

# ISOLATION AND CULTURE OF HUMAN CHONDROCYTES FOR AUTOLOGOUS TRANSPLANTATION

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## REZUMAT

**Obiectiv:** Izolarea și cultura condrocitelor pentru transplantul autolog, o nouă modalitate terapeutică în diferite afecțiuni ale cartilajului, în special cele posttraumatice.

**Material și metodă:** Fragmente de cartilaj uman au fost recoltate prin biopsie artroscopică de la nivelul condilului femural intern. Condrocitele au fost izolate prin digestie enzimatică utilizând colagenaze, timp de 12-24 ore, apoi au fost cultivate timp de 8 săptămâni în Dulbecco's Modified Eagle's Medium (DMEM) și antibiotice, cu sau fără suplimentare de albumină. Albumina umană a fost folosită în loc de ser fetal de vită deoarece obiectivul nostru a fost de a obține o cultură de condrocite umane pentru transplantare autologă. După o săptămână, la mediul de cultură a fost adăugat IGF-1 (factor de creștere insulin-like) sau dexametazonă. În final, celulele au fost resuspendate atât mecanic, cât și enzimatic prin tripsinizare.

**Rezultate:** În mediul DMEM suplimentat cu soluție de albumină umană 10%, numărul celulelor a crescut semnificativ după primele 4 săptămâni și a continuat să crească până la sfârșitul experimentului (concentrația finală la 8 săptămâni a fost de  $120 \times 10^3$  celule/ml). Când celulele au fost cultivate în DMEM fără supliment de proteină ele au supraviețuit dar nu au proliferat. Adăugarea de IGF-1 a stimulat proliferarea celulară mai mult decât dexametazona (concentrația celulară finală de  $820 \times 10^3$  celule/ml față de  $750 \times 10^3$  celule/ml).

**Concluzii:** Datele noastre sugerează că obținerea unui număr suficient de mare de condrocite umane necesită o lungă perioadă de timp iar acest proces este stimulat de anumiți factori de creștere. Adăugarea de albumină umană este considerată benefică deoarece crește rata de proliferare. Cea mai mare rată de proliferare a fost obținută prin adăugarea de IGF-1.

**Cuvinte cheie:** condrocite, IGF-1, transplant autolog

## ABSTRACT

**Objective:** To isolate and culture human chondrocytes for autologous transplantation, as a new therapeutical approach in various cartilage disturbances, especially post-traumatic ones.

**Material and Methods:** Human cartilage fragments were harvested by arthroscopic biopsy (internal femoral condyle). Chondrocytes were isolated by enzymatic digestion using collagenase (12 to 24 hours), and cultivated in DMEM for 8 weeks (Dulbecco's Modified Eagle's Medium) and antibiotics, with or without supplementation with human albumin. Human albumin was used instead of fetal calf serum because our goal was to obtain a culture of human chondrocytes to be used for autologous transplantation. After one week, either Insulin-like Growth Factor I (IGF-I) or dexamethasone were also added to the medium. Finally, the cells were resuspended, both mechanically and enzymatically by trypsinization.

**Results:** In DMEM supplemented with 10% human albumin, the cell number significantly increased after the first 4 weeks, and continued until the end of the research (final concentration at 8 weeks:  $120 \times 10^3$  cells/ml). When cells were cultivated in DMEM without protein supplement they survived, but no proliferation was noticed. Addition of IGF-1 stimulated cell proliferation more than dexamethasone (final concentration  $820 \times 10^3$  cells/ml, and  $750 \times 10^3$  cells/ml, respectively).

**Conclusions:** Our data suggest that obtaining a sufficient number of human chondrocytes requires a long a period of time, this process being stimulated by certain growth factors. Addition of human albumin is considered beneficial, increasing their proliferation rate. In case of supplementation with IGF-I the highest proliferation rate was achieved.

**Key Words:** chondrocytes, IGF-I, autologous transplantation

## INTRODUCTION

Degenerative diseases correlated with age (such as osteoarthritis) are among the ailments with a great social and economical impact, which require hospitalization, rehabilitation procedures and medical care at home. In Europe, the prevalence of osteoarthritis is approximately 12%, occurring between 25 and 50 years of age, and over 60 years reaches even 95%.<sup>1</sup>

In case of cartilage destruction, restoration and regeneration of the tissue from the adjacent perichondrium occurs mainly in young subjects, which

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are in their growth period. On the other hand, in adults, the same perichondral tissue is the source of a substitution connective tissue, sometimes accompanied by ossification. The usual therapeutical methods are mechanical ones: debridation and abrasion of irritative intraarticular factors, perforation of cartilage to vascular bone marrow in order to allow the invasion of fibrous cartilage, microfractures with periosteal/perichondral grafting. New therapeutical methods, such as cellular transplantation procedures, were followed by an accelerated functional recovery, a decrease of hospitalization period and also by the reduction of complications rate.<sup>2</sup>

In our country, new and competitive clinical and therapeutical solutions are required, especially in case of patients with chronic diseases, in which current therapy is not satisfactory. Fundamental studies concerning these new procedures, as well as evaluation of both their safety and feasibility are obligatory. Achievement of proliferating human chondrocytes in culture is absolutely necessary for autologous transplantation of chondrocytes in various cartilage disturbances, especially post-traumatic ones.

## **MATERIAL AND METHODS**

Human articular cartilage was acquired from 10 patients aged 25 to 35 years, hospitalized for various traumatic injuries in the Orthopaedics and Traumatology Department of the County Hospital in Timisoara. Cartilage fragments were removed from apparently healthy regions, internal femoral condyle respectively, by arthroscopic biopsy.

Tissue fragments had a 0.5-1 cm<sup>2</sup> area and were transported immediately after harvesting in sterile tubes containing DMEM (Dulbecco's Modified Eagle's Medium). A sterile scalpel was used to cut the cartilage into small pieces, which were digested with 0.2% collagenase (Sigma, St.Louis, MO) for 12 to 24 hours, at 37° C, in a 5% CO<sub>2</sub> humidified incubator. The time of enzymatic digestion was adapted to the size of the tissue fragment.

After digestion, the cartilage suspension was washed thoroughly with phosphate-buffered saline (PBS) and centrifuged at 1800-2000 rpm, 7-8 minutes, at room temperature. Cells were seeded in T25 flasks and then in 24-well culture plates at low density (10<sup>3</sup> cells/cm<sup>2</sup>), in DMEM, as well as in DMEM supplemented with 10% human albumin, and antibiotics (penicillin 0.1 mg/ml, gentamycin 0.05 mg/ml and amphotericin B 50 ng/ml). Human albumin was used instead of fetal calf serum because our goal was to obtain proliferating chondrocytes in culture to be used for autologous transplantation. Beginning with the seventh day of

culture, DMEM was supplemented with Insulin-like Growth Factor I (IGF-I)(Sigma, St.Louis, MO) 50-100 ng/ml or dexamethasone (Sigma, St.Louis, MO) 4 ng/ml. The cultures were examined for growth twice a week, for 8 weeks, using an inverted phase-contrast microscope. The culture medium was changed twice during the entire experiment.

These methods were adapted after those performed by Brittberg et al, and Kolletas et al.<sup>3,4</sup>

Finally the cells were detached mechanically, as well as by trypsinization, and their viability was assessed using the trypan blue test. Hematoxylin-eosin, Papanicolau and Giemsa stainings were performed in order to evaluate the morphology of cultivated cells.

## **RESULTS**

Cell isolation was considered complete only after obtaining individual cells; approximately half of the cells were in small groups, 2 to 4 cells, usually in contiguity (Fig. 1A). The initial concentration was rather low (2 x 10<sup>3</sup> cells/ml), allowing long periods of time between supplementing the medium.

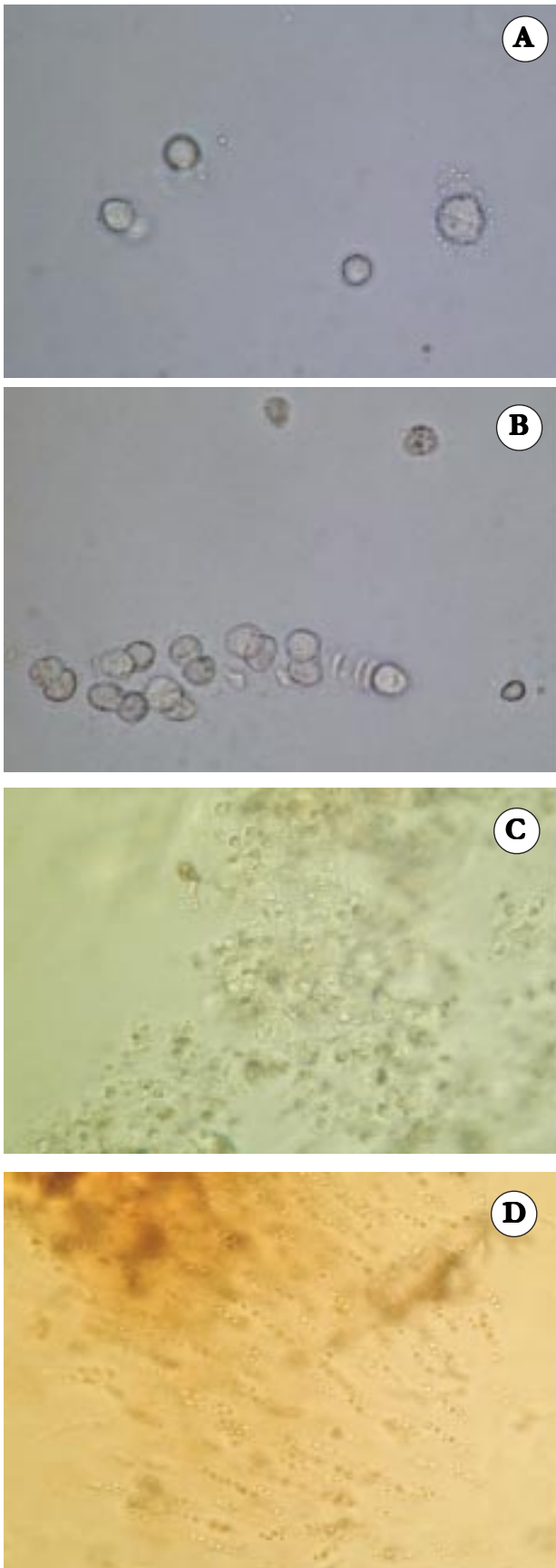
When DMEM without protein supplement was used, we noticed that cells were present without significant morphological changes, but their proliferation rate was almost 0. The final cell concentration in this case was 2.2 x 10<sup>3</sup> cells/ml (Fig. 1B).

In case of DMEM supplemented with 10% human albumin, the cell proliferation rate was good. Their number significantly increased after the first 4 weeks of the experiment, and the process continued until the end of the research (8 weeks) (Fig. 1C).

Cell groups appeared isogenous, compact, rather large, rounded, with a tridimensional architecture, consisting of rounded, large chondrocytes with a distinct nucleus, sometimes presenting nucleolus, and abundant cytoplasm. Cell concentration was 45 x 10<sup>3</sup> cells/ml after 4 weeks, and finally 120 x 10<sup>3</sup> cells/ml.

In DMEM also supplemented with IGF-1, chondrocytes presented the highest proliferation rate. Their organization remained tridimensional, with numerous isogenous groups, consisting of young chondrocytes, in a tight aposition. Occasionally, chondrocytes with a trabecular disposition, arranged in lines, were observed (Fig.1D). In this case, the final concentration was 820 x 10<sup>3</sup> cells/ml.

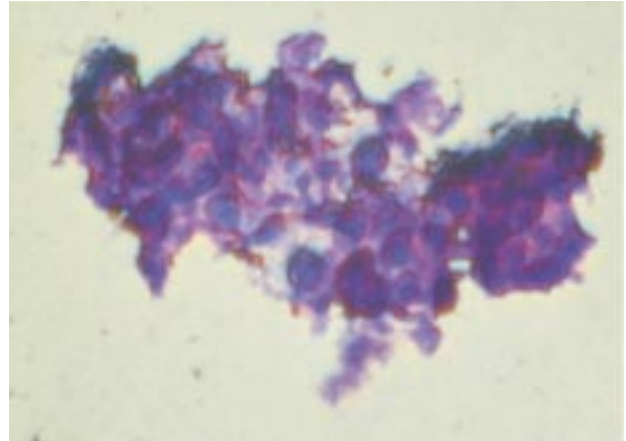
In DMEM with added dexamethasone, chondrocytes showed a good proliferation, their architecture was also tridimensional, and their groups were similar to those noted in the previous situations. Chondrocytes were not so large, generally with reduced cytoplasm, but abundant in peripheral cells. After 8



**Figure1.** Human chondrocytes culture  
**A.** immediately after seeding (x20).  
**B.** DMEM without protein supplement (x20)  
**C.** DMEM supplemented with 10% human albumin, week 8 (x10)  
**D.** DMEM supplemented with IGF-I, disposition in chains (x10)

weeks, the cell concentration was  $750 \times 10^3$  cells/ml and the cell viability, assessed by trypan blue technique, was 95%.

Examination of slides showed that chondrocytes exhibited characteristics of maturation; they were round, with a well-defined cell membrane, distinct central nucleus, with dense and homogenous chromatin. The cells were either isolated, or in groups, sometimes in apposition. (Fig.2).



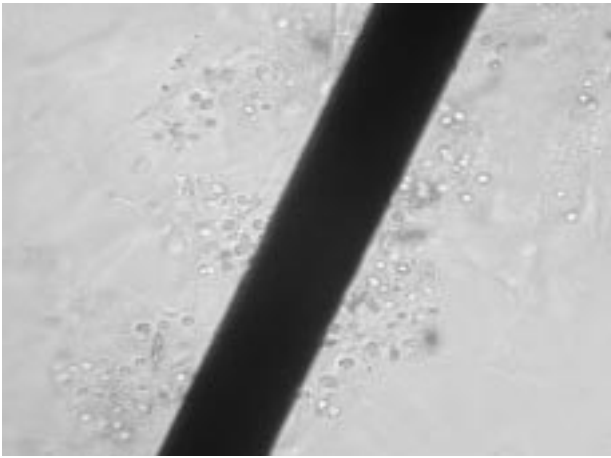
**Figure 2.** Human chondrocytes - Giemsa staining (x40)

These data suggest that at least a two-month period is necessary to obtain a high number of human chondrocytes in culture. Both addition of human albumin and growth factors such as IGF-1 or dexamethasone are required.

## **DISCUSSIONS**

Chondrocytes from isogenous groups are the result of successive cellular divisions which are not accompanied by an appropriate, supplementary secretion of extracellular matrix. Thus, the cells remain in contiguity, without space between them due to accumulation of matrix products. The same reason explains the absence of individual chondrocytes disposed in lacunae; the newly formed tissue lacks an appropriate secretion of matrix.

In our experiment, in most cases, the cell culture showed tridimensional architecture. No monolayer of chondrocytes was observed, and the cells presented no predisposition to reach confluence, as described in the literature. We noted that the proliferation process was initiated by some growth nuclei, such as plastic fragments, catgut or metal alloys. Several isogenous groups settle around them, and gradually grow until they cover the nuclei (Fig. 3). Therefore, research is focused on developing gel scaffolds based on hyaluronic polymers, which will act as a shape and guidance template, supporting a favorable environment for the development of a viable and functional cartilage.<sup>5</sup>



**Figure 3.** Human chondrocytes adhering to a nickel-titanium alloy (x10)

The comparative study of final cell densities reveals that IGF-1 supplement induced the highest proliferation rate, closely followed by dexamethasone, and then by human albumin. When no supplement was added to the medium, the proliferation rate was extremely low.

As we expected, no significant differences were noted among all the experiments. All cultures had the same behavior, and final cell densities had similar values. This phenomenon is due to the fact that all patients were young, between 25 and 35 years old. The functional capacity of chondrocytes diminishes in time, and the response to IGF-1 stimulation decreases with increased age, probably due to alterations of intracellular signal transduction mechanisms.<sup>6</sup>

In our experiment, the lack of protein supplement in the culture medium was not followed by cell death (apoptosis), as reported by Kolletas et al.<sup>4</sup> On the other hand, reduced cell concentration had a positive effect on the proliferation rate, a statement supported by our findings.

IGF-1 is a protein with a molecular weight of 7.6 kDa, which stimulates cellular growth in various tissues: muscle, bone, cartilage. Hunziker proved that at molecular level, this cytokine has the capacity to change gene transcription for matrix components, especially in case of mature, hypertrophic chondrocytes.<sup>7</sup> Moreover, Kolettas et al. identified a factor, Sox-9 transcription factor, which regulates the effect of IGF-1 on the expression of chondrocyte phenotype, being essential for modulation of matrix production.<sup>4</sup> Our experiment also reveals that the highest proliferation rate was reached when the medium was supplemented with IGF-1, but its effect was mainly upon cellular proliferation, which prevailed compared to matrix secretion.

Dexamethasone is a synthetic glucocorticoid which contributes to chondrocyte differentiation and proliferation, effects mediated by the same factor, Sox-

9, responsible for the activation of Col 2a1 (type II procollagen) gene expression.<sup>8</sup> Chondrocytes cultivated in DMEM supplemented with dexamethasone had similar aspect to those obtained when IGF-1 was added, and their similar final concentration in the two situations reinforces the hypothesis of a common action mechanism.

In order to be injected at the site of the joint defect, chondrocytes must be used as a cellular suspension. Therefore, cellular aggregates generated *ex vivo* must be dissociated again to obtain isolated cells. Consequently, we consider justified the proposal to change the implant procedure: transplantation of cellular tridimensional structures, as they develop during culture, which could adhere, multiply and deposit an appropriate matrix because of local factors (which are absent *in vitro*).

Bioengineering companies which “cultivate” chondrocytes were severely criticized for keeping the secret of working protocols, culture media, and enzymatic supplements. Therefore, standardization and quality control of cultivated products is absolutely necessary; the total cell number, cellular viability, the degree of cell differentiation, cell morphology, the levels of secretion of the main matrix components must be specified.<sup>[5]</sup> The price of the “chondrocyte culture for autologous transplantation” is of 11500\$, and there is just one company in USA (Carticel) capable to offer such services. Nevertheless, the price is higher than the price of a classical surgical procedure, and there are no studies concerning the superiority of chondrocyte transplantation, regarding life quality and safety in time. For the moment, the efficacy of this method, as well as its long term course evolution can not be confirmed.<sup>2,9</sup>

## CONCLUSIONS

Our data reveal that obtaining a sufficient number of chondrocytes requires a long period of time, the process being stimulated by protein supplementation and especially by IGF-1 addition. This process is also supported by factors which promote cell adhesion to tridimensional structures such as scaffolds. Unfortunately, cellular aggregates generated *in vitro* do not have the structure and the function of a proper cartilage. Even though autologous transplantation of chondrocytes seems to be a promising one, this method is reserved to a small category of patients, carefully selected, as a secondary therapeutical approach.<sup>9</sup>

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