Pharmaceutical Sciences

Cartilage tissue engineering: intraarticular adipose stromal cells promote chondrocyte attachment on silk fibroin scaffold

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Articular cartilage has limited repair and regeneration potential; the scarcity of treatment modalities has motivated attempts to engineer cartilage tissue constructs. The use of chondrocytes in cartilage tissue engineering has been restricted by the limited availability of these cells, their intrinsic tendency to lose their phenotype during the expansion, as well as the difficulties during the first cell adhesion to the scaffold. Aim of this work was to evaluate the attachment of adipose stromal vascular fraction from human Hoffa's Body knee fat on silk fibroin scaffold as a strategy for increasing chondrocyte adhesion and proliferation. Results show that silk fibroin scaffold allows cell attachment and scaffold colonization; moreover, adipose-derived stromal vascular fraction cells promote chondrocyte adhesion.

1 Introduction

Silk, produced by the silkworm of *Bombyx mori*, is composed of two proteins: fibroin and sericins. Besides its widespread employ in textiles, fibroin is also known for its use in several biomedical fields as suture material and as scaffold constituent in tissue engineering, in various forms, including films, hydrogels, non-woven mats, nets or membranes and 3D porous sponges [1].

The in vitro engineerized articular cartilage for transplantation has been in clinical use from many years: autologous chondrocyte were propagated in vitro and transplanted (ACI) [2,3]. During proliferation in monolayer conditions, however, chondrocytes dedifferentiate and cease producing the mechanobiologically indispensable matrix proteins: collagen type II, aggrecan and others. Therefore, a new operation procedure of matrix-associated autologous chondrocyte transplantation (MACT) has been developed: cells are seeded on scaffold in order to improve extracellular matrix production, to prevent chondrocyte dedifferentiation or to promote chondrocyte re-dedifferentiation. Moreover, scaffolds are useful for the immobilization of the cells, for a broader distribution of the cells in the defect, and for facilitating the handling during surgery: the scaffold architecture, structure and composition can dramatically influence cell adhesion, distribution, proliferation and differentiation cell-scaffold graft [4]. The use of mesenchymal stem cells has recently been proposed to improve this therapeutic approach since they represent a population of multipotential cells able to regenerate cartilage and are easier to obtain compared to chondrocyte cells. Recently, adipose tissue was identified as a source of mesenchymal stem cells [5]. Independent research groups have been studied yield and proliferation/differentiation potential of these cells respect to adipose collection sites [6,7]. Autologous mesenchymal stem cells are also an optimal support for nucleus pulposus cells, to enhance intervertebral disc regeneration, to reduce the risk of rejection, and to promote the scaffold colonization or extra cellular matrix production [8,9]. For cell therapy and regenerative medicine use, stem cells are separated from their natural niche, manipulated and transplanted in a different site: this procedure assumes that cells are equivalent, and they can substituted each other without altering development [10]. In our knowledge, this assumption is actually not yet demon-

strated and we cannot know if all the mesenchymal stem cell can influence tissues positively during regeneration, but perhaps negatively towards the pathogenesis of cancer and metastasis, as reported by Becerra et al. [10].
Recently, Wichkam and colleagues [11] have demostrated that infrapatellar fat pad of the knee (IFP) also known as Hoffa's body, could be a new source of multipotent stromal cells. The IFP is an intrarticular adipose tissue that occupies the area between the patellar ligament and the infrapatellar synovial fold of the knee joint. In this paper the use of intra-articular mesenchymal stem cells was proposed for the cartilage regeneration: ex vivo reproduction of the physiological microenvironement could mimic the natural niche and promote the tissue regeneration.
The SVF was isolated from IFP adipose tissues and employed to promote chondrocyte colonization of silk fibroin scaffold.

2 Material and methods

Subjects - The IFP samples (bioptic volume range: 3-7 mL) from 7 informed female human subjects (age: 68.8±4.90 yrs, Table 1) were obtained during surgery for knee articular diseases and washed in saline solution. Biopsies were suspended in PBS and forwarded to a GMP cell factory at a temperature of 4°C.
Preparation of silk fibroin scaffold - Cocoons of *Bombyx mori* were degummed in autoclave (120°C, 1 hour) in order to eliminate sericines, dried at room temperature and cut in small pieces. Fibroin fibers were solubilised in $Ca(NO_3)_2$/MeOH (75% w/v) [12]. The fibroin solution was dialyzed, poured into molds and freeze-dried. Silk fibroin scaffolds were immersed in ethanol overnight in order to stabilize the crystalline structure, promoting mechanical stability. Before use, the scaffolds were washed and equilibrated in PBS.
Scanning electron microscopy (SEM) - Morphological investigation of the scaffold was performed by scanning electron microscopy (JEOL JSM-6380LV) before and after ethanol conditioning. Samples were air dried, gold sputtered and observed operating at 20 kV.
Cell isolation and culture- Adipose tissue samples were digested (0.02% collagenase in PBS). SVF cells were centrifuged, washed with PBS and counted; viability was assessed by the Trypan blue exclusion technique, and cellularity was evaluated as the ratio of cell number to adipose tissue volume. Association between subject characteristics (age and BMI) and SVF yield and viability were assessed using the Spearman correlation (alpha=0.05). Data were resumed as mean±standard deviation and reported in Table 1.
Knee articular cartilage harvested from cadaver donors was digested in 0.02% collagenase in PBS overnight. The chondrocyte cell suspension was filtered, centrifuged, washed with DMEM and cultured in a T-flask (37°C, 5% CO_2) until confluence (three weeks). The cells were harvested (trypsin/EDTA) and the Trypan blue exclusion technique was employed to perform viability and cell count.
SVF was seeded on silk fibroin scaffolds ($0.5 \cdot 10^6$cells on 2x1cm^2 scaffold) and cultured in DMEM for one week (37°C, 5% CO_2); after this time, chondrocytes were seeded on the same scaffold (cell density: $0.5 \cdot 10^6$ cells/cm^2). Co-culture lasted 21 days from the start of SVF culture. At the same time, chondrocytes were seeded on silk fibroin scaffolds in the same conditions.
Samples were fixed with 35% formaldehyde in PBS medium, dehydrated by alcohol scale and embedded in paraffin. Sections were stained with hematoxylin/eosin.
Immunofluorescence staining - Immunofluorescence investigation was performed by laser confocal microscopy. Paraffin-embedded samples were cut into 5 μm thick section and mounted on immunofluorescence microscope slides. The sections were treated with a 1% Triton X-100 solution in PBS medium and 2% bovine serum albumin in PBS medium for 30 minutes at room temperature. After this period, samples were treated (37°C for 60 minutes in light proof conditions) with primary antibody against Collagen Type II (MAb clone, 1:200 dilution, Chemicon). Primary antibodies were diluted in PBS medium

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with bovine serum albumin 1% The samples were further treated with the secondary dye-coupled antibody, Goat anti Mouse IgG-Rhodamine (TRITC) conjugate (1:200 dilution, Chemicon). The slides were then treated with Vectashield liquid mounting media. Slides were observed under a Leica confocal fluorescence microscope.

Table 1 Characteristics of female enrolled subjects and results: age (y); body mass index (BMI); fat tissue SVF cellularity (SVF cell millions/mL of treated tissue) and viability (%).

Subject	Age	BMI	Cellularity (SVF cell millions/mL tissue)	Viability (%)
1	72	30.30	1.05	73
2	68	38.45	0.32	72
3	69	34.29	1.23	78
4	68	27.41	3.53	86
5	60	34.67	2.93	87
6	69	27.70	2.63	89
7	77	24.22	2.47	84

3 Results and discussion

Bioptic samples of Hoffa's Body fat yielded SVF with a suitable amount of viable cells to perform 3D scaffold cell culture (mean 2.0±1.16 million/mL tissue with vitality 81.3±6.92%). The effects of subject age and BMI are negligible, possibly due to the reduced sample size.
Silk fibroin ethanol conditioning provides a stable, uniform, smooth surface, which potentially promotes cell adhesion and proliferation (Figure 1 a and b). The treatment with ethanol induces the β-sheet transition without changing the size distribution of pores.
After 1 week of culture, SVF cells adhere in wide clusters, coating the scaffold surface (Figure 2 a), while chondrocytes seem to adhere in a lesser amount without cluster formation (Figure 2 b).
After 21 days of co-culture, SVF promotes chondrocytes adherence and proliferation on regenerated silk fibroin scaffold. To identify if adherent cells were stromal cells or pooled stromal cells/chondrocytes, an immunofluorescence microscopical investigation using an extracellular matrix marker was performed (figure 3): image indicates that on fibroin scaffold cells seem to be embedded in a type II collagen extracellular matrix.

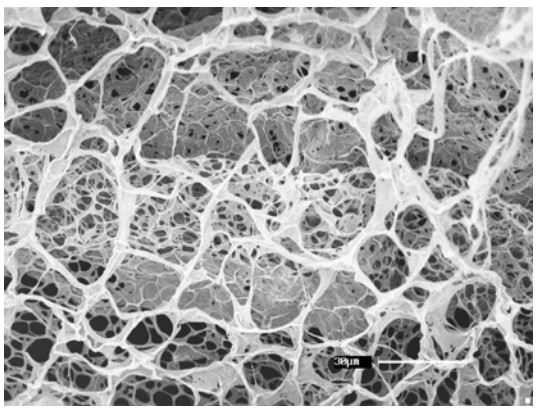
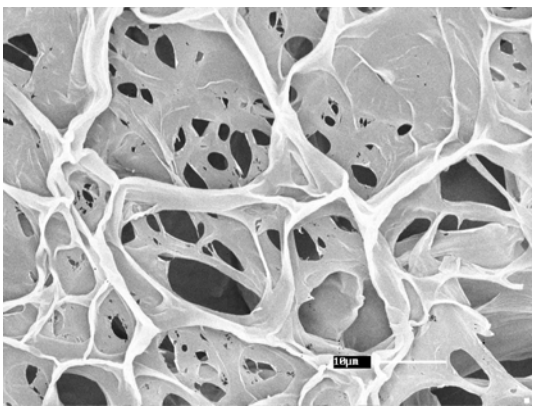
a)  b) 

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Fig. 1 Scanning electron microscopic images of fibroin scaffold: before (a) and after ethanol treatment (b).

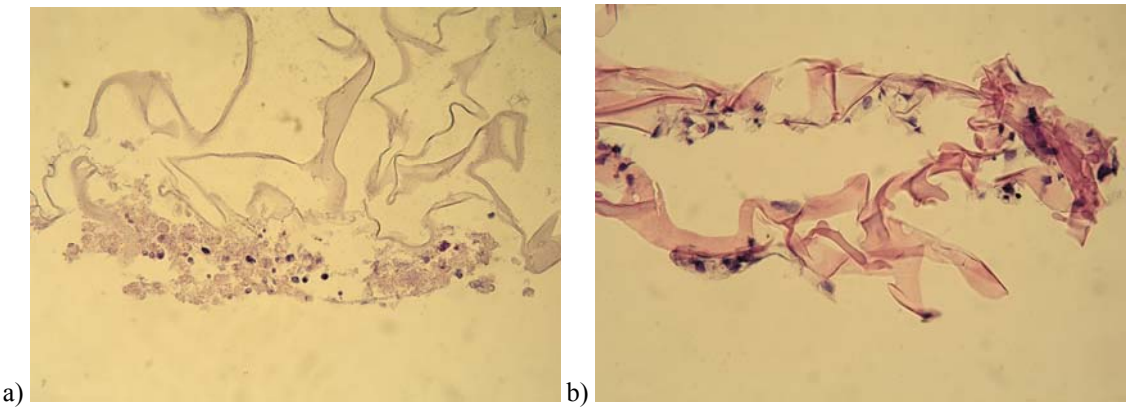

Fig. 2 Microphotograph of Hoffa's Body derived stromal vascular fraction (a) and chondrocytes (b) cultured on fibroin scaffold for 7 days. Hematoxylin/eosin staining; original magnification: 40X.

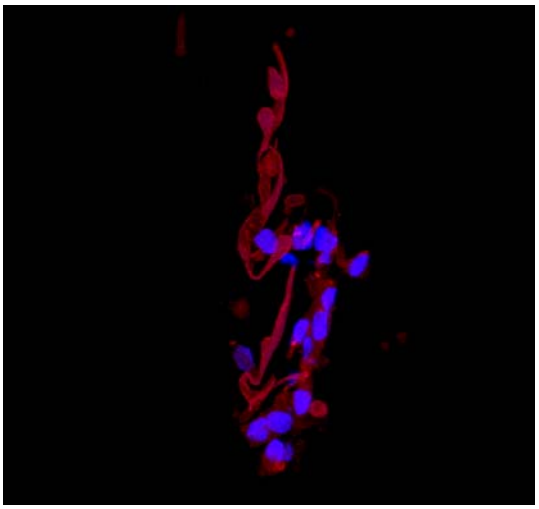

Fig. 3 Microphotographs of co-culture on fibroin scaffold after 21 days: type II-collagen/TRITC (red) and DAPI (blue).

4 Conclusions

In conclusion, three dimensional organized silk fibroin is an ideal biopolymer for cartilage tissue engineering, since it leads to cell attachment, scaffold colonization and physically cell holding in the area that must be repaired; the use of infrapatellar adipose-derived stem cells are a promising strategy to promote adhesion and proliferation of chondrocytes to the scaffold as an autologous human feeder layer.

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