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Formulation of Different Chitosan Hydrogels for Cartilage Tissue Repair

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Different formulations of Chitosan/sulphate and Chitosan/PEGDE were produced by physical and chemical reticulation to obtain hydrogels with better physiochemical properties. The hydrogels were analyzed – in terms of their non-toxicity, proliferative, differentiative, inflammatory and immunology responses.
Commercial grade Chitosan (Sigma) was solubilized and purified by progressive filtrations. Then, the polymer was freeze-dried in a water soluble cationic form. A physical hydrogel was prepared by mixing a 3 % w/w water solution of the a.m. polymer with different stoichiometric ratios of ($SO_4^{=}$) 1/0.5;1/0.75;1/1 respectively. The hydrogels were cross-linked in multiwells. The chemical hydrogel was synthesized mixing a 3 % w/w water solution of the a.m. polymer, with PEGDE. The hydrogel was purified by water diffusion, then loaded into multiwells. All gels were freeze-dried and sterilized by EtO. Primary bovine chondrocytes were extracted by collagenases (ABIEL) and Pronase (Sigma) from articular hoof tissue. MTS and Acrydin Orange assays were performed to quantify cell viability and apoptosis. CD11b, CD69 and ROS activation were evaluated in inflammatory response and CD11 expression in immune-response.
Biocompatibility analyses showed that formulated hydrogels were not toxic and didn't induce apoptosis. Chondrocyte were alive but not proliferative, inflammation and immune responses were observed but under certain acceptability threshold.
The results of this preliminary work suggest that the formulated Chitosan hydrogels are cyto-compatible and induce chondrocyte differentiation. The here tested synthesis process generated hydrogels, which are potentially useful for cartilage regenerative processes. Further mechanical characterizations are in progress.

1. Introduction

Cartilage disease such as osteoarthritis leads to serious cartilage lesions that can induce pain, immobility and joint destruction (Brittberg et al., 1994). Current therapies to treat joint disease have limited success in large cartilage defects due to the low self-healing capacity of articular cartilage. Tissue engineering techniques are proposed for cartilage repair. The ideal biomaterial should mimic the natural environment of the extracellular matrix of cartilage tissue, and promote chondrocyte attachment and proliferation. A variety of biomaterials, natural and synthetic, have been studied as potential scaffolds. However, the results obtained are inconsistent. Currently, hydrogel systems are used to rebuild the three-dimensional structure of chondrocytes in articular cartilage tissue engineering (Park et al., 2009; Cardea et al., 2013). A problem that may occur in the application of biomaterials is the possibility that they can activate inflammatory and immune responses at the site of transplantation. The production of reactive oxygen species (ROS) appears to be an early event in the inflammatory response, a phenomenon involved in the activation of lymphocytes. ROS are normally produced during cellular metabolism by the mitochondrial respiratory chain, but overproduction of ROS results in oxidative stress, which can have harmful effects on cellular omeostasis and survival. Following activation, neutrophils and macrophages produce a large amount of reactive oxygen species by phagocytic isoform of NADPH oxidase. This massive production of ROS at the site of inflammation is called "oxidative burst", and plays an important role as the first line of defense against pathogens (Droge, 2002; Reth, 2002). Some

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proteins expressed on the membrane surface, such as CD11b and CD69 are poorly expressed when the cell lines of the immune system are at rest, and their expression increases following activation, revealing activation markers of immune responses. Among these proteins there are CD11b and CD69 that we have considered in our studies.CD11b is the α_M subunit of the Mac-1 integrin, also called CR3, the receptor of C3bi, a fragment that forms in the complement cascade. This integrin, composed by CD11b/CD18, is expressed mainly on cells of the myeloid lineage, such as monocytes and neutrophils, but it is also found on T cells and its expression is up- regulated when these cells are activated. CD11b is involved in several functions such as phagocytosis, adhesion to and migration through the endothelium, regulation of apoptosis and degranulation (Ross, et al. 1993, Wagnar, et al. 2001). CD69 is a membrane glycoprotein expressed as a homodimer. It is poorly expressed in leukocytes at rest, but is rapidly induced after activation (Reddy, 2004). In fact, CD69 is considered a very early marker of activation that is expressed within 2-3 h after stimulation, reaches a peak of expression after 12 h and remains in high levels for 48 h (Gonzalez-Amaro, 2013). In particular, this molecule represents a marker for the activation of B and T lymphocytes, NK cells, neutrophils and eosinophils.
In this study, different formulations of chitosan hydrogel were synthesized and evaluated as carriers for cartilage regeneration. For this purpose, initially the citocompatibility of these 3D materials was measured. Moreover, their immunogenic power was studied to obtain more complete data for biomedical applications.

2. Materials and methods

2.1 Hydrogel preparation

Chitosan polymer (Sigma, commercial grade) was initially dispersed in purified water and filtered to remove water soluble impurities, then solubilized in a HCl 0,1 M solution to a concentration of 0.1 %, and finally vacuum filtered to eliminate impurities insoluble in water. The purified polymer in acidic solution was then freeze-dried.
Given that this form of dried polymer is water soluble, a 3 % w/w of chitosan water solution was easily prepared. Two different kinds of hydrogel were synthesized. The physical hydrogel was performed adding Na_2SO_4, in different stoichiometric ratios of Chitosan/($SO_4^{=}$) 1/0.5; 1/0.75 and 1/1. Gelation occurred instantly in a 24 multiwell plate, each well filled with 500 mg of the hydrogel. The chemical hydrogel was prepared at 70 °C by stirring the chitosan solution for 12 h with Diepoxy PEG (PEGDE, Sigma) in stoichiometric ratio of 1:1. The gel was purified after consecutive rinses of water, and 500 mg were used to fill a 24 multiwell plate . Finally the two gels were freeze-dried and sterilized with EtO.
Both physical and chemical hydrogels of Chitosan were characterized with Fourier Transformed Infrared Spectroscopy (FTIR) and rheometrical analyses. FTIR analysis allows assuring the cross-linking of the polymer.

2.2 Cell culture

Bovine chondrocytes were extracted from hoof articular cartilage by 1 h digestion in 5 mg/ml pronase (Sigma) , followed by 3 h digestion in 3,5 mg/ml collagenase (Abiel) at 37 °C, in Dulbecco's modified Eagle's medium (DMEM) low glucose containing 5 % FBS (Fetal Bovine Serum), 1 % penicillin/streptomycin and amphotericin B. After removing the undigested cartilage through a 70 mesh-filter, the chondrocytes were collected by centrifugation, resuspended in complete growth medium (DMEM low glucose supplemented with antibiotics and 10 % FBS) and cultured at 37 °C with 5 % CO_2. All freeze-dried formulations of Chitosan-hydrogels were rehydrated over-night in a 24 multiwell plate with chondrocyte growth medium at 4 °C. Two chondrocytes were harvested and seeded at two concentrations (15,000 and 30,000) on swelled chitosan-sulphate and chitosan-PEGDE hydrogels. Biocompatibility analyses were performed at different culture times (4 h, 2.5 days, 5 days and 10 days).
Leukocytes were isolated from 10 ml of heparinized blood (500 U of heparin) by using the histopaque density gradient according to the protocol (McCoy, 2001).
Mononuclear cells and granulocytes isolated were cultured at 500,000 cells/2 ml in RPMI 1640 Medium (developed by Moore et al., at Roswell Park Memorial Institute) supplemented with 10 % fetal bovine serum, 2 mM glutamine, 10,000 U/ml penicillin and streptomycin, in presence of 8.5 mg of Chitosan sulphate 1:1. The biomaterial, was weighed and was sterilized in antibiotic (10,000 U/ml penicillin and streptomycin) for 24 h and was washed three times with PBS.
Human T leukemic Jurkat cells were maintained in culture in the same medium condition at 37 °C and 5 % CO_2. The leukocytes separated from human blood were used in the analyses immediately after the separation. Stimulation of leukocytes was done with 50 ng/ml PMA (phorbol 12-myristate 13-acetate, Sigma) or 1 mg/ml ionomycin calcium salt from *Streptomyces conglobatus* (Sigma).

2.3 Viability assay
Chondrocyte proliferation was measured over time through a Cell Counting kit-8 (CCK-8) assay (Ishiyama et al., 1996). At designated times, the tetrazolium salt (WST-8) solution was added to the culture medium of each well, and the plate was incubated for 1 h at 37 °C and 5 % CO_2. The tetrazolium salt is reduced by dehydrogenases in viable cells to give a product (formazan) soluble in the cell culture medium. The amount of the formazan dye generated in samples, which is proportional to the number of living chondrocytes, was estimated by measuring absorbance at 450 nm. The rates of cell growth for each hydrogel were obtained.

2.4 Microscopic observation
The viable cells in the chitosan hydrogels were observed at selected times through fluorescent labeling with Acrydin Orange, a technique that allows to discriminate a healthy cell from one with nuclear damage. Acridine Orange is a dye which interacts with both DNA and RNA. When it labels healthy cells, nuclei emits green fluorescence and cytoplasm emits red fluorescence. This staining pattern is opposed when genomic damage occurs in cells.The hydrogels cultured were fixed with Formaldehyde 3.7 % in PBS (Phosphate Buffered Saline), labelled with Acrydine Orange (100 μg/ml) and observed by Confocal Microscopy. Fluorescent images relative to thin slices (1 – 5 μm) of the three-dimensional samples were obtained.

2.5 Intracellular ROS
ROS production was detected using 10 μM 2'-7'-dichlorofluorescein diacetate (DCF-DA), a lipophilic and a nonfluorescent compound that diffuses into the cells and in presence of ROS is rapidly oxidized to highly fluorescent 2'-7'-dichlorofluorescein (DCF), emitting a fluorescent signal that was measured using a flow cytometry. Cells were incubated with H_2DCF-DA (Sigma), for 30 minutes in the dark at 37 °C and then suspended in PBS.

2.6 Flow cytometric immunofluorescence labelling
After incubation with biopolymer chitosan sulphate, cells were washed in cold PBS supplemented with 3 % albumin (BSA) and 0.1 % sodium azide, resulting in a concentration of 500,000 cells/well. Cells were resuspended in 100 μl of PBS-3 % BSA and were incubated in the dark for 1 h at 4°C in constant shaking with mouse anti-human CD11b or FITC-conjugated mouse anti-human CD69. For CD11b analysis was used the indirect immunofluorescence method, so for CD69 analysis was used the direct method.
After incubation, cells were resuspended with 400 μl of PBS. At least 30,000 events were analyzed by flow cytometric acquisition.

3. Results

3.1 Chemical and mechanical characterization
The physical hydrogel (gCHIT+Sulfate) spectra (Figure 1 Left) presents a variation in the O-H and N-H stretching peaks: the polymer has a peak at 3265 cm^{-1} while the hydrogel has a peak at 3352cm^{-1} because of the interaction between ammonic groups of Chitosan and the $SO_4^{=}$. The chemical hydrogel (gCHIT+PEGDE) spectra (Figure 1 Right) presented the same behavior than the physical hydrogel; the polymer peak at 3265 cm^{-1} shifts to 3394 cm^{-1} due to the decrease of Chitosan's ammonic groups and the presence of two new hydroxyl groups resulting from the opening of the PEGDE's epoxidic ring. For both physical and chemical hydrogel the mechanical spectra showed that the G' (Storage Modulus) was always higher than the G'' (Loss Modulus) (Figure 2); this behavior is characteristic of a cross-linked polymer. The behavior of physical hydrogel demonstrated that higher concentrations of sulfate caused an increase of the Storage Modulus.

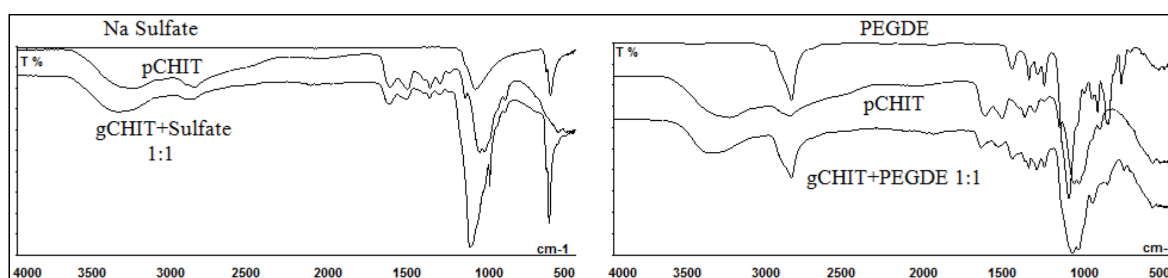

Figure 1. Left: FT-IR spectra of main reagents sulfate (Na Sulfate) and CHIT polymer (pCHIT), and physical hydrogel (gCHIT+Sulfate 1:1); Right: FT-IR spectra of main reagents PEGDE and CHIT polymer (pCHIT) and the chemical hydrogel (gCHIT+PEGDE 1:1).

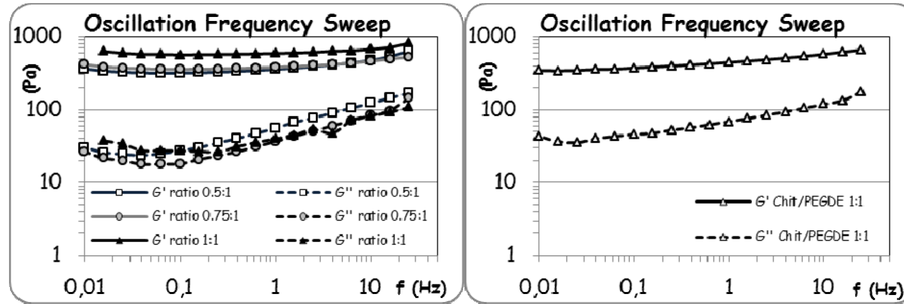

Figure 2. Left: mechanical behavior comparison for physical hydrogel CHIT+Sulfate with different stoichiometric ratio; Right: mechanical behavior of chemical hydrogel CHIT+PEGDE with 1:1 ratio.

3.2 Chondrocytes viability

The graphs in Figure 3 report the growth rate of chondrocyte cultured on dish (2D-culture) as control and on hydrogels. The data showed that Chitosan/Sulphate and Chitosan/PEGDE hydrogels are biocompatibile. Primary chondrocytes proliferated within Chitosan/Sulphate hydrogels during 2-3 days of culture, and their growth mostly stabilizes until the end of culture (10 d). There were no substantial differences in growth rate between the three Chitosan/sulphate formulations. Instead, the cells appear more proliferative when cultured with Chitosan/PEGDE hydrogel. Overall analyzed hydrogels had no cytotoxic effect, but did not induce the same proliferation rate of control (2D-culture) because they made a three-dimensional culture system.

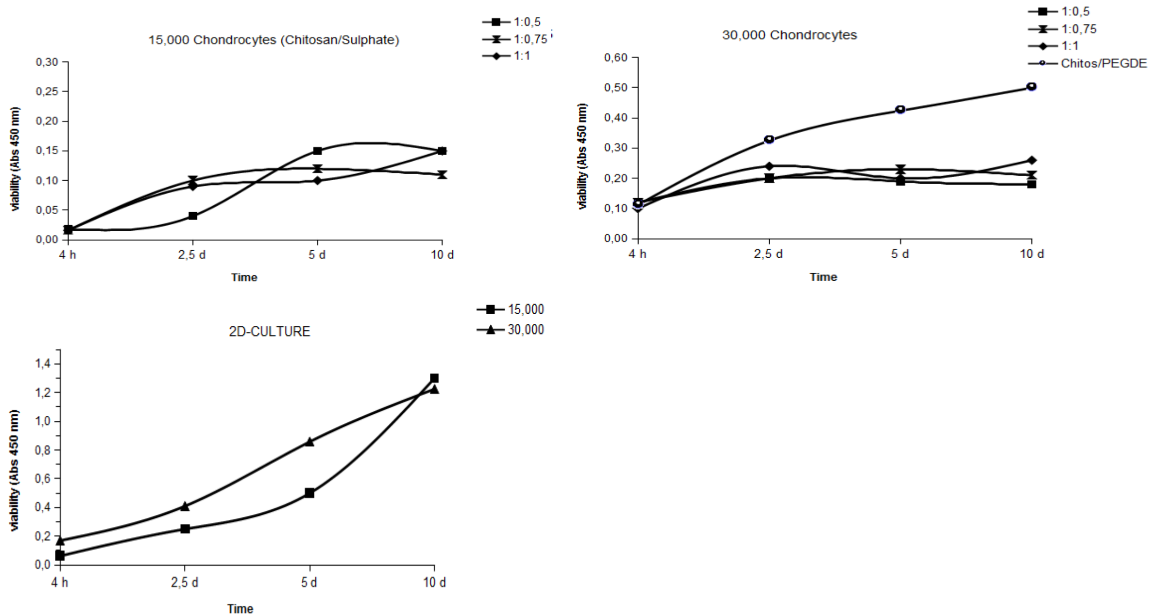

Figure 3: Proliferation rate of chondrocytes (15,000 or 30,000 cells) cultured for 10 days on Chitosan-hydrogels.

3.3 Microscopic observation

Acrydine Orange labelling were used to analyze genomic status of cells. Fluorescent images of samples were acquired and shown in Figure 4. Primary chondrocytes seeded on the different types of hydrogels showed well definite green nucleus, indicating that both sulphate and PEGDE/Chitosan formulations were not genotoxic. The confocal laser scanning showed the distribution of cells within hydrogels. Cells get inside all hydrogels and get mostly a rounded shape, such as that of the cartilage tissue. They appeared as groups of cells interacting with hydrogel's fiber. This was especially evident for Chitosan/PEGDE formulation.

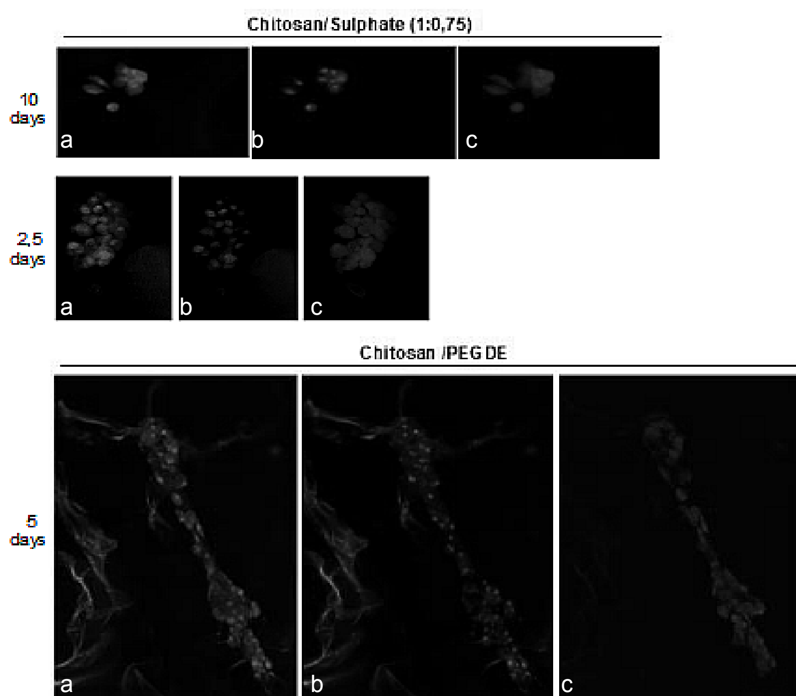

Figure 4: Fluorescent analysis of chondrocytes seeded on Chitosan-hydrogels after 2.5,5 and 10 days. The samples were fixed and labelled with Acrydin Orange. Three slides (2 μm, 20X) obtained by confocal scanning are showed, two referred to Chitosan/Sulphate 1:0.75, another to Chitosan/PEGDE. For each slide three near images are represented: merge (a), green (b) and red light (c).

3.4 Inflammatory and immune response analysis

In Jurkat cells, after 24 h of incubation, chitosan sulphate induces the production of ROS 12 times more than the control cells (established as 1 %), and chitosan PEGDE 5 times more than the control, but these values are lower than that produced by PMA (Figure 5A). Chitosan PEGDE had similar effects in leukocytes (Figure 5B) after 24 h of incubation (data not shown). Chitosan sulphate induces the production of ROS 1.5 times more than the control, but 6.5 times lower than that obtained by treating the cells with PMA. Figure 6A shows the results of CD11b and CD69 in Jurkat cells after 120 h of incubation. The flow cytometry analysis in Jurkat cells of protein markers of leukocyte activation revealed that the expression levels of CD69 induced by chitosan sulphate are 3 times higher than the control cells, while the PMA is able to induce higher levels of about 31 times. Chitosan sulphate significantly induced the expression about 19 times,in the analysis of CD11b. Figure 6B shows the results of CD69 and CD11b in leukocytes after 96 h of incubation. PMA increased the expression of CD69 of about 85 times more than the control, while the chitosan sulphate had expression levels similar to the control. Chitosan sulphate incremented 3 times the expression of CD11b.

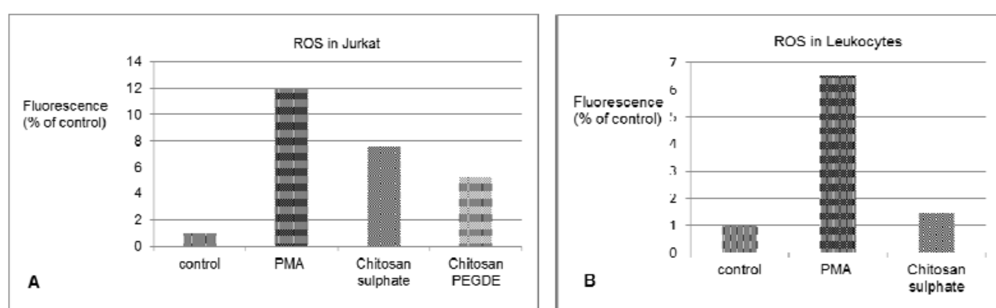

Figure 5: (A) Flow cytometric analysis of ROS in Jurkat cells after 24 h of incubation. (B) Flow cytometric analysis of ROS in leukocytes after 24 h of incubation. Results are expressed as percentage of the control. PMA 50 ng/ml is used as a positive control.

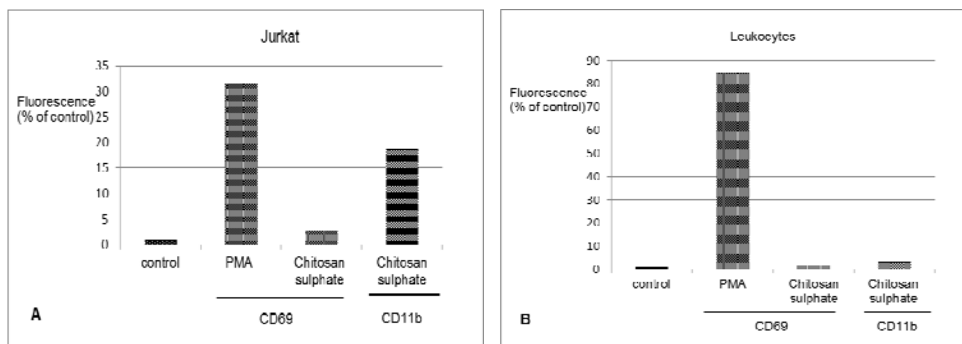

Figure 6: (A) Flow cytometric analysis of CD69 and CD11b in Jurkat cells after 120 h of incubation. (B) Flow cytometric analysis of CD69 and CD11b in leukocytes after 96 h of incubation. Results are expressed as percentage of the control. PMA 50 ng/ml is used as a positive control in CD69 analysis.

4. Conclusions

Both sulphate and PEGDE chitosans resulted cytocompatible. Bovine chondrocytes were healthy during the whole culture time, showing signs of genomic integrity. The analyzed biomaterials, especially the sulphates, had no proliferative effects on cells; rather they supported typical chondral phenotype stabilizing long-term cell growth. The analyses on the inflammatory and immune response of cells in chitosan sulphate hydrogel showed that this hydrogel was able to determine expression of CD11b, but not CD69, in both Jurkat cells and in leukocytes. Both physical and chemical hydrogels were able to determine ROS production, but levels induced by chitosan sulphate were higher than those induced by chitosan PEGDE. However, this response was not high. The results of the present study, although preliminary, indicated that chitosan sulphate and chitosan PEGDE could be applied in tissue engineering.

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