ORIGINAL ARTICLE

Study on human chondrocyte culture viability for autologous transplantation in clinical application

Estudo da viabilidade da cultura de condrócitos humanos visando aplicação clínica para o transplante autólogo*

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ABSTRACT

Objective: The limited regenerative capacity of the cartilage tissue makes the treatment of chondral lesions difficult. The techniques currently available to treat cartilage lesions may relieve symptoms, but do not regenerate the injured tissue. Autologous chondrocyte transplantation uses cell biology and cell culture techniques to regenerate the hyaline cartilage. **Methods:** In this study, we analyze chondrocyte biopsy collection and culture for autologous transplantation. Ultrastructural analyses of hyaline cartilage biopsies were performed 0, 6, 24 and 48 hours after collection. The tissue even after 48 hours. Eleven cell culture assays were performed to evaluate isolation, viability, morphology, proliferation and absence of contaminants. **Results:** The cell culture techniques used allowed chondrocyte proliferation. Rates on cell viability were maintained above the acceptable patterns (above 90). Control of cell culture laboratory conditions showed absence of contaminants, assuring safety of the process. The chondrocytes obtained presented the morphology typical of cultured cell monolayers. **Conclusion:** The results indicate viability of chondrocyte culture technique for clinical application in autologous transplantation.

Keywords: Cell culture; Chondrocytes/transplantation; Transplantation, autologous/methods; Cartilage

RESUMO

Objetivo: A baixa capacidade regenerativa do tecido cartilaginoso dificulta o tratamento das lesões condrais. As técnicas disponíveis atualmente para o tratamento de lesões de cartilagem articular podem resultar em alívio dos sintomas, mas não na regeneração do tecido lesado. O transplante autólogo de condrócitos utiliza técnicas de biologia celular e cultura de células para a regeneração da cartilagem hialina. Neste trabalho procuramos analisar a cultura de condrócitos visando o transplante autólogo. **Métodos:** Foram realizados 11 experimentos de cultura celular para avaliar os dados de isolamento, viabilidade, morfologia, proliferação e ausência de contaminantes. As condições de cultura celular utilizadas permitiram a proliferação dos condrócitos. As taxas de viabilidade celular se mantiveram acima de 90%. **Resultados:** O controle das condições do laboratório de cultura celular demonstrou a ausência de contaminantes, garantindo a segurança do processamento. As células obtidas apresentaram morfologia típica de condrócitos cultivados em monocamada. **Conclusão:** Os resultados obtidos indicam a viabilidade da técnica de cultura de condrócitos para a aplicação clínica na técnica de transplante autólogo.

Descritores: Cultura de células; Condrócitos; Transplante autólogo/métodos; Cartilagem

INTRODUCTION

The nutrition of cartilage tissue of articular surfaces is carried out by diffusion of substances found in the synovial fluid. The chondrocytes present in tissues are highly specialized and their major function is to provide biomechanical properties, by synthesizing extracellular matrix components. In the articular cartilage there are proteoglycans, especially agregan, and mostly type II collagen. The differentiated state of chondrocytes and the absence of tissue irrigation make the repair of chondral lesions difficult.

Tissue repair of chondral lesions is based on surgical techniques. Their rationale is to fill in the lesion with subchondral bone mesenchymal cells by means of abrasion, perforation and micro fractures. However, the lesions are filled in by fibrous tissue or fibrocartilage, which has different features as compared with the previous hyaline cartilage. Type I collagen prevails in the extracellular matrix of this repair tissue. The autologous transplantation of chondrocytes has been used as a biotechnological alternative to treat chondral lesions resulting from

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acute or repeated trauma and not responding to previous treatments, whether surgical or clinical[1-4].

Autologous transplantation of chondrocytes is a technique that aims at regenerating the hyaline cartilage, restoring the function of the articular surface. This technique has been studied since the end of the 80's[5-6]. In 1994, a group from the University of Gothenburg and from the Sahlgrenska University Hospital presented a pilot study with 23 patients submitted to this technique to treat cartilage lesions[7]. Sixteen patients had lesions in the femoral condyle and seven had patellar lesions. The cases were followed for up to two years, and good or excellent recovery was observed in 14 out of 16 patients with femoral condylar lesions. Biopsy was performed in 15 patients and hyaline cartilage similar to the original tissue was observed in 11 of these cases.

Other scientific reports were published later[8-11]. Improving this technique encompasses the supplementation of cell cultures with growth factors[12], the utilization of biomaterials[13-14] and the application of bioreactor systems to produce cartilage tissue *in vitro*[15].

The technique for autologous transplantation of chondrocytes for articular cartilage regeneration (figure 1) comprises removal of a sample of normal cartilage from a low-load region of the joint. This biopsy is properly prepared and sent to the cell culture laboratory where enzyme digestion is performed for cell isolation and culture[16-17]. A suspension of cultivated chondrocytes is prepared after a period of 30 to 40 days. The injection of cell suspension involves debridement of the chondral lesion, covering the lesion with a layer of periosteum usually removed from the tibia, and the injection of cell suspension between the periosteum cover and the subchondral bone[8].

The transplanted chondrocytes are able to reorganize themselves to produce hyaline matrix, regenerating a tissue with the same characteristics of the original hyaline cartilage. The recently formed tissue is completely integrated, filling in the entire site of the lesion and adjacent areas[7]. In the first eight weeks after transplantation, 71% of the area, in average, is filled in and the tissue is basically hyaline, with cell clusters and predominance of type II collagen[8].

In the present study, we sought to improve the cell culture technique aiming at autologous transplantation of chondrocytes, and evaluating cell preservation, proliferation and morphology.

METHODS

Biopsy Collection

Samples of articular cartilage were obtained from nine patients, six men and three women, age range 23-66 years (mean age = 48 years). Two samples were submitted to enzyme digestion on different days, totaling 11 cell cultures. The patients were submitted to arthroscopy for several reasons. The cartilage samples were collected in culture medium according to the protocol described above including previous patient consent[7]. Thermal containers were used to transport cartilage samples so that the temperature was kept between 4°C and 8°C.

Chondrocyte Isolation

Eleven samples of articular cartilage were taken to the culture laboratory and submitted to enzyme digestion process to isolate cells, according to the protocol of Archer et al.[16]

Chondrocyte Culture

The cells from enzyme digestions were then cultivated in culture flasks with HAMF12 medium containing penicillin/streptomycin 1% and fetal bovine serum 10%, for periods of up to 40 days, and the medium was changed twice or three times per week. Cell replication was performed as necessary.

Cell Count and Viability

Total cells and percentage of viable cells were counted, right after cell isolation and at the end of cultures, using trypan blue in a hemocytometric chamber.

Morphological Analyses

Morphological analyses of cells were routinely performed using phase microscopy in an inverted microscope (Olympus IX-50). The cultures were photographed regularly to record cell morphology.

Microbiological Analyses

Some cell culture samples were submitted to microbiological contamination tests. Testing for the presence of bacteria, fungi and mycoplasmas was performed according to ISO 11737-1 standards, 5th edition, 1995 and USP XXIII/NF XVIII, 1995.

RESULTS

Chondrocyte Isolation

Chondrocytes isolated from articular cartilage weighed between 102 mg and 417 mg (mean weight = 255 mg), resulting in an average of 140,560 chondrocytes in 100 mg of tissue (table 1). Recently isolated chondrocytes

presented rounded morphology, which is characteristic of these cells.

Cell viability counts were performed in the eleven samples, yielding values always above 90% (table 1).

Table 1. Cell culture monitoring after cartilage tissue biopsy. Specimen weight, cell count, initial and final viability values, time up to final culture

Specimen weight (mg)	Initial Number of Cells	Initial Viability (%)	Days of Culture	Final Number of Cells	Final Viability (%)
154	100,000	100	28	6,000,000	—
294	11,000	50	33	1,000,000	—
514	53,000	—	31	11,000,000	—
310	84,000	—	13	18,000,000	95
292	93,000	100	27	3,000,000	94
184	41,000	90	56	9,500,000	93
224	245,000	96	25	10,000,000	—
417	135,000	96	24	3,300,000	—
102	153,000	93	36	1,200,000	94
400	85,000	96	42	18,800,000	96
300	73,000	98	—	—	—

Chondrocyte Culture

Chondrocytes were kept in culture from 13 to 56 days in order to reach at least one million cells, resulting in an average of 31.5 days of culture. The total number of cells and their viability were counted to conclude the cultures. The values obtained for cell viability were above 90%. The final number of cells ranged from 1,200,000 to 18,800,000, resulting in an average of 8,500,000 cells, that is, a 23.7-fold increase in the number of cells (table 1).

Morphological Analyses

Cell adhesion and spreading processes on the polypropylene surface during cell culture resulted in change in the rounded shape characteristic of chondrocytes. After three to five days of culture, flattened and spread chondrocytes with typical fibroblast morphology could be seen (figure 2). Images of cell division were frequently present in cultures (figure 2D). Despite the elongated morphology, evident signs of cell degeneration or apoptosis were not observed, as proven by the final cell viability count.

Microbiological Analyses

All microbiological analyses performed in cell cultures were negative, translating the absence of bacteria, fungi or mycoplasmas in cultures.

DISCUSSION

Surgical techniques try to repair tissues based on the principle of filling in the lesion with bone marrow mesenchymal cells, by means of abrasion, perforations and micro fractures. However, the cells that fill in the chondral lesion synthesize fibrous tissue, or fibrocartilage, and have composition and properties that differ from those of the original hyaline tissue. In this case, type I collagen prevails in the extracellular matrix. The presence of fibrous tissue may cause the patient to have pain and impaired movement in the long run. To form hyaline articular cartilage similar to the original one, autologous transplantation of chondrocytes is an alternative to treat chondral lesions resulting from acute or repetitive trauma and that have not responded to previous surgical or clinical treatments[5-11].

In our laboratory we tested the processing conditions of cartilage biopsies and chondrocyte culture phases aiming at cell proliferation. We obtained fragments of femoral condyle cartilage of different sizes and regions. Our observations express the clinical reality, and it is not possible to standardize collection of material. Yet, we limited collection to the femoral condyle, always preserving subchondral bone.

The cell isolation process proved to be efficient, and enabled collecting many cells per 100 mg of tissue. It is essential to obtain a minimum number of cells to begin cultures, since we expect increased number of cells with few culture transfers in order to preserve cell characteristics and avoid apoptosis in autologous transplantation. We know that after a few transfers without culture, a cell death process may occur. Thus, the culture process was performed respecting this limit. The conditions of cell cultures, such as supplementation with fetal bovine serum, assured a high cell proliferation rate and enabled achieving a minimum of one million cells, always with cell viability above 85%, as described in the literature[7].

Chondrocyte morphology in culture translates the differentiated state of cells, as expected in the proliferative role of cultures[18-20]. Cultivated chondrocytes in monolayer cultures from some transfers acquired a flattened morphology and had high proliferation rates. Cell morphology is directly linked to protein synthesis. In this differentiated state, the cells start producing type I collagen, preferably, replacing the synthesis of type II collagen, which is characteristic of hyaline

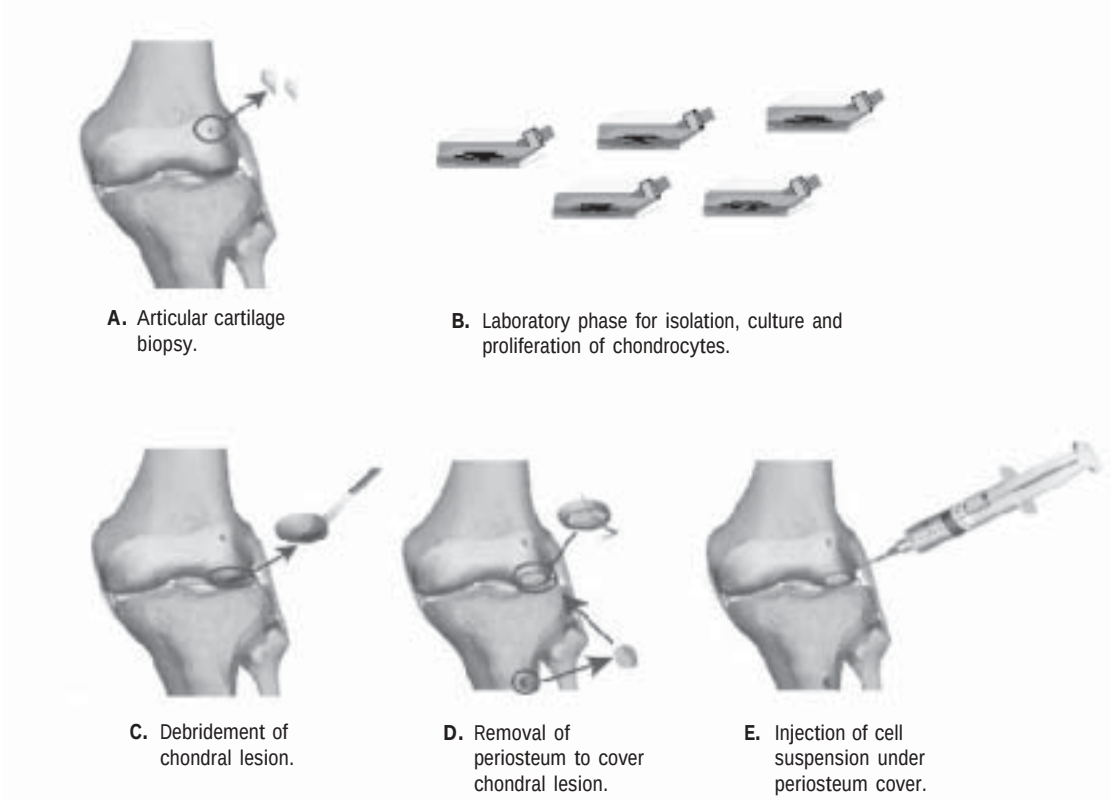

Figure 1. Autologous chondrocyte transplantation for articular cartilage regeneration. First, a cartilage specimen is excised form a region not submitted to load of the joint (A); later the biopsy specimen is taken to the cell culture laboratory where chondrocyte isolation and culture are performed (B). The cell suspension resulting from this process is sent for transplantation. The lesion should be debrided (C), and covered with periosteum (D) for cell suspension injection (E).

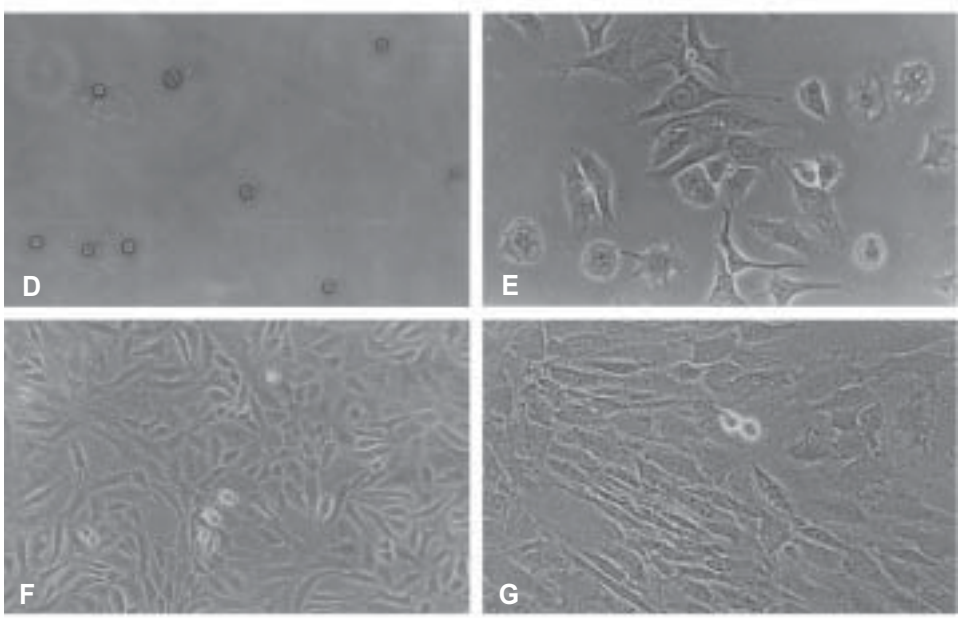

Figure 2. Phase microscopy (A). Recently isolated chondrocytes with characteristic rounded morphology are observed (B). Culture in semi-confluent monolayer, 8-day culture (C). Culture in confluent monolayer, 33-day culture (D). Cell division in 35-day culture. Bar: 100 μm.

cartilage. After some transfers it is possible to reestablish the normal chondrocyte phenotype as long as there are ideal conditions for cell re-differentiation[21-23]. The fact that cells are implanted in hyaline substrate with characteristic extracellular matrix works as a sign of cell differentiation in autologous transplantation of chondrocytes. Once implanted, chondrocytes are able to express their normal phenotype and synthesize extracellular matrix. We did not observe intense extracellular matrix synthesis in our cultures, because the target was to obtain a high cell proliferation rate.

The absence of contaminants in all cultures tested (according to quality control requirements of regulatory agencies) is an evidence of safe working conditions.

The results in the present study enabled evaluation of biopsy processing conditions to obtain chondrocytes and of culture conditions for cell proliferation. We could conclude that chondrocyte culture for autologous transplantation in our environment is a possible, feasible and safe technique for clinical application as a surgical technique of articular cartilage regeneration.

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