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FLOW CYTOMETRIC ANALYSIS OF ENCAPSULATED CHONDROCYTES CULTURED IN ALGINATE GEL FOR CARTILAGE TISSUE ENGINEERING

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ABSTRACT. In autologous cell implantation (ACI), the autologous chondrocytes recovered from the patient are amplified in tissue culture prior to re-implantation. The ability to manipulate and reconstitute tissue structure and function in vitro has tremendous clinical implications and is likely to have a key role in cell therapies in coming years. The aim of this study was to investigate and to compare by flow cytometric methods, morphological changes, cellular viability and apoptosis of human chondrocytes cultured in alginate gel and conventional cell-seeding methods for cartilage tissue engineering applications. The results obtained provide the usefulness of the gel in the culture of chondrocytes for reconstructive clinical procedures.

Keywords: chondrocytes, osteoarthritic cartilage, tissue engineering, flow cytometric analysis, microencapsulation, alginat gel, apoptosis, viability test

INTRODUCTION

Tissue engineering is part of a new wave of developments in biomedicine and as a basis for new treatments for musculoskeletal diseases. This has emerged through a combination of many developments in biology, material science, engineering, manufacturing and medicine. Because of the intrinsic biology of cartilage tissue that is devoid of nerves, blood or lymphatic vessels and contains only one cell type, the chondrocyte, articular cartilage lacks the ability for self-regeneration.

The regeneration of functional hyaline cartilage, using expanded chondrocytes and biodegradable polymers, is an important research area in the field of cell-based therapies and tissue engineering that may provide a solution for cartilage reconstruction. Tissue engineering plays an increasingly important role in the functional repair of diseased and missing cartilage that complements reconstructive and orthopedic surgery [Koch et al., 2002; Randolph et al., 2003]. A common remedial approach is to harvest cells from a small tissue biopsy from the donor, expand them in vitro, and seed the population into biodegradable and porous scaffolds, producing an enhanced semi-artificial structure for cell distribution and population in culture. The cell-cultivated construct is then implanted in vivo for extracellular matrix production and, eventually, for cartilage regeneration. It has been asserted that a high-quality cell/scaffold construct requires high spatial uniformity of seeded cells, high scaffold cellularity to enhance the tissue development rate, and sufficient nutrient and oxygen supplies to maintain cell viability [Zhao et al., 2005]. In producing high-quality cell-assisted implantable constructs, cell density may significantly affect cartilage and bone formation [Almarza et al., 2005]. Primary isolated chondrocytes from a small biopsy specimen, which may itself be diseased,

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hinder the in vitro expansion of a clinically useful number of chondrocytes and they have the tendency to de-differentiate in ex vivo culture.

Uniform cell distribution and proliferation in engineered scaffolds are also critical issues in regenerative medicine. The primary goal of research in cell seeding and cultivation is to promote cell population and uniformity in scaffolds before they are implanted in vivo. Production of uniform high cell density is limited, though, by the complexity of the scaffold assembly, insufficient migratory abilities of cells into the structure during seeding, and the tendency of cell movement to the periphery and out of the implantable materials in culture [Li et al., 2001; Radisic et al., 2003; Vunjak-Novakovic et al., 2004]. Consequently, prolonged in vitro cell culture has been required to meet effectual cell density and distribution profiles prior to implantation [Radisic et al., 2003; Vunjak-Novakovic et al., 2004; Liu et al., 2004 Almarza et al., 2005]. Unfortunately, long in vitro culture periods may induce cellular dedifferentiation, limiting the utility of the procedure for patients requiring tissue therapy under expedited terms and yet increasing the possibility of in vitro contamination leading to infection in vivo [Hamilton et al., 2005; Yang et al., 2006].To circumvent elongated cell-culture requirements and improve cell uniformity, a number of methods to increase cell-seeding density have been investigated [Radisic et al., 2003; Vunjak-Novakovic et al., 2004].

A cell-seeding device, utilizing the synergistic effects of vacuum, centrifugal force, and fluid flow, has been used with porous scaffolds [Soletti et al, 2006]. Magnetic nanoparticles have been used to guide fibroblast cells even through commercially available scaffolds; the magnetite nanoparticles were directed with a magnetic field to induce ‘‘mag-seeding,” subsequently increasing cell density and seeding efficiency [Shimizu et al., 2006]. Seeding cells on scaffolds in bioreactors to enhance cell density and uniformity has also been investigated [Radisic et al., 2003; McFetridge et al., 2004; Zhao et al., 2005].

A more simplified method was pursued to increase seeded cell density using protein gels to temporarily engage a well-distributed cell mixture with porous scaffolds [Ushida et al., 2002]. Various gels prepared as cell-encapsulating scaffolds have been investigated for high cell-density seeding. Here, we present a simple gelation method using alginate gel, a natural polymer, as gelling material to incorporate a high cell density in 3D porous scaffolds during cell seeding and restrain the cells in the scaffolds from escaping during the subsequent cell culture, aimed to achieve ultimate high cell density and uniformity in porous structures. Alginate gels have been studied for cartilage tissue engineering applications as a matrix for cellular encapsulation and culture [Guo et al., 1989; Grandolfo et al., 1993; Paige et al., 1995; Aydelotte et al., 1998]. The method involves no complex equipment, and the scaffold system is entirely constructed from natural polymers that have proven their biocompatibility and are favored for a wide spectrum of tissue engineering applications [Guo et al., 1989; Paige et al., 1995].

Previously, to understand the mechanisms underlying the process of cell death in cartilage destruction, we investigated by flow cytometry cellular viability (Cell viability calcein-AM assay) and apoptosis (Light scattering properties of chondrocytes analysis, study of chondrocytes death by Annexin-V-FITC and propidium iodide double-labeling, caspase-3 activity determination) of human chondrocytes isolated from normal and osteoarthritis cartilage [Takacs-Buia et al., 2008].

The aim of this study was to investigate and to compare by flow cytometric methods, morphological changes, cellular viability and apoptosis of human chondrocytes cultured in gel-assisted and conventional non-gel-assisted cell-seeding methods for cartilage tissue engineering applications.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Cambrex Bio Science (Verviers, Belgium), fetal calf serum, penicillin, streptomycin, amphotericin B and L-glutamine were from Gibco (Carlsbad, USA). Hyaluronidase, trypsin, collagenase from Clostridium histoliticum, alginic acid sodium salt from brown algae, CaCl2, NaCl and calcein-AM were from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein-conjugated Annexin-V (Annexin-V-FITC), propidium iodide and HEPES buffer were purchased from Pharmingen (San Diego, CA, USA). The flow cytometer was a Becton-Dickinson FACScan apparatus (San Jose, CA, USA) with CellQuest Pro software for acquisition and analysis.

Isolation of chondrocytes

Osteoarthritic articular chondrocytes were isolated using Green et al., 1971 and Kuettner et al. 1982 protocols from patients with osteoarthritis undergoing arthroplasty under sterile techniques (CF 2 Hospital, Bucharest, Romania). All enzymatic solutions were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with a mixture of antibiotics and antimycotics (penicillin 10 U/ml, streptomycin 10 mg/ml, amphotericin B 0.025 mg/ml), L-glutamine 0.002M and 10% of fetal calf serum. The fragments of cartilage were minced into small pieces and incubated with 0.1% of sheep test hyaluronidase in DMEM medium for 20 min at 37°C. The pieces were washed with PBS (Phosphate Saline Buffer pH 7.4, osmolality 320-330 mosmol kg-1) and maintained in a trypsin solution (0.25 g/100 ml PBS buffer pH 7.4) for 60 min at 37°C. The articular cartilage pieces were washed again with PSB buffer and incubated at 37°C and 5% CO2 overnight in 0.2 % collagenase from Clostridium histolyticum in DMEM medium with 10% fetal calf serum. Cells were then centrifuged for 15 min at 3,000 rpm, washed with PBS buffer and then centrifuged for 15 min at 3000 rpm. The obtained pellet was divided into three equal parts (the one for classical monolayer culture and the others for alginate microencapsulation and control samples).

Monolayer Culture of Chondrocytes

The cells were seeded into 1.5 cm chambers (Nalge Nunc International, Naperville, I L, USA) which provide enough surface area to allow 4x104 isolated chondrocytes to proliferate in DMEM medium. This medium was supplemented with a mixture of antibiotics and antimycotics (penicillin 10 U/ml, streptomycin 10 mg/ml, amphotericin B 0.025% mg/ml), with L-glutamine 0.002M and 10% of fetal calf serum. The cultures were maintained at 370 C in a humidified 5 % CO2 for 7 days.

Microencapsulation of chondrocytes in alginate gel

The chondrocytes (4x104) were suspended in 5 ml NaCl 155mM and mixed whit 0.06 g sodium alginate. The alginate/cell suspension is formed into droplets of uniform size and shape obtained by forcing the suspension through the small orifice of a needle and then breaking up the stream into droplets. The droplets fall into 70 ml calcium chloride solution 102 mM. In presence of calcium chloride the sodium alginate polymerizes and forms microcapsules in which the chondrocytes are entrapped. The obtained microcapsules (10-12 microcapsules/ml culture medium) were transferred into 1.5 cm chambers in 1 ml DMEM medium, and cultured for 7 days at 370 C in a humidified 5 % CO2.

Extraction of chondrocytes from sodium alginate gel

The microcapsules were removed from DMEM medium and washed twice with PBS buffer. The gelled alginate support was liquefied using a chelating agent to remove the calcium ions from the gel by treating with 10 ml mixture of Tris-HCl 0.01 M, 60 mM sodium citrate and 0.2M NaCl. Then the cells

were centrifugated, washed with PBS and analyzed by flow cytometry.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Morphological changes assessment of chondrocytes by light scattered measurements

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. In fact, intensity of light scattered in a forward direction (FSC) correlates with cell size and if is measured at a right angle to the laser beam (side scatter/SSC) it correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light were associated with cell shrinkage.

Flow cytometric assay of cell viability using calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin et al.2005. The membrane-permeable dye calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µM in PBS buffer pH 7.4. Chondrocytes (4 x105 in 200 µl PBS buffer) were incubated with 10 µl calcein-AM working solution (final calcein-AM concentration: 5 µM) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

Cell death assays

Cell death was determined using an annexin-V-FITC/propidium iodide apoptosis kit. Annexin-V is a Ca2+ dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from non-viable cells. Viable cells with intact membranes exclude PI whereas membranes of dead and damaged cells are permeable to PI. Cells that stain positive for annexin-V-FITC and negative for propidium iodide are undergoing apoptosis. Cells that stain positive for both annexin-V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Cells that stain negative for both annexin-V-FITC and PI are alive and not undergoing measurable apoptosis. Chondrocytes were washed with PBS buffer pH 7.4 and the were resuspended (2x105) in 100 µl of 1x binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). 10 µl propidium iodide and 5 µl annexin-V-FITC were added and incubated for 30 min at room temperature in the dark. After adding 400 µl of 1x binding buffer, the suspension was analysed in the flow cytometer and gated for biparametric histograms FL1 (FITC fluorescence) versus FL2 (PI fluorescence). The light scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. All studies were performed at least three times with three replicates each time.

RESULTS AND DISCUSSIONS

Light scattering properties of chondrocytes cultured in monostrat and alginate gel

The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure.

During apoptosis, the decrease in forward light scatter (which a results from cell shrinkage) is not initially paralleled by a

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decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents Darzynkiewicz et al., 1997.

Figure 1 shows the morphological changes of human osteoarthritic chondrocytes cultured in different ways which were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of the characteristic features of apoptosis. After 7 days of chondrocytes culture (seeding) using a standard protocol (Fig.1 B1, B2), we noticed the separation of the same regions as before, with maintenance of roughly the same percentage of viable cells (24.5%). However, when we grew the chondrocytes in alginate microspheres culture, the cells presented a more homogeneous distribution in the FSC/SSC plots (Fig 1 C1, C2), and about 64% of them remained in the region (R1) of the dot-plot, described as viable region.

Consequently, we can conclude that the three-dimensional culture system in alginate microspheres allows chondrocyte division, while normal cell morphology is retained in a single homogeneous population.

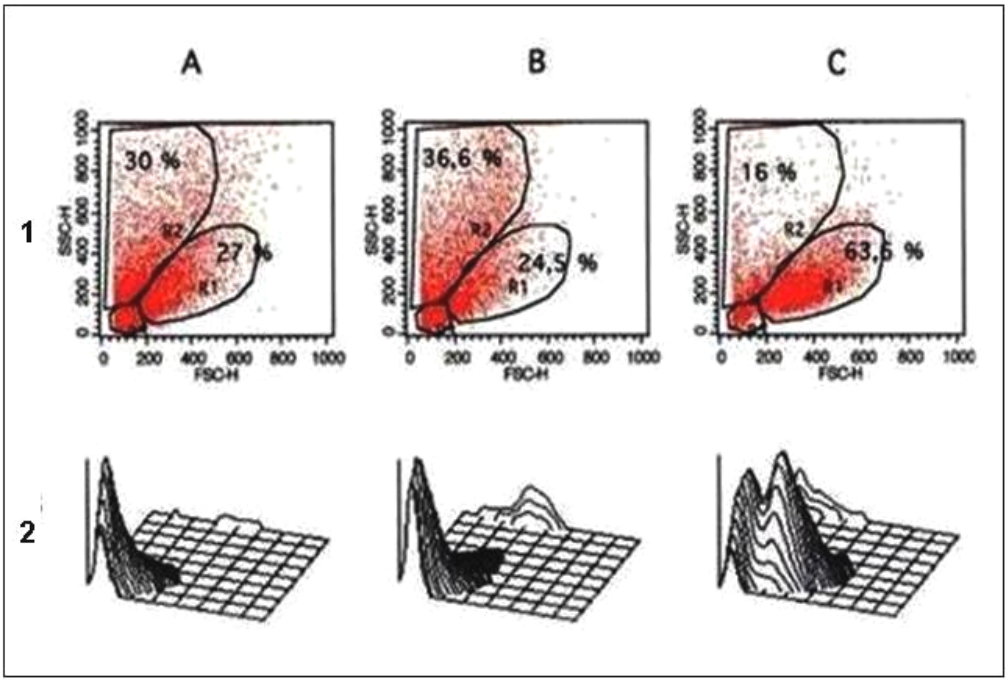

Fig. 1 Flow cytometric analysis of human osteoarthritic chondrocytes morphological changes after 7 days cultured in classical monolayer system (B) or in alginate gel microencapsulated (C). (A): Osteoarthritic chondrocytes before seeding. 1: Dot-plot; 2: 2: Tridimensional plot. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity or refractiveness). R1: viable chondrocytes; R2: apoptotic chondrocytes; R3: cellular remainders of chondrocytes. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

Flow cytometric assay of chondrocytes viability using calcein-AM

We have applied the flow cytometric assay that we recently developed for the measurement of RBCs viability using calcein-AM [21]. The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivative and non-fluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes.

Application of this assay to chondrocytes cultured in monostrat layer (Fig. 2B) or in alginate gel (Fig. 2C) showed that two regions could be clearly and unambiguously defined: (i) the region of fluorescent cells with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) and (ii) the region of non-fluorescent dead cells with damaged cell membranes (region M2). In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure [21].

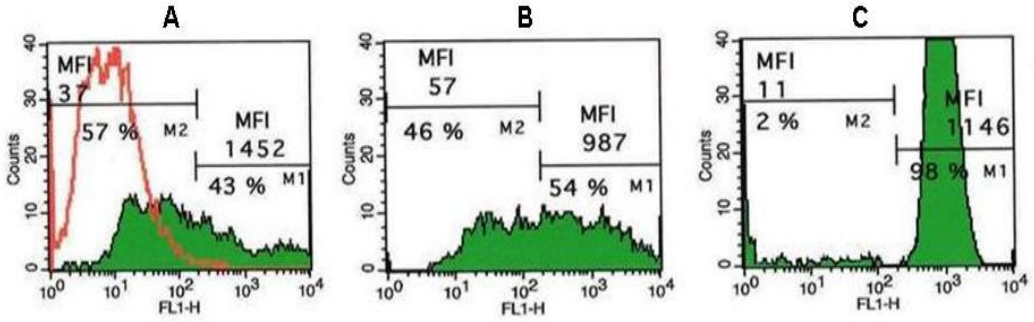

Fig. 2 Comparative flow cytometric histogram analysis of human osteoarthritic chondrocytes viability by cell esterase activity measurement using calcein-AM. A: Osteoarthritic chondrocytes before seeding B: after 7 days of monostrat layer culture and C: after 7 days of culture in alginate gel. Abscissae: log scale green fluorescence intensity of calcein (FL1). Ordinates: relative cell number. M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Numbering refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

We compared the viability of chondrocytes grown in standard culture or with alginate microspheres. When the cultures started, viable osteoarthritic chondrocytes represented 43% of the population, with a MFI of 1452. After 7 days of culture using standard method, viable chondrocytes amounted to 54% of the cell population, and had a reduced MFI of 987. In the same time, chondrocytes grown in mixture with alginate microspheres were 98% viable, with an MFI of 1146, which is closer to the initial value. In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure [Bratosin et al., 2005]

On the basis of these results we can conclude that by including the chondrocytes in alginate microspheres, in a three-dimensional culture system, cellular viability is significantly improved, as shown by both viable cell percentage and esterase activity measurements.

Study of cell death by Annexin-V-FITC / Propidium Iodide double – labeling

Chondrocytes cultured in standard culture or in alginate microspheres were analyzed by flow cytometry for phosphatidylserine (PS) exposure (annexin-V labelling) and

membrane permeabilization (propidium iodide labelling). Phosphatidylserine residues are exposed in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally observed during late apoptosis and cell necrosis. Figure 3 shows comparative flow cytometric analyses of annexin-V-FITC / propidium iodide double-stained chondrocytes cultured in different ways.

The osteoarthritic chondrocytes before seeding, as well as from monostat or alginate microsphere cultures, were analyzed by flow cytometry for externalization of phosphatidylserine (PS, labeled with annexin-V) and for cellular membrane permeability (staining with propidium iodide). Fig. 3 shows a region-based comparative analysis by which we simultaneously identified viable cells, as well as apoptotic and necrotic cells, using a double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2).

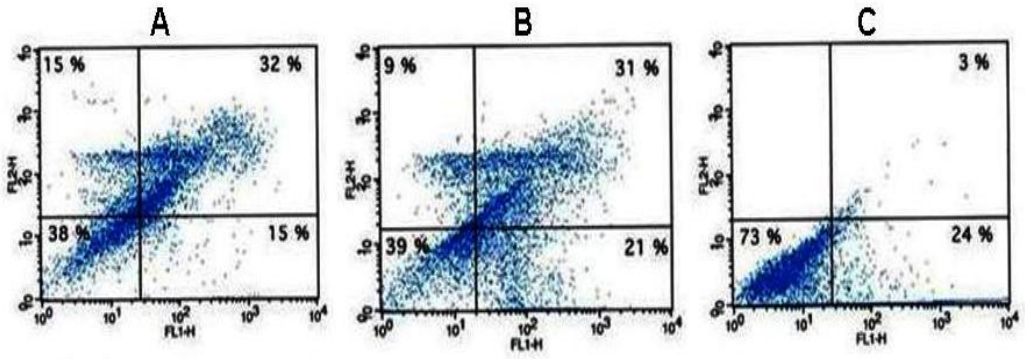

Fig. 3 Comparative flow cytometric quadrant analysis of annexin-V-FITC (FL1) and propidium iodide (FL2) double-stained to assess chondrocyte phosphatidylserine exposure and cell membrane integrity, respectively. (A): Osteoarthritic chondrocytes before seeding; (B) after 7 days of monostrat layer culture and (C) after 7 days of culture in alginate gel. Lower left quadrant: viable cells (annexin V and propidium iodide negative cells); Lower right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); Upper right quadrant: dead cells (annexin-V and propidium iodide positive cells). Abscissae: annexin-V-FITC fluorescence; ordinates: propidium iodide fluorescence. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed

The initial osteoarthritic chondrocytes had 38% viable cells, 15% cells in early apoptosis, and 32% cells in late apoptosis (necrosis), respectively. After 7 days of growth in standard culture, the population was composed of 39% viable cells, 29% early apoptotic cells, and 31% late apoptotic (necrotic) cells.

When the cells were grown in alginate microspheres, we found 73% viable cells and only 24% apoptotic cells after 7 days of culture. We were thus able to identify a better correlation with the established FSC/SSC analysis for apoptosis. This analysis allowed distinction between two populations: viable cells (high FSC and low SSC, region R1), and apoptotic cells (low FSC and high SSC, region R2).

CONCLUSIONS

Our data, in the context of other published findings, underline the advantages of culturing chondrocytes with alginate microspheres. In fact, chondrocytes cultured using this method represent a single phenotypic population with a tight cellular uniformity as shown by the FSC/SSC analyses and show a very high viability (over 90%). These findings recommend the implementation of this biotechnology in tissue engineering.

The results we obtained also emphasize the advantages of using flow cytometry to quantitatively evaluate various methods of growing chondrocytes in tissue culture. This analysis method was very useful in characterizing cell viability, which may represent the starting point for using this method to screen for growth factors and better culture conditions.

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