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Differentiation of dental pulp stem cells into chondrocytes upon culture on porous chitosan-xanthan scaffolds in the presence of kartogenin

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ABSTRACT

Adhesion, proliferation and differentiation of dental pulp stem cells (DPSCs) into chondrocytes were investigated in this work with the purpose of broadening the array of cell alternatives to the therapy of cartilage lesions related to tissue engineering approaches. A porous chitosan-xanthan (C-X) matrix was used as scaffold and kartogenin was used as a selective chondrogenic differentiation promoter. The scaffold was characterized regarding aspect and surface morphology, absorption and stability in culture medium, thickness, porosity, thermogravimetric behavior, X-ray diffraction, mechanical properties and indirect cytocompatibility. The behavior of DPSCs cultured on the scaffold was evaluated by scanning electron microscopy and cell differentiation, by histological analysis. A sufficiently stable amorphous scaffold with mean thickness of 0.89 ± 0.01 mm and high culture medium absorption capacity (13.20 ± 1.88 g/g) was obtained, and kartogenin concentrations as low as 100 nmol/L were sufficient to efficiently induce DPSCs differentiation into chondrocytes, showing that the strategy proposed may be a straightforward and effective approach for tissue engineering aiming at the therapy of cartilage lesions.

1. Introduction

Degeneration of cartilage tissue is often triggered by injury. Frequently, it leads to progressive loss of this tissue as well as to subchondral bone sclerosis, potentially resulting in chronic joint diseases such as osteoarthritis (OA). OA mainly affects the articular cartilage, which is a non-self-repairing tissue, due to its low number of cells, being considered an avascular and aneural tissue [1–3].

Osteoarthritis was considered in 2010 as the eleventh leading cause of years lived with disability, becoming then a research priority in the European Community countries [4]. Since in the coming years percentages of the world population as high as 25% may develop some type of OA, alternative approaches to the treatment of this disease may be particularly valuable.

The most commonly used treatment for OA is total joint replacement performed by reparative surgery, but it frequently results in musculoskeletal complications and fibrocartilage formation. The use of biological approaches, such as tissue engineering, to repair these injuries and restore cartilage functions would be an ideal solution. Through tissue engineering, biodegradable and biocompatible scaffolds seeded with specific cells could be implanted in the lesioned area, gradually healing the tissue and restoring its original function [5].

An ideal scaffold should meet several criteria, such as being biocompatible to reduce local immune response to its presence. It should also be biodegradable and show adequate resorption rate, providing adequate three-dimensional support for cell growth, while progressively degrading simultaneously to the new tissue formation. In addition, it should have appropriate porosity and interconnectivity, to allow cell mobility and transport of nutrients and waste products [5,6]. Particularly for cartilage substitution, the choice of biomaterials is narrow. Most of the scaffolds are produced using natural or synthetic polymers, such as Chondro-Gide (composed by collagen I and III) and MACI (third generation porcine collagen scaffold) [7].

Natural polymers are the prevalent raw materials for the production of scaffolds for cartilage tissue engineering due to their biocompatibility and potential for effective cell attachment and differentiation. The most used natural materials for this purpose comprise the carbohydrates hyaluronic acid, agarose, alginate and chitosan as well as collagen and fibrin glue [5,8].

Chitosan, produced through the desacetylation of chitin found mostly in marine crustacean shells wastes, is the only known positively charged polysaccharide. For that reason, particular attention has been given to its ability to form polyelectrolyte complexes (PEC) through electrostatic interaction with oppositely charged natural polymers, such as xanthan gum [9].

Xanthan gum is an exopolysaccharide of significant industrial importance secreted by *Xanthomonas campestris* [9,10]. Its backbone is composed of five β-D-glucose units linked at the 1 and 4 positions,

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and on each second glucose residue, a trisaccharide side chain consisting of (3,1)-α-D-mannopyranose-(2,1)-β-D-glucuronic acid-(4,1)-β-D-mannopyranose is inserted.

The PEC formed between xanthan gum and chitosan may be casted in several structures, such as hydrogels, particles and membranes. Chitosan-xanthan gum membranes, particularly, have already proved to be useful in combination with multipotent mesenchymal stromal cells for the treatment of skin wounds [11].

Mesenchymal stem cells (MSCs) of different sources can be differentiated into chondrocytes, *e.g.* adipose tissue, bone marrow and embryonic tissue cells. However, the range of cell options is limited, for instance, by ethical issues, as in the case of embryonic stem cells. This problem is aggravated in situations in which autologous stem cells are required. Thus, alternative sources of adult tissue stem cells are of major importance for the consolidation of regenerative medicine based on tissue engineering approaches. Among them, dental stem cells offer an array of advantages, as high plasticity, possibility of cryopreservation for long periods without differentiation ability loss, good interaction with growth factors and scaffolds, as well as easy harvesting [11].

There are many growth factors that promote chondrogenesis, as the transforming growth factor-beta superfamily, which includes TGF-β1, TGF-β3, BMP-2, BMP-4, BMP-7 and GDF-5, in addition to insulin-like growth factor 1 (IGF-1) and the fibroblast growth factor (FGF) family [12]. More recently, a new low molecular mass heterocyclic molecule, kartogenin (KGN), was pointed as capable of promoting robust chondrocyte differentiation of MSCs [13]. The presence of a carboxyl acid group allows direct conjugation through ionic interaction with the amine group of chitosan. Given that KGN also shows in its structure two hydrogen bond donor sites as well as three hydrogen bond acceptor groups, and since both polysaccharides used for the production of the scaffold can also participate of H bonding, kartogenin can be effectively but reversibly attached to the structure of the matrix, without the need of costly or cumbersome binding strategies, as previously reported [2,13,14].

Many successful studies focusing the field of cartilage tissue engineering are reported in the literature for different types of cells and scaffolds. Akaraonye et al. [15], for instance, evaluated the use of poly(3-hydroxybutyrate)/microfibrillated bacterial cellulose (P(3HB)/MFC) composites as 3D-scaffolds for cartilage repair, observing adequate attachment and proliferation of mouse chondrogenic ATDC5 cells. Dahlin et al. [16], on the other hand, explored the capability of co-cultures of bovine articular chondrocytes and mesenchymal stem cells isolated from the femora and tibiae of Lewis rats to repair articular cartilage when seeded onto electrospun poly(caprolactone) scaffolds and implanted into osteochondral defects of Lewis rats, reporting that this approach is indeed functional. Rodrigues et al. [17] seeded ATDC5 prechondrocyte cells on membranes made of chitosan and chondroitin sulfate obtained through a polyelectrolyte complexation sedimentation/evaporation method, noticing that the membranes sustained cell adhesion, inducing their rearrangement in typical chondrogenic aggregates and the expression of specific cartilage markers.

Given that no studies were found in the literature focusing the culture and differentiation of dental pulp stem cells into chondrocytes using chitosan-xanthan gum matrices as scaffolds, the objective of this study was to analyze this possibility.

2. Materials and methods

2.1. Materials

Chitosan-xanthan gum (CX) scaffolds were prepared using chitosan with medium molecular weight (190–310 kDa) and deacetylation degree around 75% (Sigma-Aldrich Co.; Lot # STBF3507V; product reference: 448,877), xanthan gum from *Xanthomonas campestris* (Sigma-Aldrich Co.; lot # 108 K0038, product reference: G1253), Poloxamer 188 solution at 10% (Sigma-Aldrich Co.) and acetic acid (Synth). The water used was purified in a MilliQ system from Millipore. All other reagents used were of analytical grade.

2.2. Scaffold preparation

The CX scaffold was prepared according to adaptations of the procedures described by Bellini et al. (2012) [18]. The scaffold was prepared using a xanthan to chitosan mass ratio of 1:1.

A peristaltic pump (model minipuls 3, Gilson) was used to add 200 mL of a 1% Ch solution (*w*/*v*) in 1% (*v*/v) acetic acid, at a flow rate of 10 mL/min, to 215 mL of a 0.93% Xn aqueous solution (*w*/*v*) at pH 7.7 containing 7% of Poloxamer 188. During the addition, which took place in a reactor with 10 cm internal diameter and 20 cm height maintained at 25 °C, the mixture was stirred at 1000 rpm with a mechanical stirrer (model Q-251 D, Quimis). The suspension obtained was transferred to two polystyrene Petri dishes (15 cm in diameter) and dried in an oven with air circulation (model 410D, Nova Ética) at 37 °C for 48 h. After that, the dried scaffolds were immersed twice in 200 mL of deionized water for 30 in, to remove the residual acetic acid and Poloxamer 188. Then, the scaffolds were washed twice by immersion in 200 mL of 10 mM HEPES buffer for 30 min and dried again in the same conditions mentioned, but for 24 h.

The scaffolds were cut into appropriate sizes and sterilized with ethylene oxide (EO) by exposure to Oxyfume-30 (30% EO and 70% carbon dioxide) for 8 h at 40 °C and relative humidity of 30–80% at Acecil Central de Esterilização Comércio e Indústria (Campinas, SP, Brazil) [19,20]. The resulting material was stored in a desiccator with silica gel.

2.3. Scaffold characterization

The scaffold was characterized as described below regarding morphology, thickness, mechanical properties, absorption and stability in culture media. Thermogravimetric analysis and determination of crystallinity were also performed. All quantitative results are expressed in terms of mean values attained for at least three individual repetitions accompanied by their respective standard deviations.

2.3.1. Morphology

The scaffold morphology was analyzed using a scanning electron microscope (Leo 440i model, Leica). The material was cut in samples of 1 cm × 1 cm and coated with an ultra-thin gold layer (92 Å) in a mini sputter coater (model SC 7620, VG Microtech) before the analysis.

2.3.2. Thickness

The thickness of 5 cm × 2.54 cm samples was measured at five different regions using a digital micrometer (model Mitutoya, MDC-25S, Japan).

2.3.3. Porosity

Membrane porosity was determined by gravimetric analysis based on previously described methods [15,21–23]. Basically, the membrane was weighed before and after immersion in culture medium for 24 h, and porosity was calculated considering both the volume of solved absorbed and the swelling of the matrix, according to Eq. 1:

$$\varepsilon(\%) = \frac{\frac{w_w - w_d}{\rho_{cm}}}{V_t} \times 100 \quad (1)$$

where w_w is the weight of the wet membrane, w_d is the weight of the dry membrane, ρ_{cm} is the culture medium density and V_t is the final volume of the membrane after culture medium absorption. All experiments were performed in at least triplicate.