Evaluation of Different Seeding Methods for Cell-Seeded Collagen Matrix-Supported Autologous Chondrocyte Transplantation

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Abstract

Purpose   The aim of the present study was to evaluate different methods for the intraoperative seeding of chondrocytes on commercially available collagen I/III matrix, in the context of cell-seeded collagen matrix-supported autologous chondrocyte transplantation (ACT-CS).

Methods   Human chondrocytes were enzymatically isolated from cartilage portion of discarded femoral heads of patients who underwent total hip replacement. Chondrocytes were cultured until passage 3, and then used for the experiments. The cells (5.0×10^5) were suspended in two different volumes, 75 and 250 µL, and seeded on a matrix sample with a surface of 1 cm^2 by means of a micropipette. Moreover, the direct immersion of the matrix in the cell suspension was evaluated as a possible protocol for chondrocyte seeding. Cell adhesion was allowed for 10, 30, or 60 minutes in all samples before evaluation.

Results   Data showed that the seeding time did not affect cell viability and distribution, but there was a great difference between the two volumes of injection. In fact, the use of 75 µL significantly reduced cell viability with respect to both 250 µL seeding volume and the immersion protocol. Indeed, cell distribution resulted homogeneous in the samples seeded with the larger volume and with the immersion protocol.

Conclusion   The use of 250 µL/cm^2 volume or the immersion protocol for 10 minutes are valuable methods for chondrocyte seeding on collagen matrix in an intraoperative scenario.

Clinical Relevance   The protocol of chondrocyte seeding in ACT-CS is extremely variable among available literature reports. Chondrocytes adhesion to the matrix represents a crucial step in this methodology, and the present study provides in vitro indication for the choice of the seeding protocol in the context of ACT-CS.

Keywords
► ACT-CS
► matrix
► chondrocytes
► cartilage
► intraoperative application

Introduction

Cartilage is a nonvascularized connective tissue made of chondrocytes immersed in a dense extracellular matrix, mainly composed of collagen fibers and proteoglycans.[1] Traumatic or degenerative events could lead to chondral and osteochondral lesions that may affect cartilage and the subchondral bone.[2,3] The low grade of vascularization and the poor migration ability of chondrocytes within the tissue represent the two major limitations to cartilage self-repair, leading to healing failure and thus to pain, loss of function, and long-term complications.[3–5] Hence, it is important to rely on a valid therapeutic approach to enhance tissue repair and avoid fibrotic scar formation. For this purpose, different

surgical strategies have been proposed to restore or preserve hyaline cartilage, even though a successful treatment of these lesions remains challenging.[6]

The treatment of cartilage lesions comprises traditional operative approaches, such as joint debridement,[7] drilling, and microfractures of the subchondral bone.[8] However, these approaches are feasible in small chondral lesions only, while different techniques should be applied to treat larger defects. Indeed, for these kinds of lesions, a regenerative approach is preferred. For instance, matrix-assisted microfracture techniques like autologous matrix-induced chondrogenesis or autologous chondrocytes implantation (ACI), either associated with a collagen matrix, seem to provide more satisfactory and durable results in the treatment of medium-large chondral defects.[9–12] In this context, matrices are used with different applications and purposes. Indeed, in matrix-induced ACI chondrocytes may be injected in the lesion before suturing or directly cultured on the matrix for weeks before implantation, while in cell-seeded collagen matrix-supported autologous chondrocyte transplantation (ACT-CS) the cells are seeded on the matrix right before implantation, intraoperatively. These techniques represent an evolution of ACI and require a two-step surgery, the first where a cartilage biopsy is collected for chondrocytes isolation and culture, followed by a second procedure for cell and matrix implantation in the recipient site.[13,14]

One of the limitations of the techniques combining cells and matrices is the low reproducibility of the methods, mainly due to the differences in chondrocytes viability, number of cells, and seeding protocols, sometimes leading to conflicting outcomes.[10,15–17] Thus, in the context of ACT-CS, different methods were evaluated to define the most effective protocol for chondrocytes seeding on a commercially available collagen I/III matrix.

The main purpose of this study was to in vitro investigate the effect of different protocols for chondrocytes seeding on the matrix, to identify the most effective approach, and provide valuable indications for the standardization of the procedures. The hypothesis of the study was that different seeding methods would influence cell adhesion and distribution of chondrocytes on the matrix in the context of ACT-CS.

Methods

Human Chondrocytes Isolation and Culture
Autologous human chondrocytes were enzymatically isolated from discarded hip joint cartilage portions of patients who underwent total hip arthroplasty at the Galeazzi Orthopedic Institute, Milan, Italy. All patients gave written consent to the collection of waste biological material. Briefly, cartilage tissue was harvested from the femoral head, minced, and incubated overnight at 37°C with 0.15% Collagenase type II (Worthington Biochemical Corporation, New Jersey, United States) in Dulbecco's modified Eagle medium (Sigma-Aldrich, Missouri, United States). Then, cells were counted and seeded at a density of 5.0×10^3 cell/cm^2 and cultured in a humidified atmosphere at 37°C and 5% of CO_2. When at 80% confluence, cells were harvested by trypsin (Lifetechnologies, California, United States) treatment and then seeded for

further expansion until passage 3, when the experiments were performed.

Human Chondrocytes Seeding on Collagen Matrix
The bilayer collagen matrix used is composed of biocompatible porcine collagen type I/III (Chondro-Gide, Geistlich, Switzerland). The device has a compact layer which has a smooth surface and prevents the diffusion of the injected cells; the other layer of the matrix consists of a porous surface with a more loosely arrangement of collagen fibers that favors cell invasion and attachment.

To evaluate the suitable volume for cell seeding on Chondro-Gide, a preliminary absorption test was performed on 1 cm^2 of the dry matrix.

The following volumes of blue color pigment solution (3% in phosphate-buffered saline), were tested: 50, 75, 100, 150, and 250 μL.

Three different seeding methods were adopted: small volume (75 μL) or large volume injection (250 μL) on the porous side of the matrix with a micropipette and matrix immersion in the cell suspension. According to the literature,[11,18] we decided to use 0.5×10^6 cells/cm^2 suspended in the two volumes of interest. The more concentrated cell suspension (5×10^5 cell/75 μL) was used for matrix immersion. Three seeding times, 10, 30, and 60 minutes, were tested with the purpose to identify the best time allowing for a complete and homogeneous cellular adhesion on the matrix in view of intraoperative seeding technique as usually done by many surgeons.[19] For each condition, a qualitative and quantitative analysis of adhered cells were made by 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) fluorescent staining and MTT assay (Sigma-Aldrich), respectively. Not-seeded matrices were prepared as controls.

Viability Assay
Metabolic activity of human chondrocytes seeded onto Chondro-Gide was measured by the MTT assay. After cell seeding with different protocols, the matrices were transferred in a 0.5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) and incubated for 4 hours at 37°C and 5% CO^2, to allow the formation of formazan crystals in the proximity of viable cells. Then, the medium was removed and substituted with the same volume of dimethyl sulfoxide (Sigma-Aldrich) to solubilize the formazan crystals. Finally, the absorbance of the resulting solution was read at 570 nm (Victor X3, Perkin Elmer microplate, Massachusetts, United States).

Immunofluorescence Assay
Matrix specimens were fixed in 10% formalin for 24 hours. Then, they were dehydrated in an increasing alcohol scale before embedding in paraffin and cutting into 3.5 μm longitudinal sections. The slides were rehydrated in a decreasing alcohol scale and then permeabilized with a 0.5% Triton X-100 (Sigma-Aldrich) solution at room temperature (RT) for 10 minutes. After washing passages, the slides were incubated for 10 minutes at RT in the dark with DAPI (Sigma-Aldrich) to stain cells nuclei, and mounted with an aqueous mounting

agent (Enzo Life Sciences, New York, United States). Photomicrographs of the matrix were captured through an Olympus IX71 fluorescence microscope and an Olympus XC10 camera (Olympus Corp, Tokyo, Japan).

Statistical Analysis

The experiments were conducted in triplicates. Results were analyzed by GraphPad Prism 5.0 software (California, United States). Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used for the comparison of samples in the different groups. Two-way ANOVA test with Bonferroni's post hoc test was applied to test the influence of seeding method and incubation time in all samples. A p-value of < 0.05 was considered statistically significant.

Results

The results of the absorption test showed that 1 cm^2 of the matrix was completely wet already by the use of 75 μL; increasing the volume of the blue color pigment solution lead to a progressive swelling of the matrix size till it started to leak noticeably when a volume of 250 μL was used. Consequently, it was decided to compare the same number of cells seeded in a small (75 μL) and a large volume (250 μL) in the following experiments (►**Fig. 1**).

Cell Viability

The results of cell viability assay revealed that the time of seeding did not significantly affect cell viability, although a slight increasing trend with time was observed for each protocol. Indeed, the analysis of the contribution given by seeding method and incubation time revealed that they accounted for 87.21 and 4.30% of the total variance, respectively. Despite both being statistically significant ($p < 0.0001$ and $p < 0.01$, respectively), the influence given by time resulted negligible. On the other hand, samples seeded with high volume (250 μL) and by immersion demonstrated significantly higher values compared with the 75 μL seeding protocol (►**Fig. 2**). An evaluation of gross appearance of the matrix showed that in this case, the cells were not able to colonize the whole matrix, but rather generated a spot of concentrated cells in the middle (►**Fig. 3**), negatively affecting cell viability.

Distribution of Human Chondrocytes on Collagen Matrix

To evaluate cells distribution and density on the porous surface of Chondro-Gide, DAPI staining was performed on

Fig. 1 Absorption test. Chondro-Gide matrices injected with different volumes of blue pigment solution.
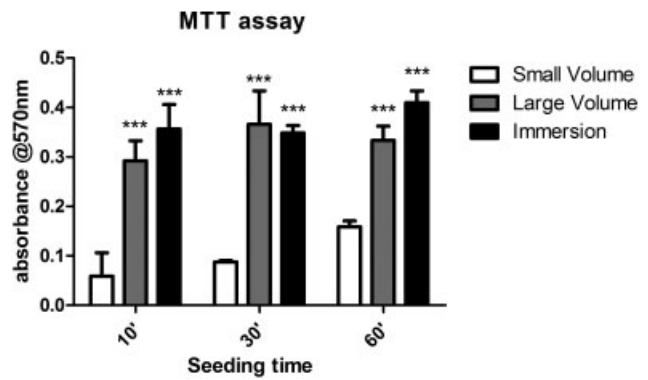

Fig. 2 Cell viability. The picture shows the results after 4 hours' incubation wit MTT assay of the seeded matrices. $^{***}p < 0.001$ vs. small volume.
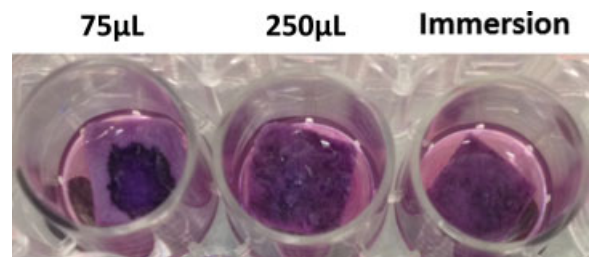

Fig. 3 Gross appearance of membranes after MTT assay.

paraffin-embedded samples. No differences among seeding time were observed. In particular, the results indicated that the immersion seeding protocol allowed for a uniform seeding on both sides of matrix, while the small and large volumes were injected on the porous surface of Chondro-Gide, thus resulting in a one-side seeding due to the bilayer structure of the matrix. Small volume injection appeared to increase the depth of seeding, and, indeed, it enhanced the concentration in the center of the matrix with respect to both large volume and immersion protocols. Conversely, the large volume allows for a more uniform and less concentrated seeding on the matrix (►**Fig. 4**).

Discussion

The present study aims to describe a reliable and reproducible method to seed chondrocytes on surgical devices in the contest of ACT-CS. Indeed, one of the limitation of this technique is represented by the different protocols adopted throughout the literature, limiting the comparison and the evaluation of surgical outcomes.[5,19–21]

Nowadays, the importance to improve the strategies of regenerative medicine to enhance cartilage healing is compelling. Among the different surgical and regenerative procedure proposed in the last decades, ACT-CS represents a valid strategy to overcome the limitations of traditional techniques[11,13,19,20,22] and to partially restore the complex architecture of the hyaline cartilage, mitigating pain by reducing the friction surface phenomena.[23]
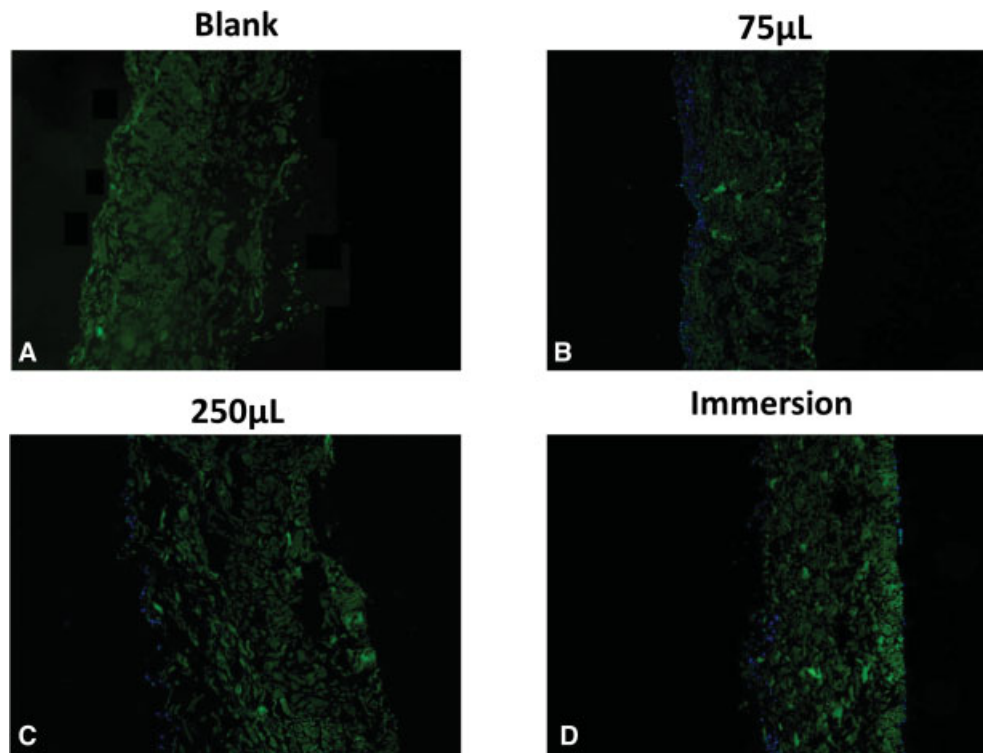
Fig. 4 4′,6-Diamidino-2-phenylindole (DAPI) staining at 30 minutes. (**A**) A nonseeded membrane used as control. The small (**B**) and large (**C**) volumes (75 and 250 μL) allowed for cell distribution on the porous surface of the membrane, resulting in one-side seeding. (**D**) Immersion method determined a homogenous cell seeding on both sides of Chondro-Gide.

In our experiments, we used Chondro-Gide, a porcine collagen I/III matrix that represents a popular scaffold for this application.[13,24–26] This device presents two different surfaces made of collagen type I/III, one porous intended to allow cell infiltration, and the other more compact, aimed to contain the cells within the lesion site, preventing their migration in the joint space. However, the compact side of the matrix also allows for cell adhesion as demonstrated by our results.

The optimal number of chondrocytes per square centimeter may be product-specific and thus our results might not be generalizable. According to the literature,[11,18,27] in this study 5.0×10^5 cells/cm^2 were used, suspended in two different volumes of injection (75 and 250 μL). Our results confirmed that, with this method, chondrocytes were distributed on matrix surface, as previously reported by other authors.[28] Moreover, these findings showed that the small volume was not suitable to allow a uniform and effective cell adhesion on the matrix surface, while the use of the largest one increased the distribution of cells on the porous face of Chondro-Gide. Nevertheless, we applied the lower limit of the cell density reported in the literature, thus it is possible to hypothesize that increasing this parameter would enhance cell concentration and penetration within the matrix with the larger volume, similarly to what observed in the central portion of the matrix seeded with the smallest one. On the other hand, the immersion of the matrix in the cell suspension would represent a valid method for homogenous seed-ing, especially if the adhesion on both side of the matrix is desirable. Notably, negligible differences were observed among seeding times, suggesting that 10 minutes represents a sufficient time to allow for cell adhesion, both when cells were distributed by means of needle or immersion. This observation is particularly significant since the reduction of operative time can reduce costs and risks associated with surgeries.

Limitations of the present study are given by the use of laboratory equipment and techniques for cell seeding on the collagen matrix whose translatability in the context of a surgery room is yet to be confirmed. Moreover, the number of replicates was limited, even if the obtained results were statistically consistent.

In conclusion, in the context of ACT-CS, our results suggest using a volume of 250 μL containing at least 5.0×10^5 cells per cm^2 of matrix, for 10 minutes. Similarly, the immersion of the matrix in a cell suspension of 6.7×10^6 cells/mL (corresponding to 5.0×10^5/75 μL) for the same seeding time would be a reliable method, but the adhesion of chondrocyte on both side of the matrix, and a lower level of penetration within the matrix, should be acknowledged.

Conflict of Interest
None declared.

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