area of 30 x 30 μm and scan rate of 0.3 Hz. The mean surface roughness (R_a) was calculated from the surface image, using a Nanoscope IIIa software, by averaging of height deviations over the 30 x 30 μm scan area (256 x 256 pixels), with the following equation:

$$R_a = \frac{\sum_{i=1}^{N}|Z_i|}{N},$$

where Z_i is represents the height deviation from the mean plane in a pixel and N is the number of pixels over the scanned area.

Stiffness measurements were carried out on the repaired tissue and normal cartilage through a nano indentation mode of the AFM. The force-indentation depth curves were obtained for the surface of the specimens at an indentation rate of 1 Hz (5.12 μm/s). The stiffness was calculated from the slopes of force-indentation depth curves in 300-400 nm of depth.

2.6 Statistical analysis

The results are presented as mean±SD. Statistical analysis for surface roughness and stiffness were performed using t-test and significant level was set at $p<0.05$.

3. Results

3.1 Histological observation

At 6 months after implantation, the TEC-untreated groups showed an evidence of osteoarthritic changes with loss of cartilage and erosion of subchondral bone in both immature and mature groups (Fig.3C and D). In contrast, in the TEC-treated groups, the defects were repaired with a hyaline cartilage-like tissue with positive Safranine O staining, regardless of animal maturity (Fig.3A and B). However, the superficial layer of the repaired tissues, negatively stained with Safranine O, was thicker than those of the adjacent normal cartilage in the TEC-treated groups. The thickness of the superficial layer was 0.03-0.05 mm in normal cartilage in both immature and mature groups, while that was 0.35 mm and 0.17 mm in immature and mature groups, respectively in the TEC-treated groups.
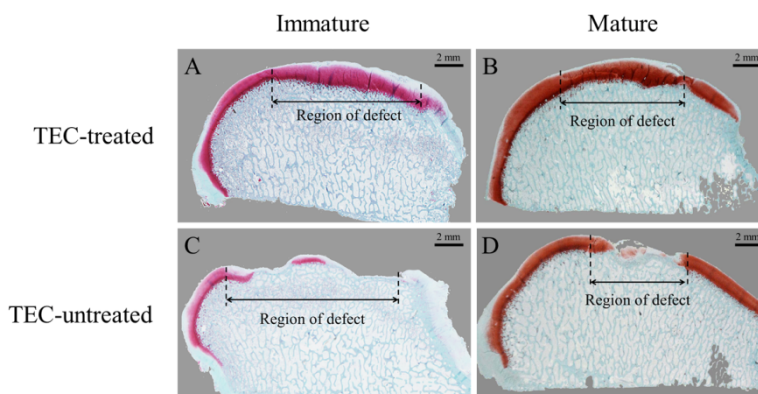

Fig.3 Safranine O staining of TEC-treated (A, B) and TEC-untreated tissues (C, D) (A, C: Immature groups, B, D: Mature groups).

3.2 AFM analysis

The surface morphology of normal cartilage showed consistently smooth surface both in immature and mature groups (Fig.4A and B). The TEC-treated and TEC-untreated groups seemed to be more irregular surface, regardless of animal maturity (Fig.4C-F). The surface roughness of the TEC-treated groups (297.9±161.3 nm and 415±131.5 nm in immature and mature groups, respectively) were slightly lower than those of TEC-untreated groups, regardless of animal maturity (353.9±61.7 nm and 557.5±17.9 nm in immature and mature groups, respectively) (Fig.5).
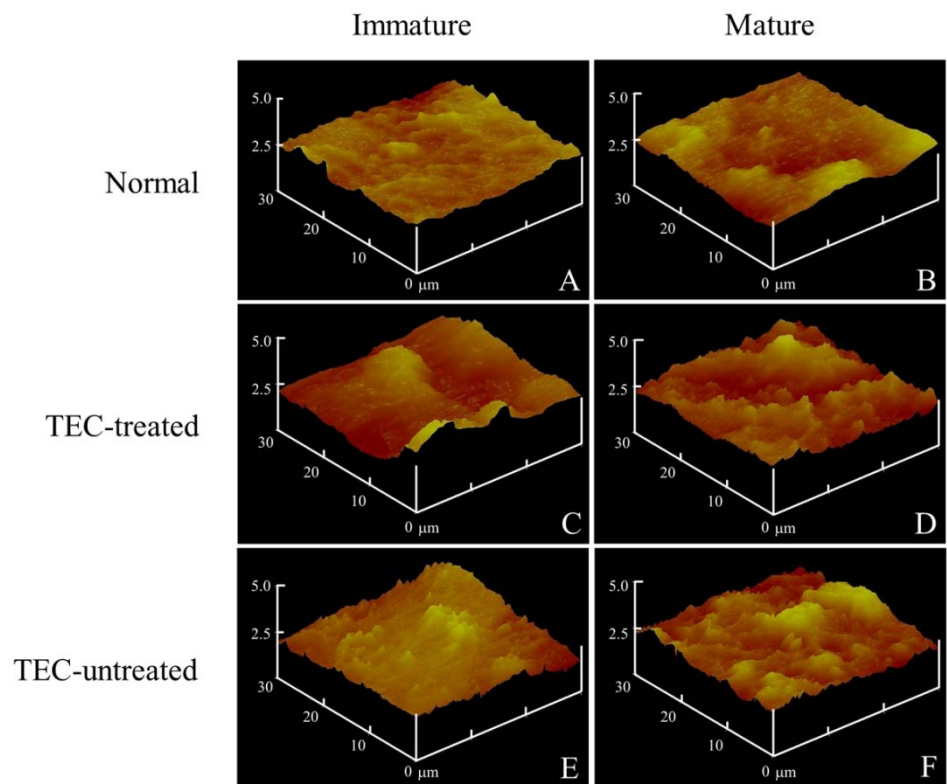

Fig.4 Surface morphology of normal cartilage (A, B), TEC-treated (C, D), and TEC-untreated tissues (E, F) (A, C, E: Immature groups, B, D, F: Mature groups).
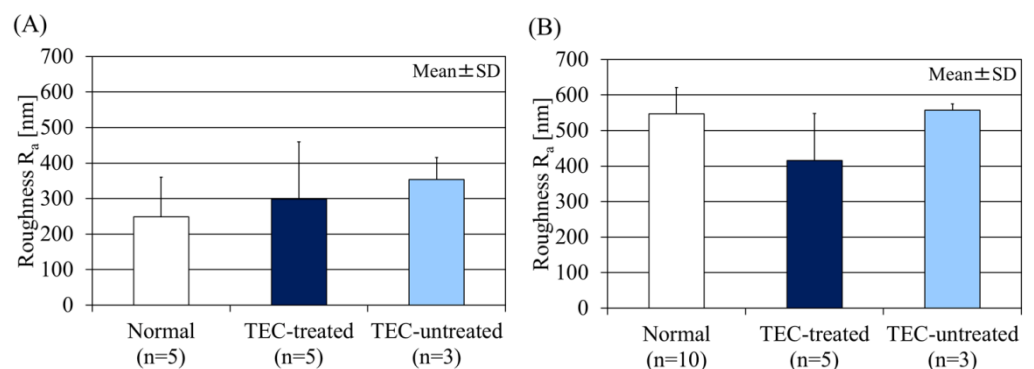

Fig.5 Surface roughness of normal cartilage, TEC-treated, and TEC-untreated tissues (A: Immature groups, B: Mature groups).

The typical indentation curves of normal cartilage and repaired tissues in the indentation test are shown in figure 6. The normal cartilage exhibited non-linear, J-shaped responses in the force-indentation curves in both the immature and mature groups; in

particular, the slopes of the curves gradually increased in the indentation range less than 250 nm, and rapidly increased in the indentation range more than 200 nm in the immature group. In contrast, both the TEC-treated and TEC-untreated tissues exhibited almost linear responses in the indentation range up to 600 nm. The stiffness of the superficial layers of normal cartilage in the immature and mature groups were 23.8 ± 1.7 mN/m and 15.0 ± 6.0 mN/m, respectively (Fig.7). The stiffness of the superficial layer of the TEC-treated group was significantly lower than those of the normal cartilage, indicating 6.8 ± 2.3 mN/m in the immature group and 8.8 ± 2.3 mN/m in the mature group. The TEC-untreated groups indicated slightly larger stiffness as compared with the TEC-treated groups in both the immature and mature groups although no significant difference was observed.
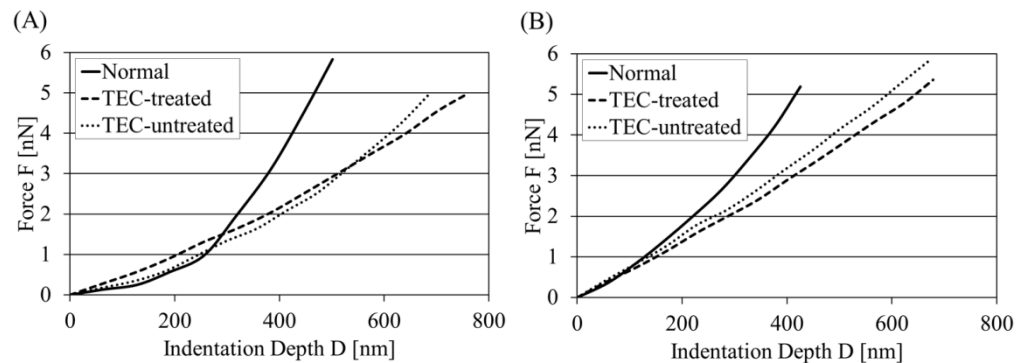

Fig.6 Typical indentation curves of normal cartilage, TEC-treated, and TEC-untreated tissues (A: Immature groups, B: Mature groups).
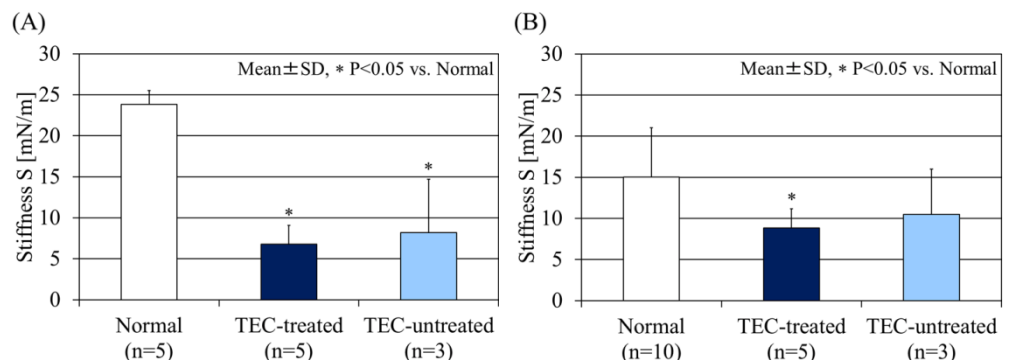

Fig.7 Surface stiffness of normal cartilage, TEC-treated, and TEC-untreated tissues (A: Immature groups, B: Mature groups).

4. Discussion

In experiments as regard with cartilage repair, cell-seeded scaffolds have been often used. Wakitani et al. implanted MSCs-seeded collagen gel into a square-shaped, full thickness cartilage defect of 6 x 3 mm in the rabbit femur [18]. They found that the repaired tissue consisted of hyaline cartilage stained with toluidine blue at only two weeks after implantation. Schaefer et al. implanted a composite of chondrocyte-seeded scaffold and an osteoconductive sponge into a square-shaped, osteochondral defect of 7 x 5 mm in the rabbit femur [19]. Six months after implantation, Young's modulus of repaired cartilage (0.80 MPa) recovered closely to that of normal cartilage (0.84 MPa). In those studies, cartilage repair has been successfully achieved. However, it is concerned that a long-term safety and efficiency of animal-derived or synthetic scaffolds still remain unclear. Our previous studies demonstrated that the TEC successfully repaired the chondral defects and compressive property of the cartilage-like tissue recovered close to that of normal cartilage regardless of animal maturity [7]-[9]. However, the superficial layer of the tissue was negatively stained with Safranine O. For improving the cartilage repair technique, it is required to precisely

investigate the morphology and mechanical properties of the superficial layer of the TEC-treated cartilage-like tissues.

Many investigators used AFM to determine the surface morphology and elastic modulus of superficial layer of normal articular cartilage [11]-[17]. Chan et al. used an AFM apparatus and silicon nitride probe (MSCT-AUNM, Veeco instruments, Santa Barbara, CA, USA) to perform a micro-indentation test on the medial chondyle of bovine femoral cartilage[17].
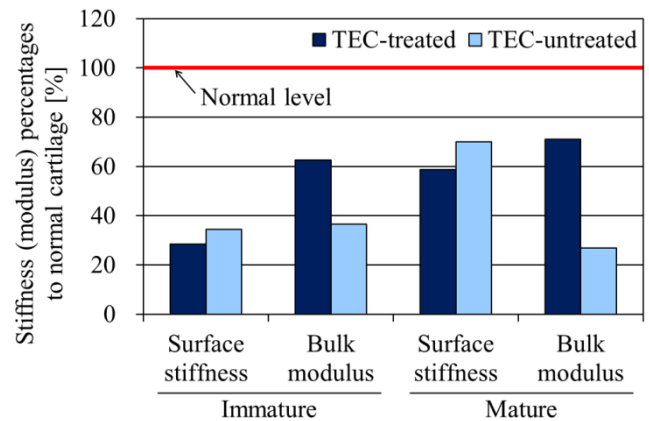

Fig.8 Stiffness (modulus) percentages of the TEC-treated and the TEC-unterated tissues to normal cartilage.

They reported that the stiffness of the cartilage surface was 28.6 ± 0.1 mN/m and 15.8 ± 0.1 mN/m in the load-bearing area and non-load-bearing area, respectively. Their results agree well with the result of immature normal cartilage (23.8 ± 1.7 mN/m) obtained in the present study. However, the AFM has been hardly used so far to determine the morphology and mechanical properties of repaired cartilage tissues in previous biomechanical studies. The present study indicated that the surface morphology of the cartilage-like tissue repaired with the TEC was different from normal cartilage. Moreover, the surface stiffness of the repaired cartilage was softer than the normal cartilage regardless of the treatment of the TEC. The stiffness (modulus) percentage of the TEC-treated and the TEC-untreated cartilage-like tissues to those of the normal cartilage are indicated in Figure 8. The results of macro-scale unconfined compression data were adapted from our previous reports [7]-[9]. Unconfined compression tests in our previous studies showed that the bulk modulus of the TEC-treated cartilage-like tissue was higher than that of the TEC-untreated tissue and increased with no significant difference against normal cartilage [7]-[9]. Taking into account the previous findings, the recovery of the superficial layer is delayed in both immature and mature animals although the treatment with TEC enhances the repair of partial chondral defects. This agrees well with a finding by Imura et al. that the permeability of the TEC-treated cartilage-like tissues recovered to normal level for 6 months except the superficial layer [10]. The histological observation indicated that the TEC-treated cartilage-like tissue was hyaline cartilage-like at intermediate-to-deep area but remained fibrous cartilage-like at the superficial layer. Nansai et al. indicated that the integration strength of cartilage-like repaired tissue to surrounding host cartilage was depth-dependently increased in the TEC-treated cartilage-like tissue [20]. These results suggest that the repair process of the cartilage-like tissue is promoted by the TEC in conjunction with extrinsic factors possibly permeated from the bottom of the chondro-defect. This may be a reason for the delay of repair in the TEC-treated tissues. For further improvement of cartilage repair technique using the TEC, a novel strategy may be required to enhance the mechanical and morphological properties of the superficial layer.

In summary, we performed an atomic force microscopic analysis on the surface morphology and stiffness of cartilage-like tissues repaired with scaffold-free, tissue engineered construct (TEC) bio-synthesized from allogenic synovial mesenchynmal stem cells, and obtained the following conclusions.

(1) As compared with normal cartilage, the surface roughness of the TEC-treated cartilage-like tissues is slightly higher and lower in immature and mature animals, respectively.

(2) The stiffness of the superficial layer of the TEC-treated repaired tissue is significantly lower than that of the normal cartilage, regardless of maturity.

(3) The repair speed of TEC-treated tissue is depth-dependent; the recovery is slower in the superficial layer than in bulk tissue.

Acknowledgment

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