CHONDROCYTE CULTURES IN TRIDIMENSIONAL SCAFFOLD: ALGINATE HYDROGEL

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ABSTRACT

Objectives: The aim of this study was to culture chondrocytes from knee joint cartilage of rabbits encapsulated in alginate hydrogel (HA) and to characterize the production of extracelular matrix (ECM). Methods: Joint cartilage was obtained from rabbits' knees, three to six months old, fragmented into 1-mm pieces and submitted to enzymatic digestion. A concentration of 1x106 cells/mL were re-suspended into a 1.5% (w/v) sodium alginate solution, followed by gel formation process with CaCl2 (102 mM), allowing HA to build for culturing it into a DMEM-F12 medium for four weeks. The distribution of cells and ECM were assessed from histological slices stained toluidine blue and hematoxyline-eosin (HE).Results:There was an increase of the number and viability of the chondrocytes during the four weeks of culture. By assessing the histological sections stained with toluidine blue and HE, we could note the definitive distribution of chondrocytes in the hydrogel, similarly to isogenous groups and territorial matrix formation. Conclusion:In this study, the alginate was shown to be an effective scaffold for use in chondrocytes culture, constituting an alternative for repairing joint cartilage defects.

Keywords: *Articular Cartilage. Chondrocytes. Alginate. Hydrogel. Regeneration. Tissue engineering.*

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INTRODUCTION

Once damaged, cartilage never recovers spontaneously, probably due to the little effective repair of the collagen meshwork, since proteoglycans deficit is reversible.[1] About one million people a year are estimated to require treatment for cartilage defects, particularly those on knee joints.[2] Experimental studies have shown that there is a reduction of growth activity on cartilage with age. However, tissue engineering offers new opportunities for functional and structural restoration of an injured tissue.[3]

In literature, chondrocytes are reported to require a 3-D scaffold in order to grow and preserve the morphology and production of natural chondrocyte matrix components when in cell culture.[4] Otherwise, when cultured in monolayers, cells tend to adhere to the bottom of culture plates, and go through a dedifferentiation process, where they acquire morphologic characteristics and start to produce fibroblastic matrix components, such as type-I collagen.[5-7] In order to produce a functional cartilaginous tissue, it is crucial to avoid chondrocyte dedifferentiation during cartilage engineering process.[8]

Chondrocyte culture in 3-D scaffold is based on culturing these cells in an artificial biodegradable matrix able to stand cartilage growth for some months, while chondrocytes and the matrix are being established. The principle consists of producing cells through matrix in order to implant them on a joint defect. The matrix gradually degrades within 8-10 weeks after cartilage implant.[9]

Recently, a variety of hydrogel and synthetic polymers matrixes have been investigated for in vitro chondrocyte expansion for repairing an injured cartilage. Such matrixes include collagen-based scaffolds: type-I and II collagen gel, type-II collagen sponges, polylactic acid and polyglycolic acid, fibrin, polyethylene oxide, peptides and alginate.[10,11]

Chondrocyte culture in alginate hydrogel constitutes the most frequently recommended method for isolating those cells.[12] Alginate is a linear anionic polysaccharide (n - guluronic acid - manuronic acid), with ability to reversibly transform itself into gel in the presence of calcium or other 2-valent cations.[13,14]

The advantage of this polymerized polysaccharide is its non-toxic, biocompatible constitution that can be injected into animal models. Alginate-encapsulated chondrocytes do not adhere to the matrix, enabling an easy retrieval after cultured, allowing the study of protein and genetic expression. By this method, we can

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keep the expression of a differentiated phenotype, restore non-differentiated chondrocytes, and form a similar extracellular matrix to that found on joint cartilage.[12,15]
This study targeted the culture of alginate hydrogel-encapsulated chondrocytes, the characterization of extracellular matrix (ECM) production of a tissue produced in vitro so that this hydrogel could possibly be used in autologous implants aiming to regenerate injured joint cartilages.

MATERIALS AND METHODS

Materials

The culture mediums used in this study were the following: DMEM: Ham's F12 (Dulbecco`s modified Eagle's medium) (1:1), Ham's F12 medium, bovine fetus serum (BFS), antibiotics: 10U/ml penicillin G, 10μg/ml streptomycin and 25μg/ml amphotericin B, trypsin 0.25%/EDTA, type-I collagenase, all of these obtained from GIBCO Invitrogen Corporation. Alginic Acid Low-Viscosity Sodium Salt obtained from Macrocisti sp, hyaluronidase, ascorbic acid, all of these from Sigma . 2-hydrated calcium chloride (Fluka) and sodium chloride (LABSYNTH).

Animals

For this study, 10 3-6 month-old Botucatu rabbits were used. The animals were weighted and then anesthetized with sodium pentobarbital (30 mg/ Kg) administered by central or marginal veins of the ear. This research was approved by the Committee of Ethics in Animal Research of Botucatu Medical School, according to protocol nr. 345.

Chondrocyte isolation

Joint cartilage was removed from the femoral condyle, fragmented into ± 1mm pieces and submitted to extracellular matrix digestion with the following enzymes: trypsin (0.25%), hyaluronidase (2mg/mL) for 45 minutes each enzyme and type-I collagenase (0.45%) at 37°C for 16 hours.

Chondrocyte culture in Alginate Hydrogel

After enzymatic digestion, the cell suspension was filtered with a 70-μm nylon filter, centrifuged, and the cells were re-suspended at a density of 1x106 cells/mL into 1.5% (w/v) sodium alginate solution. The cells suspended into non-polymerized alginate were transferred to a 10 cc syringe with a built-in 21 G needle, and subsequently dispensed from the syringe drop by drop into the gel-forming solution ($CaCl_2$ - 102 mM) with moderate magnetic movement, allowing alginate polymerization during 10 minutes until the "hydrogels" were formed. The gel-forming solution was discharged and the hydrogels were washed 3 times with 5 vol. NaCl at 0.15 M. The hydrogels were cultured into DMEM-F12 medium supplemented by ascorbic acid (50 μg/mL) and 10% BFS. The culture was kept in a heater at 37°C in a wet atmosphere at 5% CO2 and 95% air for four weeks. The culture mediums were refreshed at each 2 days.

Chondrocyte retrieval after hydrogel culture

Chondrocytes retrieval from the hydrogel to access cell viability was performed by dissolving the hydrogel in sodium citrate (155mM), for 20 minutes in a heater at 37°C. Subsequently, the sample was centrifuged and dissolved into trypan blue solution.

Cell Count and Viability

Cell count and the determination of cell viability were conducted by using a Neubauer chamber. The number of vital cells was determined by the technique of exclusion of the non-vital cells stained with trypan blue solution, as recommended by Freshney, 2001.[12]

Morphological Analysis

Morphological analysis of the cells was routinely performed using phased microscopy under inverted microscope (Axiovert 200 – Carl Zeiss). Cell cultures were photographed on a weekly basis.

Extracellular Matrix Characterization

In order to assess the production of extracellular matrix components, the samples were fixated in 4% formaldehyde, exposed to increasing alcohol solutions (70%, 95% and 100%), included into resin Historesina - Leica and then sectioned at the Leica microtome. The obtained sections were stained with 0.3% toluidine blue pH 3.65 and Hematoxylin and Eosin (HE).

RESULTS

Chondrocyte isolation

We experienced difficulties on standardizing the enzymatic digestion of the joint cartilage. On the first assays, digestion was provided with agitation using two enzymes: trypsin and type-I collagenase; low cellularity and viability was achieved, at an average of 60%. After some standardization attempts, we managed to determine the use of three enzymes for the digestion process: trypsin, hyaluronidase and collagenase at different concentrations and times, using the static digestion model. We achieved a cell viability above 90% in most of the samples digested by this protocol. In all collections, we could get cartilage samples weighting 170mg - 475 mg. (Table 1)

Table 1 – *Weight values of cartilaginous tissue biopsies, number of cells and viability at baseline.*

Biopsy weight (mg)	Number of Cells at Baseline/mm^3	Viability at Baseline
221	1.6x10^5	99%
300	1.8 x10^5	94%
391	1.0 x10^5	63%
475	1.9 x10^5	97%
402	2.8 x10^5	93%
326	3.7 x10^5	92%
473	2.8 x10^5	99%
170	5 x10^5	96%
234	6 x10^5	84%
288	1.2 x10^5	94%

Chondrocyte culture

When cultures were made as monolayers, after 12 hours in culture, chondrocytes adhered to the Petri plate, forming cell colonies with fibroblastoid morphology, indicating that they have lost its rounded phenotype (Figures 1A and 1B). At first, non-differentiated chondrocytes present long phylopodes. After one week in culture, cell confluence was found on the culture plate with fibroblastoid morphology (Figure B).

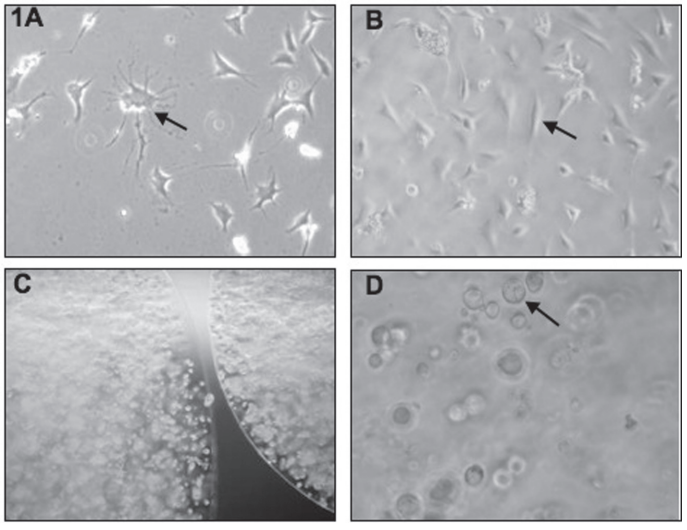

Figure 1 – *Phase contrast microphotography. (A) Chondrocyte culture as monolayers. Note the elongated cells with several protrusions (arrow) (Magnification: 1,000x). (B) Culture as monolayer, confluent after 2 weeks in culture, showing chondrocytes with fibroblastoid morphology (arrow) (Magnification: 1,000x). (C) Chondrocyte culture in alginate hydrogel. The arrow indicates translucent alginate hydrogel boundaries. Within alginate hydrogels, note the uncountable spherical and rounded whitish structures representing high cellularity at the beginning of culture (Magnification: 100x). (D) Note the rounded phenotype of chondrocytes (arrow) within the alginate hydrogel (Magnification: 1,000x).*

Forty alginate hydrogels were obtained from a mean amount of 400 mg of collected joint cartilage. Each hydrogel measured 3 mm in diameter. (Figure 2) After alginate hydrogel polymerization, we could see under microscope a homogenous distribution of the chondrocytes in each hydrogel. (Figure 1C). The chondrocytes maintained a spherical morphology similar to the original cartilage (Figures 1C and 1D) when compared to monolayer culture (Figures 1A and 1B), remaining fixed at the polymer. Since the first days of culture, the presence of cell agglomerates on alginate pearls had demonstrated strong cell division, similarly to isogen groups of the newly-formed hyaline cartilage. (Figure 1D) By weekly accessing cell viability on alginate pearls stained with trypan blue, the increased cellularity and viability maintenance can be confirmed by comparing it to the number of baseline cells (Figure 3). On the fourth culture week, high cellularity was observed by microscopy, compared to the first week, and all hydrogels remained intact, in addition to the maintenance of a rounded phenotype of the chondrocytes.

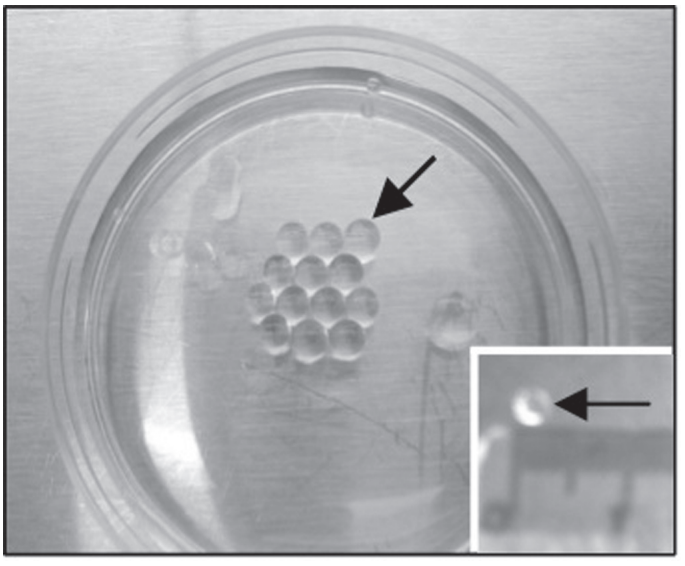

Figure 2 – *(A) Several alginate hydrogel spheres submitted to gel-forming process (arrow). (B) One alginate hydrogel sphere with ~3mm in diameter (arrow).*

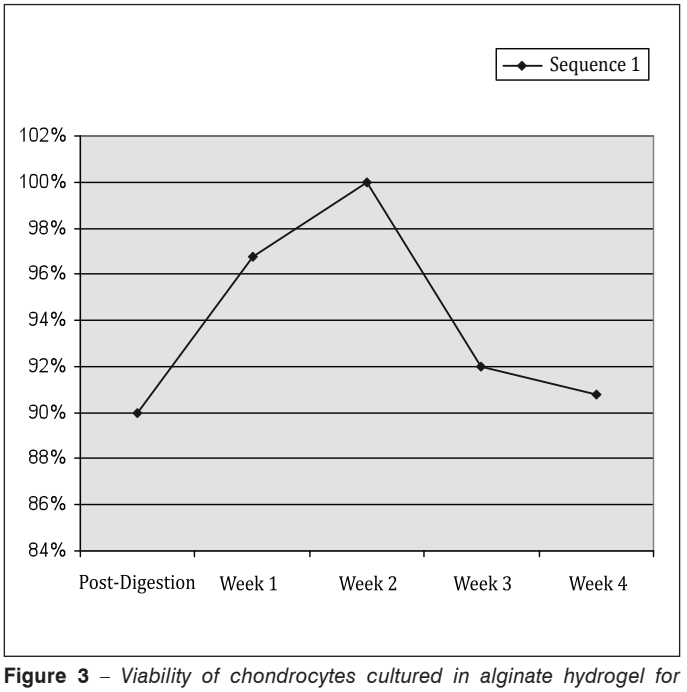

Figure 3 – *Viability of chondrocytes cultured in alginate hydrogel for 4 weeks*

Extracellular Matrix Characterization

Figure 4A shows a broad view of the whole alginate hydrogel stained with toluidine blue, where it demonstrates the high cellularity and the organized distribution of chondrocytes within the hydrogel.

On histological sections (Figures 4B and 4C) we could see a stronger color on pericellular and/or territorial matrix areas around chondrocytes, calling them as chondrocyte groups (isogen groups). On Figure 4B pericellular matrix formation is quite evident with gap forming around the chondrocytes and maintenance

of a round morphology similar to that of the original cartilaginous tissue. On Figure 4C, a group of chondrocytes with high cellularity was found indicating the multiplication of these cells on the hydrogel, with pericellular and territorial matrix production, as stained with toluidine blue. Pericellular and territorial matrix stained with toluidine blue revealed the production of proteoglycans, main component of that region.

As shown on Figure 4D, HE-stained sections showed no inter-territorial matrix production, but chondrocytes stuck into their gaps producing pericellular and territorial matrix.

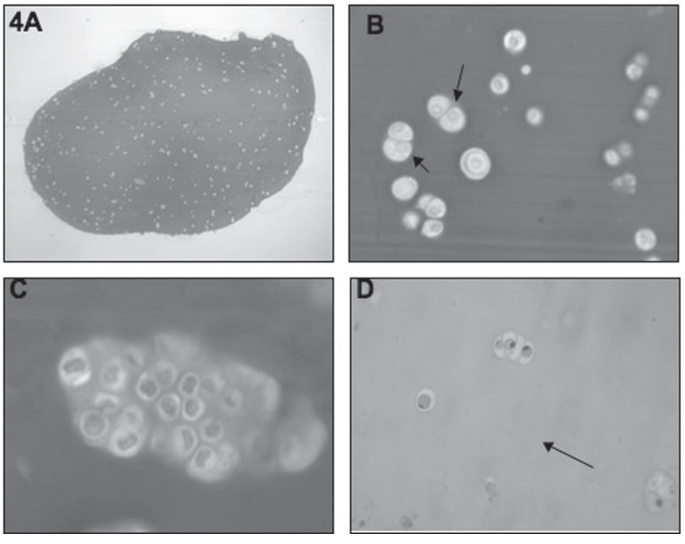

Figure 4 – *Histological analysis of chondrocyte culture in alginate hydrogel (A, B and C); toluidine blue stain, (D) HE stain. (A) Histological section of the whole alginate hydrogel, showing an uniform distribution of chondrocytes within the hydrogel (Magnification: 25x). (B) Evidence of pericellular and inter-territorial matrix formation (arrow) (Magnification: 1,000x). (C) Dividing chondrocyte groups (Isogen groups) (Magnification 1,400x). (D) Absence of territorial matrix (arrow) (Magnification 1,000x).*

DISCUSSION

Tissue engineering represents a promising method for building autologous chondrogenic grafts in reconstructive orthopaedic surgery. The existence of several matrixes and specific growth factors causes the production of cartilaginous tissue in vitro and in vivo.

Chondrocytes are cells that need 3-D culture in order to maintain their original phenotype, because, as monolayers, these cells dedifferentiate, producing matrix components such as type-I collagen characteristic of fibrocartilage and not of the hyaline cartilage, therefore resulting from a change on gene expression.[3] When we work with joint cartilage, only the hyaline cartilaginous tissue is of interest, because, in a joint implant, the production of type-I collagen indicates fibrosis, i.e., the change of hyaline tissue into fibrous tissue.

Alginate matrix can be used both in implants and in chondrogenesis studies, an important factor for understanding the physiology of the cartilaginous tissue.[10]

According to Masuda[17], chondrocyte culture in alginate stabilizes for up to eight months the chondrocytic phenotype of these cells. In the present article, we showed the results of 4-week culture, but, in other experiments, we managed to culture chondrocytes for up to 4 months without losing these cells' phenotype.

Alginate strength much influences cell metabolism and the diffusion of required components from the culture medium. The optimal concentration found throughout the experiment was found to be 1.5% in a range of 1.0%, 1.2%, 1.5% and 2%. Another key factor is that the preparation of alginate must be made on the same day, because experiments using alginate previously prepared showed lower gel consistence and the appearance of elongated cell groups, showing a fibroblastoid phenotype. A study conducted by Domm et al.[18] evidenced by experiments that the kind of alginate may influence chondrocyte culture, consistently to our findings.

Cartilaginous tissue consists of chondrocytes sparsely distributed surrounded by extracellular matrix (ECM). Chondrocytes are responsible for maintaining ECM and consequently cartilaginous tissue. ECM can be divided into pericellular, territorial, and inter-territorial compartments containing type-II collagen and the aggrecan proteoglycan.[3] Alginate hydrogel was effective in maintaining chondrocytes phenotype, in cell proliferation, in producing pericellular and territorial matrix; however, it was regarded as ineffective for producing inter-territorial matrix.

Many researchers have used alginate hydrogel as chondrocyte-sustaining scaffold for implants in injured joints on animal models. The advantage of this hydrogel is its non-toxic property and its use as injection. That scaffold can be injected together with in situ chondrocytes, enabling its change into gel with calcium chloride at injury site.[3,15] Alginate is degraded through enzymatic paths on the tissue itself into two monomeric sub-units: glucoronic acid and manuronic acid.

In this study, we could obtain data about alginate hydrogel in vitro. For a clinical application of alginate hydrogel, further implants in pre-injured areas of knee joints will be required, using rabbits as animal models in order to confirm its effectiveness in producing in vivo hyaline cartilaginous tissue, which indeed constitutes the next phase of this study.

CONCLUSION

Chondrocyte culture in alginate hydrogel showed high cellularity and an increased production of pericellular and territorial matrix when compared to the inter-territorial.

Alginate hydrogel constituted an effective chondrocyte culture and sustaining scaffold, keeping its rounded phenotype, similar to the original cartilage, being an important scaffold for use in joint implants.

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