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Effect of electrical stimulation on chondrogenic differentiation of mesenchymal stem cells cultured in hyaluronic acid – gelatin injectable hydrogels

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Abstract

Electrical stimulation (ES) has provided enhanced chondrogenesis of mesenchymal stem cells (MSCs) cultured in micro-mass without the addition of exogenous growth factors. In this study, we demonstrate for the first time that ES of MSCs encapsulated in an injectable hyaluronic acid (HA) – gelatin (GEL) mixture enhances the chondrogenic potential of the hydrogel. Samples were stimulated for 21 days with 10 mV/cm at 60 kHz, applied for 30 minutes every 6 hours a day. Mechanical properties of hydrogels were higher if the precursors were dissolved in Calcium-Free Krebs Ringer Buffer (G' = 1141 ± 23 Pa) compared to those diluted in culture media (G' = 213 ± 19 Pa). Cells within stimulated hydrogels were rounder (55 %) than non-stimulated cultures (32 %) ($p = 0.005$). Chondrogenic markers such as SOX-9 and aggrecan were higher in stimulated hydrogels compared to controls. The ES demonstrated that normalized content of glycosaminoglycans and collagen to DNA was slightly higher in stimulated samples. Additionally, collagen type II normalized to total collagen was 2.43 times higher in stimulated hydrogels. These findings make ES a promising tool for enhancing articular cartilage tissue engineering outcomes by combining hydrogels and MSCs.

Keywords: Chondrogenic differentiation; Electric fields; Injectable hydrogels; Hyaluronic acid; Gelatin, Mesenchymal stem cells.

1. Introduction

Cartilage tissue engineering has focused up to now on the development of novel therapeutic techniques to restore articular cartilage by combining biomaterials, cells and external biophysical stimuli [1]–[5], although finding a well-established biomimetic structure to help in the recovery of injured cartilage is still a challenge. In this context, we propose the use of injectable hydrogels as the tridimensional matrix and electric fields (EFs) as an external stimulating factor to improve MSCs' chondrogenic differentiation.

Injectable hydrogels are produced from biopolymers and have several advantages, such as similarity with native ECM, the capacity to perfectly fill irregularly shaped injures, and the ability to encapsulate cells for homogeneous distribution within the scaffold [6].

In cartilage tissue engineering the material should enhance the expression of chondrogenic markers, the modulation of a round morphology and the differentiation of cultured MSCs [7]. Collagen and HA are native ECM constituents of articular cartilage, which makes them perfect candidates for cartilage tissue engineering approaches. HA is a polysaccharide with a proven ability to enhance chondrogenesis [8], while GEL, a polymer obtained by collagen denaturation, contains RGD sequences for integrin-mediated cell adhesion [9]. Previous studies have shown that injectable HA-GEL hydrogels maintain chondrocyte morphology and enhance gene expression of type II collagen and aggrecan [10], [11]. For instance, *Pfeifer et al.*, found that human MSCs embedded in HA-GEL hydrogels synthetized cartilage-related molecules such as type II collagen, glycosaminoglycans (GAGs), TGF-beta and the melanoma inhibitory activity protein (MIA) [12].

Other studies have shown that chondrogenesis is improved in the absence of growth factors such as TGF-β3, as in the case of the collagen-HA scaffolds developed by *Murphy et al*., [13] and the decellularized cartilage matrices investigated by *Burnsed et al*., [4]. Another outstanding feature of HA-GEL hydrogels is their ability to precisely control physiochemical and mechanical properties such as shear modulus, stiffness, water permeability, swelling and crosslinking [9], [14]–[16], although it has been shown that 3D culture environments alone cannot maintain prolonged chondrogenesis [17]. For this reason, cartilage tissue engineering has brought in new technologies such as applying biophysical stimuli to enhance the flow of nutrients, cell attachment, molecular synthesis and prolong chondrogenesis [18].

EFs have been shown to play an essential role in controlling different cellular functions such as morphology, migration, proliferation and secreted molecule synthesis [19]. For example, it has been reported that MSCs cultured either in monolayer or 3D constructs respond to external EFs. Some *in vitro* studies have shown that EFs applied to MSCs cultured in monolayer changed the alignment and cell morphology [20], [21], improved cell migration [22], [23], and increased cell proliferation [24]. It has also been shown that micro-mass MSCs in 3D cultures experienced an increase in collagen type II, aggrecan and SOX-9 expression after stimulation with EFs [25]–[27]. Electrical signals have an effect on the cell membrane, activating the Voltage-Gated Calcium Channels (VGCC) leading to an increase in the intracellular Ca^2+ levels [28]–[30]. The influx of Ca^2+ activates calmodulin, a cytoskeletal protein that regulates the signal transduction of calcium intracellularly, allowing the production of the SOX-9 transcription factor. Thus, SOX-9 triggers the gen production of collagen type II and aggrecan [28], [31], [32].

In view of the above, we hypothesized that EF stimulation would improve the positive results we observed with MSCs in HA-GEL hydrogels [9]. In a previous study we demonstrated that injectable HA-GEL mixtures promote the chondrogenic phenotype of MSCs in the optimal composition of 70% HA and 30% GEL, without the addition of specific growth factors and avoiding any possible risks to the patient [9]. In the present study a capacitively coupled system was implemented to generate and homogeneously distribute 10 mV/cm EFs at 60 kHz (sine wave-form) in the HA-GEL with the optimal hydrogel composition. MSCs encapsulated into the hydrogel were stimulated by this electric field for 30 minutes four times per day for 21 days of culture. The cells were also cultured in the absence of exogenous factors in order to observe the chondrogenic potential of electrical stimulation in the 3D hydrogel.

2. Materials and Methods

2.1 Materials

The following reagents were used for hydrogel synthesis: HA sodium salt from *Streptococcus equi*, GEL strength 300 type A, 2-(*N*-morpholino) ethanesulfonic acid (MES), tyramine hydrochloride (HCl-Tyramine), N-hydroxysuccinimide (NHS), dialysis tubing of 12'400 molecular weight cutoff (MWCO) for gelatin-tyramine conjugates dialysis [9], sodium chloride (NaCl), hydrochloric acid (HCl),sodium hydroxide (NaOH), hydrogen peroxide solution (H_2O_2), horseradish peroxidase (HRP) and paraformaldehyde. All the reagents were purchased from Sigma-Aldrich. 3'500 MWCO Dialysis Tubing for hyaluronic acid-tyramine dialysis [9] was acquired from Spectrum labs and carbodiimide hydrochloride (EDC) was obtained from Iris Biotech GMBH.

For the biological experiments, DMEM (high glucose, GlutaMAX Supplement), Fetal Bovine Serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), penicillin/streptomycin (P/S) and trypan blue stain antibiotics were obtained from Gibco. All the other reagents used in the experiments were CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) from Promega, recombinant human fibroblast growth factor basis (FGF-2) from Eurobio, saccharose from Panreac Química, Tween-20, DAPI dilactate, Triton X-100 and bovine serum albumin (BSA) from Sigma-Aldrich, QIAzol lysis reagent from Qiagen, chloroform from Fluka Analytical, absolute ethanol for molecular biology (100%) from Fisher Bioreagents, isopropanol from Scharlab, OCT from Tissue-Tek, mouse monoclonal aggrecan primary antibody (BC-3) from Novus Biologicals, rabbit polyclonal anti-SOX-9 primary antibody from Abcam, secondaries antibodies for aggrecan (Alexa Fluor 555 goat anti-mouse), SOX-9 (Alexa Fluor 488 goat anti-rabbit), cell viability/toxicity kit and phalloidin (Alexa flour 488) from Invitrogen, Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase and PowerUp™ SYBR™ Green Master Mix from ThermoFisher Scientific. The buffers used were

Calcium-Free Krebs Ringer Buffer (CF-KRB), Tris-Buffered Saline (TBS 10X) with pH of 7.6 and Papain Buffer Extract (PBE). The preparation protocols are described in [33].

2.2 HA – GEL hydrogel synthesis

The tyramine conjugates of hyaluronic acid (HA-tyr) and gelatin (GEL-tyr) required for the hydrogel preparations were synthesized following the protocols described in [9], [34] and [35]. Briefly, low molecular weight HA (350 kDa) was obtained by acid degradation and then modified by grafting tyramine molecules through carbodiimide/succinimide chemistry, as described previously [34]. Gelatin tyramine substituted polymer was obtained following the protocols described in [34], through carbodiimide/succinimide chemical reaction to graft tyramine hydrochloride in the polymer backbone. Molecular weight of the HA produced was measured by Gel Permeation Chromatography (GPC) on a Waters Breeze system (Waters Corporation, MA, USA), and following the protocol described in [36]. Tyramine content was determined by spectrophotometry at 275 nm using tyramine hydrochloride as reference in a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

Hydrogels 2% w/v in a proportion of 70% HA and 30% GEL were prepared according to the protocol described in [9], [34]. First, HA was dissolved in CF-KRB for 5 h at 4 °C, while GEL was dissolved in CF-KRB for 30 min at 37 °C. The hydrogels were prepared by mixing 80% (v/v) of the 2% w/v HA–GEL solution, 10% (v/v) of a 12.5 U/mL HRP solution and 10% (v/v) of a 20 mM H_2O_2 solution. They were incubated at 37 °C and 5% CO_2 for 20 min to ensure hydrogel crosslinking.

2.3 Rheological characterization of HA–GEL hydrogel

First, an oscillatory time sweep was performed to register the gelation dynamics of the hydrogel. Second, a dynamic strain sweep was carried out to calculate the range of strain amplitudes in which the material maintains linear viscoelasticity. A dynamic frequency

sweep test was then made to determine the frequency dependence of the dynamic shear storage modulus and loss factor. The input parameters used for each measurement are reported in [9]. Parameters such as solvent loss and temperature were controlled according to [9], [34], except that hydrogel dynamics weres measured by a gap between the plates around 1100 μm. Measurements were carried out by crosslinking 2% w/v HA-GEL mixtures either in CF-KRB or DMEM to determine how the medium affects the crosslinking reaction and modifies the mechanical properties of the resulting hydrogel. Rheological experiments were performed on a Discovery HR-2 strain-controlled rheometer from TA Instruments (New Castle, USA).

2.4 Dielectric constants determination of HA–GEL hydrogel and culture medium

Dielectric experiments measured the hydrogel and culture medium conductivity and permittivity. A volume of 350 μL of hydrogel precursor mixture (2% w/v) dissolved in CF-KRB and culture medium (DMEM) were placed in a liquid parallel plate sample cell (BDS1308). The hydrogels were *in situ* crosslinked on the electrode by adding the required amount of HRP enzyme and H_2O_2 peroxide. The electrode gap was adjusted by silica spacers. The measurements were carried out at room temperature and a frequency window from 1.1×10^{0} to 1×10^{7} Hz using a Novocontrol BDS system comprising a frequency response analyzer (Solartron Schlumberger FRA 1260) and a broad-band dielectric converter with an active sample head. The measurement error was shown to be less than ±3%.

2.5 Estimation of EFs applied to the hydrogel by computational analysis

A computational simulation was implemented to estimate the EF in the capacitively coupled system and within the hydrogel. The coupled capacitive system was composed of two parallel stainless-steel electrodes at the top and bottom of a 48 well-culture plate. The EF generated between the electrodes is perpendicular to the hydrogels and is

influenced by the differential potential applied between the electrodes (100 V at 60 kHz) and the dielectric constants of the substrates (**Table 1**). A single well-plate from a 48 well-plate was modeled as an axisymmetric configuration and the electrodes were big enough at the top and bottom of the single well to ensure a homogeneous and isotropic distribution of the EF during the simulation. The computational simulation was implemented by a finite element analysis using an electromagnetic simulation software (COMSOL Multiphysics, Comsol Inc. Los Angeles, USA).

Table 1. Dielectric properties and measurements used in the simulation.

Component	Parameter	Value
Stainless-steel	Electrode separation	20 [mm]
	Electrode radius	50 [mm]
	Thickness	5 [mm]
	Relative permittivity	1
	Electric conductivity	1.7 [MS/m]
Well plate	Length and height	11 and 20 [mm]
	Thickness	1 [mm]
	Relative permittivity	3.5
	Electric conductivity	6.9 [nS/m]
Air	Relative permittivity	1
	Electric conductivity	0 [S/m]

2.6 Mesenchymal stem cell culture in the hydrogel

Porcine MSCs were obtained from bone marrow using a modified protocol for human MSC isolation [33], [37], [38]. Before MSC encapsulation in the hydrogels, the cells were expanded until passage 4 in expansion medium containing DMEM supplemented with 10% (v/v) FBS, 1% (v/v) of P/S and FGF-2 (50 ng/mL).

Hydrogels in a proportion of 70% HA (2% w/v) and 30% GEL (2% w/v) were prepared. Once HA-tyr, GEL-tyr and HRP solutions were mixed, the resulting solution was filtered through a 0.22 µm syringe filter for sterilization. Then, 1×10^6 cells/mL from passage 4 to 5 were added to the HA-GEL–HRP solution [9]. Thereafter, a drop of 45 µL of the HA–GEL cell suspension was crosslinked with 5 µL of 20 mM H_2O_2 on each well of a 48 culture well-plate. The well-plate was left in the incubator for 20 min to ensure hydrogel crosslinking. 300 µL of basal medium (expansion medium without FGF-2) were then added. The hydrogels were cultured for 21 days and the cell culture medium was replaced every 2 days.

2.7 Electrical stimulation of hydrogel cell culture

ES was performed using a coupled system that delivers homogeneous EFs [39]. The electrodes were placed on Teflon supports to eliminate any contact with the incubator surface. The positive and negative terminals of the electrodes were connected to an electronic circuit (oscillator) that generated the voltage and frequency required to create the EF. The oscillator was energized with a dual source (Lendher – HY3003D-3, Shenzhen, China) and signal verification before and after ES was monitored by an oscilloscope (Keysight – DSO1052B, Santa Rosa, CA, USA). The stimulated cultures were placed between the electrodes, while the non-stimulated cultures were incubated in the same way, except that the electrodes were not connected to the oscillator. The cell culture wells containing the hydrogels with encapsulated cells and culture medium were exposed to an EF of 10 mV/cm at 60 kHz sine wave-form for 21 days [39], [40]. According to a previous report, the EF was delivered on the first day of culture at an exposure time of 30 min four times per day, which corresponds to 30 min of stimulation and 5.5 h without stimulation [39], [40].

2.8 Hydrogel digestion for biochemical analysis

Five stimulated and five non-stimulated hydrogels were digested enzymatically with papain (3.875 U/mL) for 18 h at 60 °C to solubilize the ECM content. The remaining HA was digested with hyaluronidase (12.5 U/mL) for 3 h at 37 °C. After digestion the samples were stored at 4 °C for DNA, total collagen and GAG quantification.

2.9 Morphology, cell viability and cell proliferation assays

Hydrogel cell viability after crosslinking either in CF-KRB or in DMEM (both with 1% (v/v) P/S) was performed by a Live/Dead assay. Four hydrogels with encapsulated cells were used as negative control (living cells), while four samples of monolayer cultured cells were the positive control (dead cells), which were obtained by incubating the cells in the presence of DMSO at 50 % (v/v) for 1 h at 37 °C and 5% CO_2. After incubation, the hydrogels and monolayer cells were washed three times with DPBS 1X. The samples were then stained with 200 µL of the Live/Dead assay mixture (1 µL Calcein-AM, 4µL Ethidium-homodimer1, and 2 mL DPBS 1X) and incubated in darkness for 30 min at 37 °C. Immunofluorescence images were taken on a microscope with a built-in camera (Nikon Eclipse 80i, Melville, NY USA).

Cell morphology was assessed by microscopic examination after DAPI/actin fluorescent staining. After culture time the samples were washed with DPBS fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature and then washed twice with DPBS 1X. The hydrogels were submerged overnight in 30% w/v saccharose embedded in OCT and stored at -80 °C. For DAPI/actin staining, the cryopreserved hydrogels were segmented at 30 µm in a cryostat (Leica CM 1860 UV) and placed on SuperFrost slides. The slices were washed twice with DPBS 1X, after which the samples were permeabilized with Triton X-100 at 0.1% v/v in DPBS 1X for 10 min. The permeabilizer solution was then removed and two washes with DPBS 1X were performed. DAPI at 1:500 and Alexa Fluor 488 Phalloidin at 1:100 were then added to the samples, which were incubated for 1 h at

room temperature. The slides were washed twice with DPBS 1X and stored at 4 °C for further examination under a fluorescence microscope (Nikon Eclipse 80i, Melville, NY USA). Round and elongated cells were counted on 20X-magnification immunofluorescence pictures. The percentages of round and elongated cells were calculated from a total of 140 cells for the 14 and 21-day culture times. The cells were recognized and counted on Image J software (NIH Image software, Bethesda, MD USA). Proliferation was determined on days 2, 7, 14 and 21 by analyzing the total deoxyribonucleic acid (DNA) of samples digested as previously described in Section 2.8. DNA was quantified on a Quanti-iT Picogreen dsDNA Reagent Kit, as described in a previous work [33].

2.10 Immunofluorescence study

The cells cultured in hydrogels were immunostained for aggrecan and SOX-9 after 21 days of culture. First, hydrogel slices were obtained following the protocol described in Section 2.9. For immunoassaying, the slides were washed twice with TBS for 4 min, permeabilized with 0.1% v/v Tween-20 for 15 min and blocked in 2% BSA in TBS for 15 min, both at room temperature. After, aggrecan at 1:500 and SOX-9 at 1:250 primary antibodies were diluted in blocking solution (2% (w/v) BSA in TBS) and incubated overnight at 4 °C in separate sets of samples. The samples were washed four times with 0.1% v/v Tween-20 in TBS for 3 min, after which Alexa Fluor 555 goat anti-mouse (1:300) and Alexa Fluor 488 goat anti-rabbit (1:350) secondary antibodies were diluted in blocking solution and incubated at 37 °C for 1 h in the dark in separate sets of samples. The slides were then washed four times with 0.1% v/v Tween-20 in TBS for 3 min and stained with DAPI (1:500). Immunofluorescence images were taken on a microscope with a built-in camera (Nikon Eclipse 80i, Melville, NY USA).

The aggrecan and SOX-9 expression was quantified from the immunofluorescence images. The percentage of cells expressing molecules was calculated from 20X-magnification images (n=9). Aggrecan and SOX-9 positive cells were recognized and counted on Image J software (NIH Image software, Bethesda, MD USA).

2.11 Gene expression assay

Total RNA isolation was carried out on the samples at days 14 and 21. Two hydrogels of the same group were mixed and mechanically processed in the presence of 700 µL of QIAzol lysis reagent to disintegrate the hydrogel and homogenizethe the RNA, after which 140 µL of chloroform were added to the mixture, which was centrifuged at 13000 rpm for 10 min at 4 °C to separate the RNA from the organic phase. The liquid phase, which contained the RNA, was then carefully removed and transferred to a nuclease-free tube, and a second wash with 140 µL of chloroform was performed, centrifuging the sample at 13000 rpm for 10 min at 4 °C. Thereafter, the upper aqueous phase was carefully removed and transferred to a new nuclease-free tube and an equivalent proportion of isopropanol was added to the sample to precipitate the RNA. The sample was stored at -80 °C for 30 min to ensure correct RNA precipitation, and then centrifuged at 14000 rpm for another 30 min. The isopropanol was removed and 1 mL of pure ethanol was added to wash the RNA. The sample was centrifuged at 14000 rpm for 1 min and the ethanol was removed from the sample. The RNA was then resuspended in 30 µL of nuclease-free water and the RNA quantity was determined in a UV spectrophotometer Q3000 (Quawell, San Jose, CA, USA).

For reverse transcription of the isolated RNA a Maxima First Strand cDNA Synthesis Kit for RT-qPCR was used following the manufacturer's protocol in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Expression of collagen types I and II, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene was assessed by RT-qPCR

(**Table 2**) using PowerUp™ SYBR™ Green Master Mix. For each experiment corrected ΔC_T values (ΔC_T [non-stimulated control - stimulated]) were obtained for pooled pairs of non-stimulated and stimulated samples and analyzed in triplicate at the same time. The gene expression was analyzed in a 7500 Fats Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).

Table 2. Forward (F) and Reverse (R) primers for pig targets of collagen type I (Col IA1), collagen type II (Col 2A1) and GAPDH.

Target	Primers	Ref
Col IA1	F: 5'-AAGACATCCCACCAGTCACC-3' R: 5'-CAGTTCTTGATTTCGTCGCA-3'	
Col 2A1	F: 5'-TGAAAAAGGTGCTCCTGGAC-3' R: 5'-CCTTCTCATCGAATCCTCCA-3'	[41]
GAPDH	F: 5'-TGGTGAAGGTCGGAGTGAAC-3' R: 5'-TGTAGTGGAGGTCAATGAAGG-3'	

2.12 Biochemical analysis

Sulphated GAGs content and total collagen of non-stimulated and stimulated samples were quantified at 14 and 21 days of culture according to the protocol described in [33]. GAGs were processed on a Blyscan assay kit, and the signal was measured at 656 nm. GAGs content was calculated from a standard curve performed using chondroitin sulphate standard. Total collagen was quantified analyzing the hydroxylation of hydroxyproline and the signal was measured at 570 nm. Total collagen content was calculated from a standard curve of known concentrations of trans-4-hydroxy-L-proline. The absorbance of the samples was read on a Victor3 microplate reader (Perkin Elmer). Collagen type II content in the sample was measured through an Enzyme-linked immunosorbent assay (Collagen Type II (COL2), ELISA Kit). Non-stimulated and

stimulated 21-day culture samples were digested by 100 µL of hyaluronidase (50 U/mL) in 0.9% of NaCl for 3 h at 37 °C. The samples were then mixed with 450 µL of pepsin at 1.1 mg/mL in 62.5 mM of acetic acid and incubated for 72 h at 4 ºC with gentle mixing, after which 50 µL of TBS 10X and 50 µL of pancreatic elastase were added to each sample. The pH was adjusted to 8 and the samples were incubated for 24 h at 4 ºC with gentle mixing, centrifuged at 10000 rpm for 5 minutes and the supernatant was collected and stored at -80ºC for collagen type II quantification. Collagen Type II ELISA was performed following the kit's user manual. Samples were read at 450 nm on a Victor3 microplate reader (Perkin Elmer).

2.13 Statistical analysis

Statgraphics software was used for the statistical studies and a Shapiro-Wilks test to verify data normality. Paired samples were studied by a t-student assay after determining their homoscedasticity. Sample groups were analysed by one-way ANOVA and the Tukey post-test was applied. When the Levene test indicated sample non-homoscedasticity a Kruskal-Wallis test was also performed.

3. Results

3.1 Mechanical properties of hydrogels

The rheological properties were measured in hydrogel precursors dissolved in either CF-KRB or DMEM. Gelation time as a function of the storage modulus (G') was 20 min for both media (**Figure 1A)**. The polymers dissolved in CF-KRB had a $G'$of 1141 ± 23 Pa, in comparison with those dissolved in DMEM (G'= 213 ± 19 Pa) (**Figure 1B**). The frequency-dependence of the G' and the hydrogel's loss modulus (G'') are shown in **Figures 1B** and **1C**, respectively. The storage modulus is the dominant contribution to $|G^*|$, since $G' >> G''$, verifying that the gels indeed display an elastic behavior. As the complex modulus ($|G^*|$) of the already crosslinked hydrogels remained independent of

amplitude strain (**Figure 1D**), we confirmed that the measurements performed at 1% strain were within the linear viscoelastic region of the materials. The Live/Dead assay showed that cell deaths were slightly higher in hydrogels crosslinked in CF-KRB than in DMEM (**Figure 1E**).

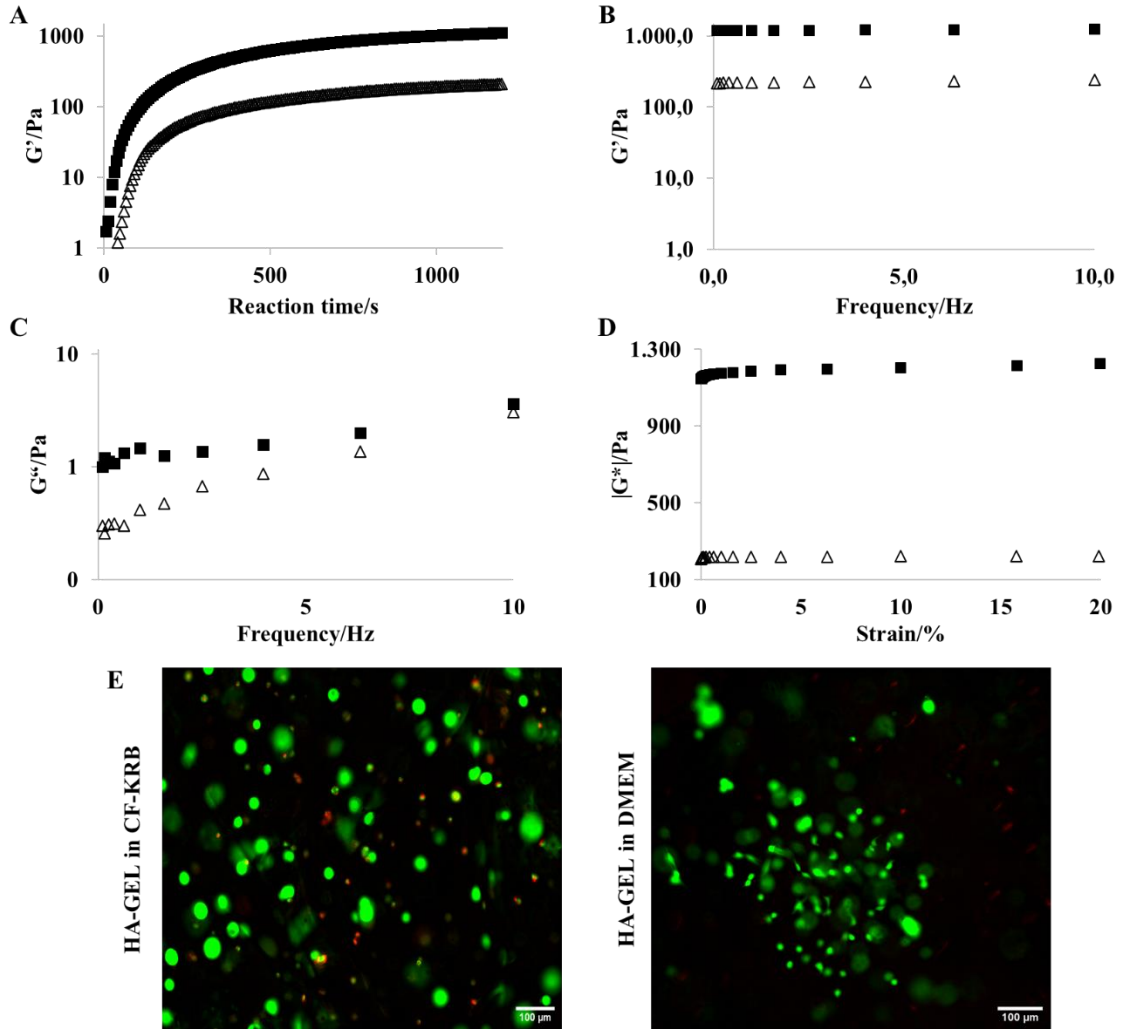

Figure 1. Gelation dynamics and strain amplitudes of hydrogels and dynamic frequency sweep test to determine the dependence of the dynamic shear modulus and loss factor on the frequency. **A)** Oscillatory time sweep to register the crosslinking kinetics of hydrogels. Storage modulus as a function of reaction time. **B)** Storage modulus ($\boldsymbol{G'}$). **C)** Loss modulus ($\boldsymbol{G''}$). **D)** Dynamic strain sweep to calculate the range of strain amplitudes. Complex modulus magnitude ($|\boldsymbol{G^*}|$) of crosslinked hydrogels as a function of strain. The black squares represent the data of HA-GEL dissolved in CF-KRB, while the white

triangles show the data of HA-GEL diluted in DMEM. **E)** Live/Dead staining of hydrogels cultured in presence of CF-KRB and DMEM. Viable cells shown in green, while dead cells are in red (scale bar = 100 µm). Each curve is the average of three different samples.

3.2 Electrical properties of hydrogels

The hydrogels and culture medium's (DMEM) permittivity (ε') and conductivity (σ) are shown in **Figure 2**. The ε', which is the medium's ability to store an EF, was found to be higher in the DMEM, ($\varepsilon' = 2.67\,E+04$), than in the hydrogel ($\varepsilon' = 8.03\,E+03$) at frequencies of 60 kHz (**Figure 2A**), which means that the hydrogels have more electric flux than the DMEM and thus higher EF. At frequencies of 60 kHz, the DMEM presented a similar ($\sigma = 7.20\,E-04$) to the hydrogel ($\sigma = 7.10\,E-04$) (**Figure 2B**), indicating that both the polymers and the medium have the same electrical conductivity.

3.3 Distribution of EFs

An axisymmetric computational simulation was carried out to calculate the EFs in the capacitively coupled system. **Figure 2C** represents a drop of hydrogel surrounded by the cell culture medium (DMEM) in the culture well, plus the calculated EF distribution. Considering that the cells are immersed in the hydrogel, it is important to know the EF intensities within the entire volume of hydrogels, which is homogeneous at a given height from the bottom of the well (**Figure 2C**). However, slightly different EFs (between 9 and 12 mV/cm) are obtained from the bottom of the well to the top of the hydrogel drop (see **Figure 2D)**.

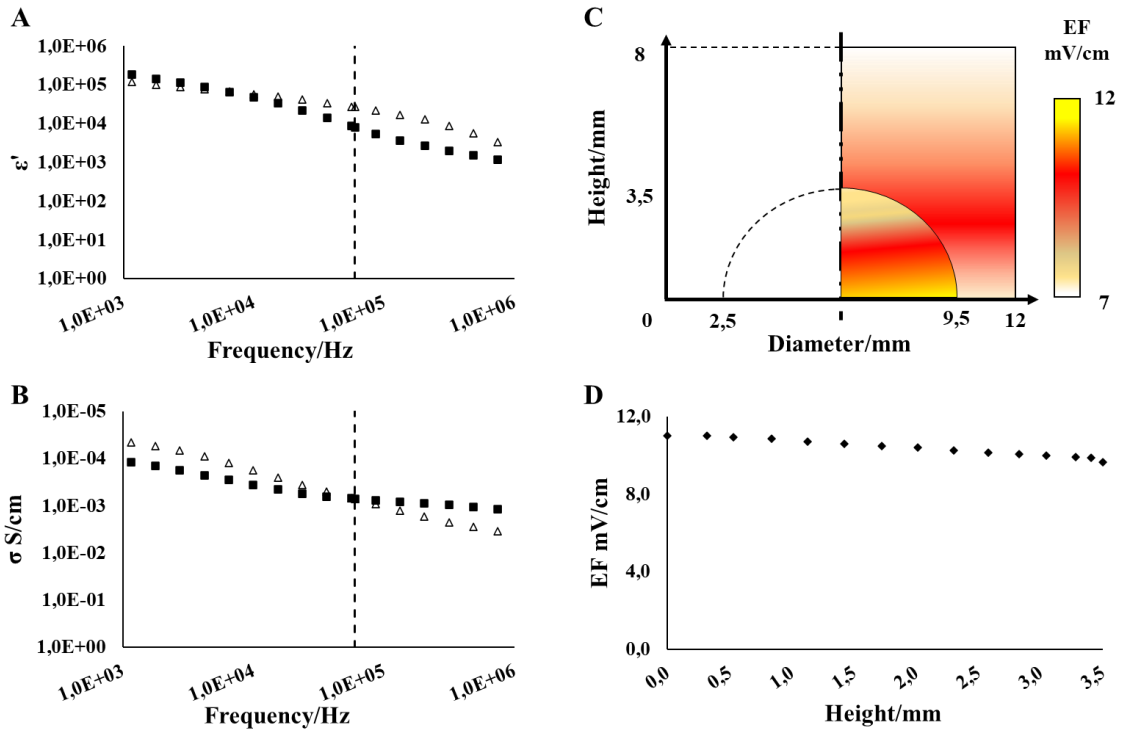

Figure 2. Dielectric properties of hydrogels and culture medium and EF distribution in the cell culture. **A)** Permittivity (ε') of hydrogels and culture medium. **B)** Conductivity (σ) of hydrogels and culture medium. The curves in A and B are the average of three different samples. **C)** EF distribution in drop of hydrogel in the well surrounded by culture medium DMEM. The semicircle represents the drop of HA-GEL hydrogel, while the square illustrates the DMEM around the drop. **D)** EF values in the hydrogel. The black squares represent the data of the HA-GEL mixture, the white triangles show the data of the DMEM, the dashed lines illustrate the measurement of dielectric properties at 60 kHz, and the black diamonds describe the EF within the HA-GEL hydrogel.

3.4 Morphology and cell proliferation

Considering that chondrocyte differentiation is usually observed after 14 days of culture, especially at day 21, a DAPI/actin fluorescence assay with was performed on these days to stain the cytoskeletons and identify the cell morphology. Cell morphology is an indirect indicator of chondrogenic differentiation when mesenchymal stem cells (MSCs) are cultured in three-dimensional constructs [9], as it is generally accepted that rounded

morphology corresponds to chondrocytic differentiation. For this reason, we assessed the morphology of MSCs through a DAPI/Actin staining. The cells were found to keep their rounded morphology on these days, except for some that acquired an elongated shape inside the hydrogels (**Figure 3A**). The cultures stimulated for 14 days had 86% of round and 14% elongated cells, while the non-stimulated cultures had 67% of round and 33 % of elongated cells ($p = 0.13$). Similarly, the cultures stimulated for 21 days had 55 % of round and 45 % elongated cells, while non-stimulated cultures showed 32 % of round and 68 % of elongated cells ($p = 0.005$). The non-stimulated hydrogels had a higher proliferation rate on days 2 and 7 than the stimulated hydrogels (**Figure 3B**). The higher proliferation continued up to day 21, but with no significant differences between the non-stimulated and stimulated hydrogels.

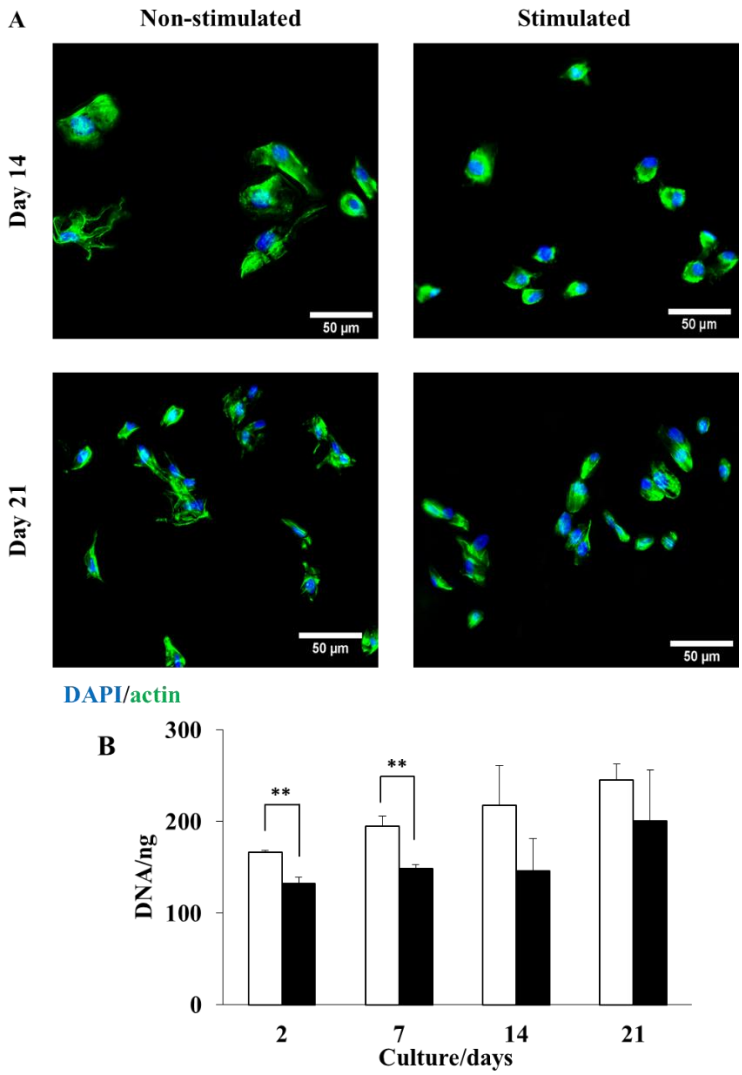

Figure 3. Morphology, cell viability and cell proliferation. **A)** DAPI/actin staining of non-stimulated and stimulated hydrogels on days 14 and 21 of culture. Scale bar = 50 µm. **B)** Cell proliferation of non-stimulated and stimulated hydrogels. Statistically significant differences were found on days 2 and 7. The DNA concentration of non-stimulated and stimulated hydrogels are illustrated by the white and black bars, respectively. (n=5, $p < 0.05$**).

3.5 Chondrogenic differentiation

Chondrogenic differentiation was assessed by immunofluorescence on day 21. The MSCs cultured in hydrogels expressed both SOX-9 and aggrecan (**Figure 4**). The percentage of cells expressing aggrecan in non-stimulated hydrogels on day 21 seems to be lower than

the stimulated ones, as seen in the immunofluorescence images in **Figure 4A**, in which yellow arrows indicate non/positive cells for this marker. SOX-9 expression seems to be similar in both non-stimulated and stimulated hydrogels in the immunofluorescence images. Immunofluorescence images at day 14 did not show changes in the expression of Agrecan and SOX-9 (Data not shown). Aggrecan and SOX-9 were quantified and expressed as the percentage of positive cells as shown in **Figures 4B** and **4C**. Aggrecan expression was higher in stimulated hydrogels on days 14 and 21 than in non-stimulated cultures (**Figure 4B**), while SOX-9 synthesis was higher on day 21 in stimulated cultures than in non-stimulated hydrogels (**Figure 4C**).

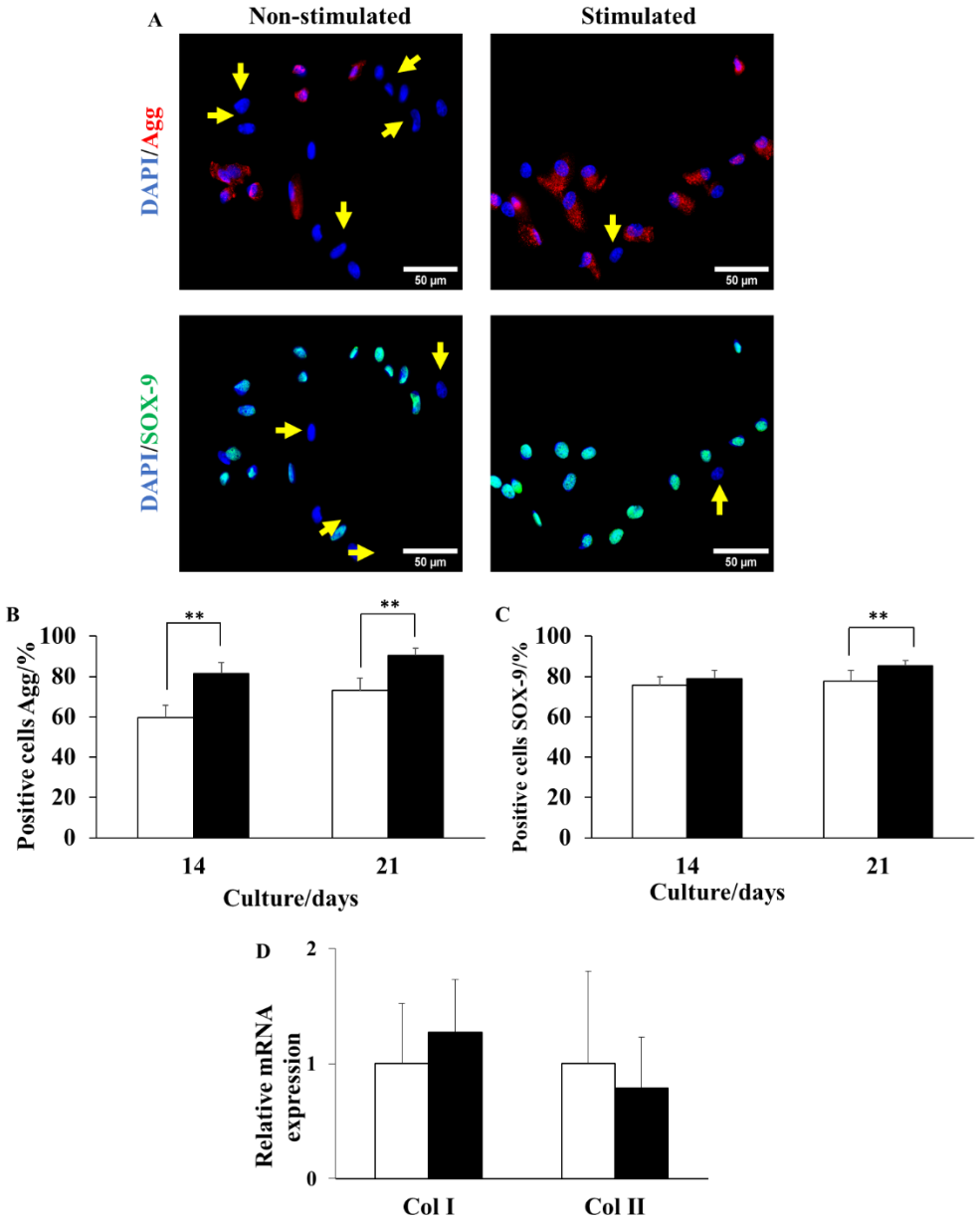

Figure 4. A) Immunofluorescence images and SOX-9 and Aggrecan quantification of MSCs cultured in HA–GEL hydrogels for 21 days. Scale bar = 50 µm. **B)** Percentage of positive cells for aggrecan on days 14 and 21 of culture. Statistically significant differences were found on days 14 and 21 ($p < 0.05$**). **C)** Percentage of positive cells for SOX-9 on days 14 and 21. Statistically significant differences were found on day 21 ($p < 0.05$**). **D)** Relative gene expression of collagen type I and II from qPCR on 21 days of culture. The data of non-stimulated and stimulated hydrogels are represented by the white and black bars, respectively.

The GAG biochemical analysis, total collagen and collagen type II are shown in Table 3. Although the GAGs values in stimulated were higher than non-stimulated hydrogels at 7 days of culture, after this time the amount of GAGs in the stimulated samples was below the non-stimulated control samples (Table 3). Total collagen levels were slightly higher in stimulated samples than control samples for all the time points studied (Table 3). It is noteworthy that collagen type II normalized to total collagen is 2.43 times higher in the stimulated hydrogels than the control samples, which is again an indication of the chondrogenic potential of electrical stimulation (Table 3). Here, it is noteworthy to mention that the collagen type II normalized to total collagen was measured until day 21 because chondrogenic differentiation tests are at least 21 days to make sure that the cells have differentiated.

The results showed that there were no significant differences in the gene expression of collagen type I and II between the non-stimulated and stimulated hydrogels after 21 days of stimulation (**Figure 4D**).

Table 3. Normalized content of GAGs and collagen to DNA and collagen type II to total collagen.

Normalized values	Day 7	Day 14	Day 21
GAG/DNA	2.62 ± 0.69	0.56 ± 0.26	0.74 ± 0.51
COLL/DNA	1.20 ±0.51	2,17 ± 1.15	1,10 ± 0,79
COLL II/COLL TOT	-	-	2.43 ± 1.14

4. Discussion

The aim of the study was to evaluate the effects of electric stimulation on chondrogenic phenotype of MSCs cultured in HA-GEL injectable hydrogels in the absence of growth factors. These biomaterials were selected for their ability to promote cell chondrogenic characteristics [9], their well-established stiffness [34] and their biocompatibility, which allows them to be injected into the body and crosslinked via non-cytotoxic reactions [35].

The hydrogels obtained showed similar mechanical properties to those obtained in previous studies [34]. In the present study, the polymers crosslinked in CF-RKB had higher G' than those crosslinked in DMEM. As stiffness depends on the degree of crosslinking [9], the use of a growth medium leads to unstable hydrogels with a poorly reticulated mesh and a lower mechanical modulus. Our hypothesis was that the chemical composition of the DMEM interferes with the polymer crosslink. The culture medium is a defined mixture of amino acids, sugar, salts and other organic compounds, besides aromatic amino acids such as tyrosine, which includes a phenol group that can be activated through peroxides. The tyrosine may compete with tyramine activation and lead to blocking the chain bond formation. The culture medium also contains antioxidant compounds like folic acid or thiamine that could neutralize either the peroxide or the free radicals and protect tyramine molecules from activation [42].

The dielectric properties of the cell culture medium and hydrogels were characterized in order to appropriately establish the ES conditions. This was the first attempt to calculate the dielectric properties of the HA–GEL hydrogels. Even though a study has measured the dielectric constants of a HA – collagen mixture, the results showed lower $\varepsilon' = 34.15$ and $\sigma = 1.0\text{E} - 7$, than the $\varepsilon' = 8.03\text{E} + 03$ and $\sigma = 7.10E - 2$ found in this study [43]. This discrepancy may be explained by the concentration of the polymers used to prepare the hydrogels. The aforementioned study used 0.5% (w/v) of HA and 0.5% (w/w) of collagen, while the concentration used in this work was 2% (w/v) for both HA and GEL. In this context, it is possible to conclude that higher concentrations of polymer increase the hydrogels' ε' and increase molecule polarization to override the internal EF when an external ES is applied. Based on the σ found in this work, it can be concluded that hydrogels with higher concentrations of polymers have low electric resistance, which leads to efficient current flow. In fact, the σ of the hydrogels depends on the degree of

crosslinking, as the more hydrogen bonds formed the greater the density of the charge carriers [43].

HA–GEL injectable hydrogels have shown great potential as 3D cell culture scaffolds since the hybrids stimulate chondrogenic differentiation in the absence of growth factors [9]. Both HA and GEL are widely used to upregulate the gene expression of chondrogenic genes in the presence of growth factors [2], [44]–[48], but the cells of pure GEL hydrogels acquire a fibroblast-like morphology due to their strong adhesion [47], while HA has very poor cell adhesion and cell proliferation [9]. The mixture of HA and fibrillar proteins may increase the chondrogenic markers' expression in basal conditions [49]. Our hydrogel combines the advantages of both materials and provides a hydrated environment with high resistance to shear loading plus anchoring points to favor cell adhesion while reducing the fibroblast-like morphology acquired by the cells.

In this work ES was evaluated as a means of improving our hydrogel and its natural ability to induce the expression of chondrogenic markers. The ES method applied to cell culture is divided into two possible application schemes: the former uses electrodes in direct contact with the biological material, while the latter consists of an indirect coupling system with external parallel electrodes, which was the one we selected, because the former type has insufficient biocompatibility and alters the physicochemical features of the cell culture medium [19].

On the other hand, indirect contact electrodes increase cell population and molecular synthesis of different types of cells [19], and provide a non-invasive system similar to medical stimulation devices used to treat osteoarthritic problems [50]. In this context, a specialized bioreactor was used to stimulate the hydrogels, together with a computational analysis to estimate the EFs generated by the capacitive coupling system. Computational

modeling can not only calculate the electric magnitude produced by the bioreactor, but can also estimate the EFs that are stimulating the cells embedded in the hydrogel.

Similar computational approaches had previously been implemented to calculate the EFs stimulating chondrocytes cultured in monolayer [39] and MSCs encapsulated in collagen-based scaffolds [5]. The combined experimental and computational approach thus allowed both the generation and prediction of the EF intensity stimulating the hydrogels. This computational model is a promising tool that can not only estimate the EFs stimulating the biological samples, but can also modify parameters such as electrode size, well plate types, scaffold morphologies, frequencies and voltages in order to find the electric mechanism that best fits the required cellular responses. It can be used for different device configurations and stimulation parameters to trigger different molecular mechanisms within the cell [29]. Another of its advantages is its capacity to simulate both the cells and their different morphologies during growth *in vitro*. Although this was not the aim of our study, we have implemented similar computational approaches to assess the effect generated by external EFs on the cell membrane of different cell morphologies [51]. This type of theoretical model can be extrapolated to investigate the interaction of EFs with cells cultured in 3D scaffolds in order to create aligned and orientated biomimetic materials for tissue recovery. In fact, these findings open up a new research area in organ and tissue engineering focused on the design of specialized tissue-engineered constructs modulated by external biophysical stimuli such as EFs [19], [29].

The computational model had a key role in designing the bioreactor by simulating different electrode configurations, well plate dimensions and hydrogel morphologies. It also estimated the minimum electrode dimensions and the optimal cost-efficient material to distribute the EFs equally over the entire surface of the cell culture and identified the relation between well plate size and hydrogel morphology to obtain the correct EF flow.

It was thus possible to simulate the EF flow direction in the entire capacitively coupled system, especially around and inside the hydrogel.

These simulations were helpful in designing and building the bioreactor, since it was possible to model: 1) the appropriate size of the electrodes to avoid the edge effect and distribute the EFs equally in the cell culture, 2) the frequency and the voltage needed to stimulate the cells immersed in the hydrogel, and 3) the shape and size of the culture well plate to obtain the right electric field flow in the hydrogel droplet. A non-contact electrode stimulation system was simulated and implemented because this type of mechanism avoids changes in the physicochemical features of the cell culture medium and the biological material, such as pH variations and reduced levels of molecular oxygen [19]. From the medical point of view, the use of capacitively coupled systems improves clinical trials for patients' comfort and treatment compliance, avoiding the placement of electrodes directly on the patient's skin [19], [29].

The experimental analyses showed that the cellular dynamics of MSCs embedded in hydrogels were stimulated by 10 mV/cm EFs applied for 30 min every 4 h per day. A decrease in DNA content was observed after 2 and 7 days of stimulation, which agrees with previous reports that found that MSCs cultured either in micro-mass or collagen scaffolds do not proliferate after 7 days of ES [5], [25], [27]. However, it has also been found that short ES exposure times increased the MSCs' proliferation rate in monolayer in the presence of a chondrogenic medium [24]. In terms of molecular MSC synthesis, different chondrogenic markers were analyzed in this study as indicators for chondrogenic differentiation. It was observed that stimulated hydrogels experienced an increase in SOX-9 and aggrecan expression after 14 and 21 days of stimulation. Some studies have reported that micro-mass cultures of MSCs stimulated with 20 mV/cm at 1 and 60 kHz experienced an increase in SOX-9, aggrecan and collagen type II; however,

the increase in gene expression was obtained under chondrogenic conditions using TGF-β3 [25], [27].

A recent study found that MSCs encapsulated in bovine collagen-based scaffolds and stimulated with direct coupled EFs for 7 days had a weaker response as regards the expression of cartilage matrix proteins [5]. While we also observed that the combination of hydrogels and ES has a positive effect on chondrogenic differentiation due to the higher aggrecan and SOX-9 positive cells and the higher collagen type II/total collagen ratio in stimulated samples compared to non-stimulated ones. These results suggest that HA–GEL injectable hydrogels are a good alternative for inducing chondrogenic differentiation, not only in the absence of exogenous factors but also in presence of an ES, indicating that this biocompatible material can be used not only to treat cartilage injures, but also to improve the current alternative therapies without causing any undesirable local or systemic effects in the injury zone.

5. Conclusion

The aim of this study was to develop a novel framework to enhance the chondrogenic differentiation of MSCs encapsulated into injectable hydrogels and stimulated with external EFs without the need for growth factors. The results obtained show that EFs affect the proliferation rate and stimulate the synthesis of chondrogenic markers such as SOX-9 and aggrecan. ES could therefore be used to improve the cell dynamics of MSCs during *in vitro* cultures and to enhance therapies such as the autologous implantation of scaffolds in injured cartilaginous tissue.

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Conflict of interest

The authors declare that they have no conflict of interest.

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