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Thermosensitive Hydrogel Development for Its Possible Application in Cardiac Cell Therapy

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***Abstract*—**Ischemic events can culminate in acute myocardial infarction with irreversible cardiac lesions that cannot be restored due to the limited regenerative capacity of the heart. Tissue engineering proposes therapeutic alternatives by using biomaterials to resemble the native extracellular medium combined with healthy and functional cells. This research focused on developing a natural thermosensitive hydrogel, its physical-chemical characterization and *in vitro* biocompatibility determination. Hydrogels' morphological characterization was carried out through scanning electron microscopy and its chemical characterization by employing Infrared Spectroscopy technic. In addition, the biocompatibility was determined using fetal human ventricular cardiomyocytes cell line RL-14 and the MTT cytotoxicity test according to the ISO 10993-5 standard. Four biocompatible and thermosensitive hydrogels were obtained with a three-dimensional internal structure and two gelation times. The results show the potential of the hydrogel to increase the cell survival rate to the cardiac cell therapies under investigation and lay the foundations to continue with its characterization and biological evaluation both *in vitro* and *in vivo* models.

***Keywords*—**Cardiac cell therapy, cardiac ischemia, natural polymers, thermosensitive hydrogel.

I. Introduction

CARDIOVASCULAR diseases are the leading cause of morbidity and mortality worldwide. These diseases include coronary heart disease, also known as ischemic coronary disease, which is related to the diseases that affect the blood vessels that supply the heart muscle and lead to Acute Myocardial Infarction (AMI) [1].

Traditional treatment after an ischemic event includes a revascularization procedure and the combined administration of fibrinolytic and anticoagulants [2]. Despite advances and efforts to improve current treatments [3], their effects are always palliative rather than curative since they cannot reverse fibrosis and cardiac remodeling [4]. In this sense, tissue engineering seeks to develop scaffolds that mimic the extracellular matrix's microarchitecture and incorporate cells or different bioactive molecules to restore, maintain, and improve affected ischemic heart tissue [5]. Scaffolds have been developed mainly from polymeric compounds due to their diversity and versatility to be molded and chemically modified [6] and have been used to develop thermosensitive hydrogels, which can change from a sol to a gel state when increasing the temperature. This characteristic makes them suitable to be administered through catheterization in an *in vivo* model [7].

The purpose of this work was to develop a natural thermosensitive hydrogel as an alternative strategy to favor the regeneration of the cardiac tissue and determine their physical-chemical and biocompatible properties.

II. Materials and Methods

A. Materials

Low molecular weight chitosan (Ch) (50-190 KDa, 75-85% DDA, Sigma-Aldrich 901276-4), beta-glycerophosphate (β-GP, Merkmilipore 35675), bovine hydrolyzed collagen (BHC, Sigma-Aldrich 9000-70-8 G9391), porcine hydrolyzed collagen (PHC, Sigma-Aldrich 9000-70-8 G1890), glacial acetic acid (HAC, PanReac AppliChem 141008.1211), ethanol (Merkmilipore 1.00983.2500), cell line RL-14 (ATCC PTA-1499, ventricular human embryonic cardiomyocytes), Dulbecco's modified Eagle medium (DMEM Lonza BE12-604F) and fetal bovine serum (Gibco A4766801) were used.

B. Natural Hydrogel Development

The methodology was adapted and modified from Dang *et al*, 2017 [8]. Four types of hydrogels were made with different concentration of β-GP and different type of HC. A1: Ch 2.5% w/v/β-GP 8.5% w/w/BHC 6.5% w/v. A2: Ch 2.5% w/v/β-GP 8.5% w/w/PHC 6.5% w/v. B1: Ch 2.5% w/v/β-GP 10.5% w/w/CHB 6.5% w/v. B2: Ch 2.5% w/v/β-GP 10.5% w/ w/PHC 6.5% w/v. These final study concentrations were determined in previous experimental designs, carried out by the Cardiovascular Dynamics research group (unpublished data).

Ch was mixed with CH, and under constant stirring, at 4 °C x 15 min. Then β-GP was added dropwise and mixed under stirring at 4 °C x 30 min. Once the hydrogels were obtained in sol state, they were stored at 4 °C overnight for degassing. The following day the samples were incubated at 37 °C in a thermal bath, and their gelation time was determined using the inverted tube and turbidity method.

C. Physicochemical Characterization

1. Scanning Electron Microscopy (SEM)

The hydrogels' morphological characteristics were determined using a SEM (Jeol NeoScope JCM-6000plus) by a

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magnification of 2000X, 15 kV, and a high vacuum. Previously, the samples in gel state were subjected to successive dehydration with ethanol between 50 and 100% v/v. Subsequently, the samples were dried for three consecutive days. Finally, the hydrogels were cryofractured by immersion in liquid nitrogen for 3 min. In this test, three batches were evaluated for n = 3, where the results are shown as representative micrographs of each type of hydrogel. The batches evaluated for n = 3 and the results are shown as representative micrographs of each type of hydrogel.

2. Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (ATR) Module

To determine the changes in the functional groups present in the developed biomaterial, the hydrogels in gel state were dried for three days to remove the water content. Then, they were characterized by an FT-IR spectrophotometer (Nicolet iS50 with ATR module) at a resolution of 4 cm^{-1} and 32 scans. In this test, the experimental n was three, and the results shown are representative absorption spectra of each sample.

D. Sterilization

The hydrogels in the sol state were exposed to ultraviolet radiation for 30 min for their sterilization and subsequent use in biological tests.

E. In vitro Model: RL-14 Human Ventricular Cardiomyocytes

1. Hydrogel´s Precursors Cytotoxicity

To determine the degree of cytotoxicity of the precursors (Ch, BHC, PHC and β-GP), the cell line RL-14 was cultured and evaluated for metabolically active cells, quantified through the MTT method, according to the ISO 10993-5 standard [9]. This method consists of the metabolic reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole] Bromide (MTT) carried out by the mitochondrial enzyme succinate dehydrogenase, turning it into a purple-colored compound called formazan. For the assay, $3x10^3$ RL-14 cardiomyocytes/well of cardiomyocytes were seeded in 96-well dishes, in 80 µl of DMEM culture medium supplemented with 10% FBS and incubated at 37 °C with 5% CO_2. After 24 h cell growth, 10 µl of each precursor was added to each well, and after 24 hours of post-treatment, 10 µl of MTT was added at a concentration of 5mg/mL and incubated for 4 hours at 37 °C and 5% CO_2. Next, 100 ul of DMSO/well was added, and the plate was incubated overnight at 70 rpm and 37 °C. Finally, the absorbance was read in a spectrophotometer (Multiskan FC) at a wavelength (λ) of 570 nm.

The cytotoxicity of the hydrogel was presented in terms of cell viability, using (1):

$$\%Cellviability = \frac{(\lambda P - \lambda PM)}{(\lambda CC - \lambda M)} x100 \quad (1)$$

where P: precursor, PM: precursor + medium, CC: Cell control and M: medium.

2. Hydrogels' Cytotoxicity

The cytotoxicity of the hydrogels A1, A2, B1 and B2 was evaluated through the viability of cells in contact with the surface of the hydrogel according to ISO 10993-5. For that, 150 µl/well of hydrogel was placed in a 96-well plate and brought to 37 °C for 3 hours. Once gelled, the hydrogels were washed with NaCl 0.9 % w/v and supplemented DMEM. Then, $3x10^3$ RL-14 cardiomyocytes/well of were seeded in 80 µl of supplemented DMEM. The cytotoxic effect of each hydrogel was evaluated at incubation periods of 24 h, 48 h and 72 h, using the MTT assay, using the protocol described for the precursors.

The data were presented as the increase in optical density (OD) over time, since the greater the formazan amount in the culture medium, the greater the light beam absorbance and, therefore, more OD. Each hydrogel was evaluated in triplicate with three production batches (n = 3).

3. RL-14 Cardiomyocytes Viability Post Interaction with Hydrogels

The viability of RL-14 cardiomyocytes in contact with the hydrogels was determined, placing 150 µl/well of each hydrogel in a 96-well plate and brought to 37 °C for 3 h. Once gelled, the hydrogels were washed with NaCl 0.9% w/v and not supplemented DMEM. Then, $3x10^3$ RL-14 cardiomyocytes/well of RL-14 were seeded in 80 µl of DMEM with 10% FBS and incubated at 37 °C with 5% CO_2. After 13 days, the supernatant from each well was collected and placed in new wells.

Subcultured supernatants photomicrographs were taken in an inverted light microscope (IM3FL4) starting from day 2 and every 4 days until the well had a confluence between 80 to 100%, in order to record cell growth.

F. Statistical Analysis

An experimental design using a multifactorial ANOVA in the Statgraphics 18® Centurion program was carried out to establish the best results in terms of cell viability. In addition, T-test statistics analyzed the means between paired samples. For the MTT test, three replicates were used at three independent times with n = 3.

III. Results and Discussion

A. Natural Hydrogel Development

Four types of hydrogels were developed denominated A1, A2, B1 and B2, each with a different concentration of β-GP and different nature of CH, as shown in Fig. 1. The gelation time of samples A1 and A2 was 40 min, and for B1 and B2 was 30 min, which indicates that the higher the concentration of β-GP, the shorter the gelation time.

The gelation time was determined qualitatively through the inverted method and the turbidity of the solution. A study conducted by Berger *et al*, [10] indicated that in commercial chitosan, deacetylated areas in polysaccharide chains do not occur throughout the entire carbon chain (random) but are distributed in blocks due to the chitin type deacetylation. Therefore, in the gelling process, there is an interaction between the acetylated areas forming large hydrophobic domains, which

due to their block distribution and their size generate a scattering of light that is observed as turbidity in the solution, which does not happen with hydrophilic domains (between deacetylated areas) [10].

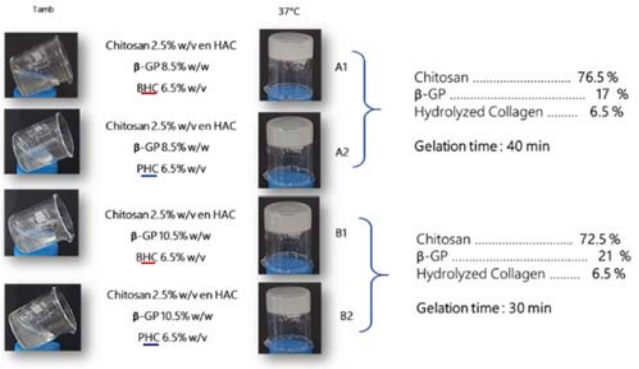

Fig. 1 Thermosensitive hydrogels. The volumetric proportions of the two groups of hydrogels are specified. Tamb: room temperature

The pH of the hydrogels was evaluated in the sol and gel state and are shown in Table I, denoting that all the pH values are in the physiological range, suitable for application in cardiovascular tissue engineering.

TABLE I
HYDROGELS' PH

Sample	Sol state pH	Gel state pH
A1	7.0	7.0
A2	7.0	7.0
B1	7.0	7.0
B2	7.0	7.0
Ch 2.5% w/v	5.0	N/A
BHC 2% w/v	4.0	N/A
PHC 2% w/v	4.0	N/A
β-GP 50% w/w	10.0	N/A
Ch + β-GP 8.5% w/v	7.0	N/A
Ch + β-GP 10.5% w/v	7.0	N/A

B. Physicochemical Characterization

1. SEM

The electron micrographs of each type of hydrogel are observed in Fig. 2. All the samples presented a three-dimensional internal microarchitecture with interconnected pores. For tissue engineering applications, it is necessary to develop structures that mimic the microarchitecture and composition of the ECM of native tissue, which is a network of polymers that communicate to the cell through its cytosolic membrane with the surrounding environment [7]. The samples with BHC type B (see Figs. 2 (a) and (c)) presented a network of homogeneous porosity and regular pore sizes. On the other hand, a laminar morphology and heterogeneous pores were observed in the samples with PHC type A (see Figs. 2 (b) and (d)). These differences may be since BHC has short polypeptide chains (40-90 kDa) due to the production treatment, which could favor the generation of a porous homogeneous microstructure. On the contrary, PHC exhibits long polypeptide chains (100-700 kDa), which could favor a framework with larger and lax pores, generating a heterogeneous microarchitecture. On the other hand, the concentration of β-GP can influence the microarchitecture of the hydrogel, as shown in Figs. 2 (e) and (f), where at a higher concentration of β-GP tiny pores are formed, probably because of the higher molecules of β-GP in the solution, the better interaction with the positively charged deacetylated chains of Ch. Which in turn promote the formation of more hydrogen bonds, generating an increase in hydrophobic interactions and a greater electrostatic attraction [11].

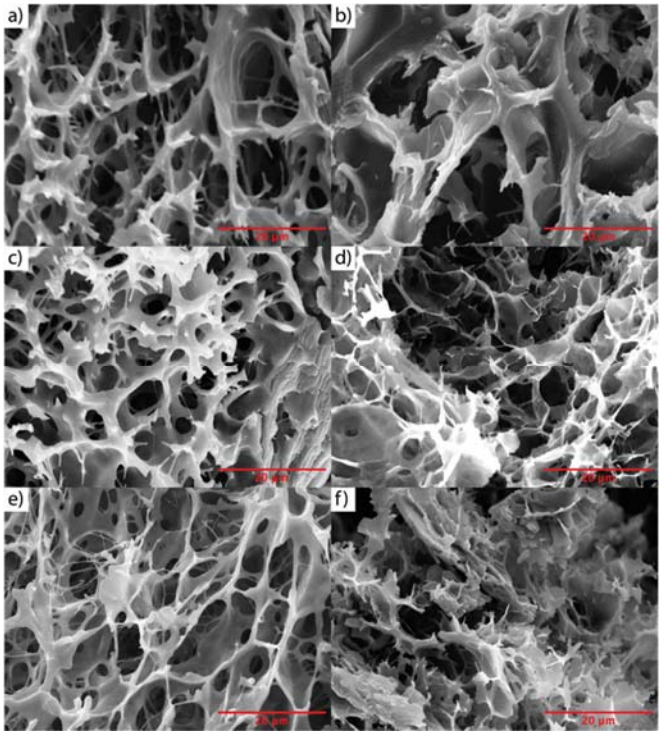

Fig. 2 Cross section of hydrogels microarchitecture where (a) A1 (QB 2.5% w/v/β-GP 8.5% w/w/CHB 6.5% w/v); (b) A2 (QB 2.5% w/v/β-GP 8.5% w/w/CHP 6.5% w/v); (c) B1 (QB 2.5% w/v/β-GP 10.5% w/w/CHB 6.5% w/v); (d) B2 (QB 2.5% w/v/β-GP 10.5% w/w/CHP 6.5% w/v); (e) QB 2.5% w/v/β-GP 8.5% w/w); (f) QB 2.5% w/v/ β-GP 10.5% w/w; 20 µm scale bar

2. Fourier Transform Infrared Spectroscopy with ATR Module

The absorption spectra of samples A1, A2, B1 and B2 and the precursors of Ch, BHC, PHC and β-GP, are observed in Fig. 3. The four types of hydrogels showed a similar absorption behavior, with a change in the intensity and shape of the bands that correspond to OH and NH groups compared to the precursors, indicating the formation of hydrogen bonds [8]. The amide bands I (C = O), II (N-H and C-N) and III (N-H and C-N) were conserved in wavelengths close to the controls of BHC and PHC; however, in the gelling process, these bands decrease in intensity. That means that the amides participate in the gelling reaction, forming intramolecular or intermolecular hydrogen bonds with β-GP, Ch and water, or electrostatic attractions, between amino and carboxyl groups [12]. These interactions are corroborated by the slight shifts of the original

β-GP bands with respect to the hydrogel samples, corresponding to the P = O bonds (from 1130 cm^{-1} to 1128 cm^{-1}), OPO (from 1057 cm^{-1} at 1053 cm^{-1}), POH (from 1057 cm^{-1} to 105 cm^{-1}), PO (from 983 cm^{-1} to 982 cm^{-1}) and OPO (from 544 cm^{-1} to 524 cm^{-1}). These results showed the interaction that occurs between the three compounds.

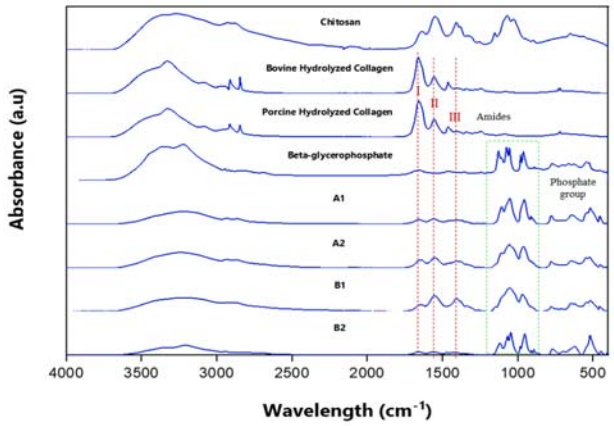

Fig. 3 FTIR-ATR absorption spectra of hydrogels and their precursors, where A1 (QB 2.5% w/v/β-GP 8.5% w/w/CHB 6.5% w/v). A2 (QB 2.5% w/v/β-GP 8.5% w/w/CHP 6.5% w/v); B1 (QB 2.5% w/v/β-GP 10.5% w/w/CHB 6.5% w/v); B2 (QB 2.5% w/v/β-GP 10.5% w/w/CHP 6.5% w/w); Representative images of n = 3

C. In vitro Model: RL-14 Human Ventricular Cardiomyocytes

1. Hydrogels' Precursors Cytotoxicity

Fig. 4 shows the response of cells in contact with hydrogel precursors, which is expressed as cell viability and evaluated in 24 hours. All the precursors presented a viability percentage higher than 75% and a cytotoxicity percentage lower than 25%; that means all the concentrations used are within the accepted range as non-cytotoxic, according to ISO 10993-5 [9].

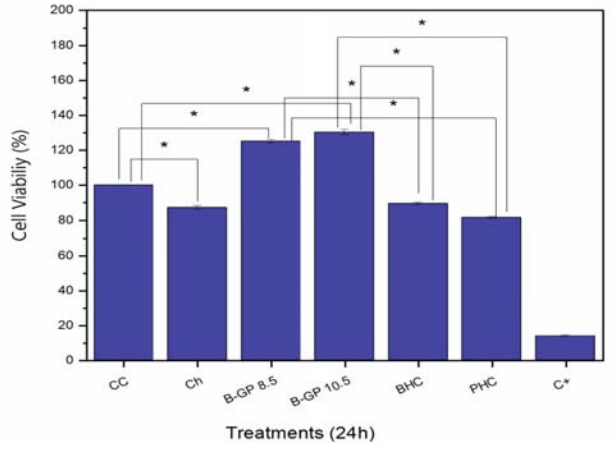

Fig. 4 Cytotoxic effect of hydrogel precursors on RL-14 cardiomyocytes treated for 24 h, where CC: cell control, 3 batches with n = 3 were evaluated, each in triplicate

It was found that Ch decreases cell viability to 86.9% compared to the control cells. This behavior is because as the concentration of Ch increases, the degradation products of the polysaccharide also increase, which modifies the osmolarity of the medium, generating an imbalance in the cellular homeostasis of cardiomyocytes [13]. Although the difference in cell viability was statistically significant ($p < 0.05$) compared to the control, it was not less than 75%, as indicated by the ISO 10993-5 standard. On the other hand, the effect of β-GP at both concentrations was to enhance the percentage of cell viability (125% for β-GP at 8.5% w/w and 130% for β-GP at 10.5% w/w), which exceeded 100% compared to the cell control with statistically significant differences ($p < 0.05$). This behavior was reported by Ahmadi & Bruijn, where bone marrow derived mesenchymal cells increased their proliferation by 20% by contact with 2% Ch and 10% β-GP [13]. Furthermore, β-GP has also been used in cell cultures as a source of inorganic phosphate (Pi) for the osteogenic differentiation of bone marrow stromal cells [14] while, in the *in vivo* model, Pi is an essential nutrient for living organisms since it is a component of energy metabolism, kinases and phosphatases signaling pathways [15] and synthesis and function of lipids, carbohydrates and nucleic acids [16]. Therefore, in RL-14 cardiomyocyte cell culture, β-GP could be a source of Pi and play a role in intracellular reactions necessary for cell proliferation (see Fig. 4).

Both BHC and PHC decreased cell viability statistically significantly (89% and 81%, respectively, $p < 0.05$) compared to the control cells. Qualitatively, it was observed that PCH solution was more viscous than BCH solution, both being at the same concentration of 6.5% w/v. Thus, the difference in apparent viscosity could be associated with the decrease in cell viability. Furthermore, the viscosity of HC increases the viscosity of the culture medium, affecting the availability of nutrients in the medium and modifying the mass transfer, limiting the proliferation of cellular metabolic responses [17].

2. Hydrogels' Cytotoxicity

The OD is directly related to the absorbance of the sample, which indicates the formazan compound's concentration in the medium.

Samples A1, B1 and B2, had a statistically significant increase ($p < 0.05$) in OD between 24 h and 72 h, so that A1 went from 1.16 at 24 h to 1.30 at 72 h, B1 went from 1.14 at 24 h to 1.42 at 72 h, and B2 went from 1.1 at 24 h to 1.40 at 72 h (see Fig. 5). These results indicate that cells in contact with the hydrogel for 72h did not lose their viability but instead could proliferate over time and be metabolically active to transform the compound MTT.

Hydrogels B1 and B2 exhibited the highest OD values compared to A1 and A2, which could be related to the fact that hydrogels B had a β-GP concentration of 10.5% w/w, related to a greater increase in cell proliferation compared to the β-GP concentration of 8.5% w/w (see Fig. 4). On the other hand, A2 OD did not present a statistically significant difference ($p > 0.05$) over time since it went from 1.2 at 24 h to 1.25 at 72 h. Correlating these results with the viability of the PHC precursor (see Fig. 4), it showed a lower percentage of cell viability compared to sample A1, which contains BHC.

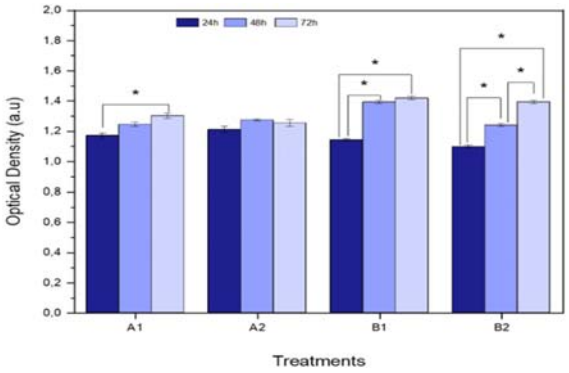

Fig. 5 Cytotoxic effect of hydrogels on RL-14 cardiomyocytes, where A1 (Ch 2.5% w/v/β-GP 8.5% w/w/BHC 6.5% w/v); A2 (Ch 2.5% w/v/β-GP 8.5% w/w/PHC 6.5% w/v); B1 (Ch 2.5% w/v/β-GP 10.5% w/w/BHC 6.5% w/v); B2 (Ch 2.5% w/v/β-GP 10.5% w/w/PHC 6.5% w/v); Three lots were evaluated for a n = 3, each in triplicate

3. RL-14 Cardiomyocytes' Viability Post Interaction with Hydrogels

The supernatants subculture was carried out to observe the proliferation of RL-14 cardiomyocytes, which had been in contact with hydrogels A1, A2, B1 and B2 for 13 days. The assessment time was determined, considering the differences found between a 2D culture and a 3D culture, including the slowing down of cell division since the availability of oxygen and nutrients is not homogeneous throughout the culture [18].

Fig. 6 shows the microphotographic record of the RL-14 cardiomyocyte cell cultures in interaction with each hydrogel, and the following aspects are highlighted: i) The supernatant of the 3D cultures contained cells and hydrogel fragments (yellow arrows), which indicates that the hydrogel begins its degradation process over time in a biologically active microenvironment where are living cells without alteration of their metabolic processes. ii) The RL-14 cardiomyocytes conserved the ability to proliferate in the *in vitro* subculture, having been in contact with the hydrogels for 13 days, and iii) The hydrogel fragments in the cell subculture acted as ECM areas where cell clusters (blue arrows) proliferated until they joined with each other (purple arrows) and covered the entire surface of the culture flask (black arrow).

Morphologically, long extensions of cell membranes (red arrows) such as lamellipodia and filopodia were observed, and they are not usually seen in 2D cultures. HC and Ch may generate these cell membrane extensions in the hydrogel fragments since it has been shown that Ch components such as N-acetylglucosamine and D-glucosamine induce type I collagen synthesis *in vitro* [19], increasing the components of the ECM and, therefore, the cell-ECM interactions. Furthermore, the cellular phenotypes are diversified as in a native cellular environment (green arrows) [20], [21].

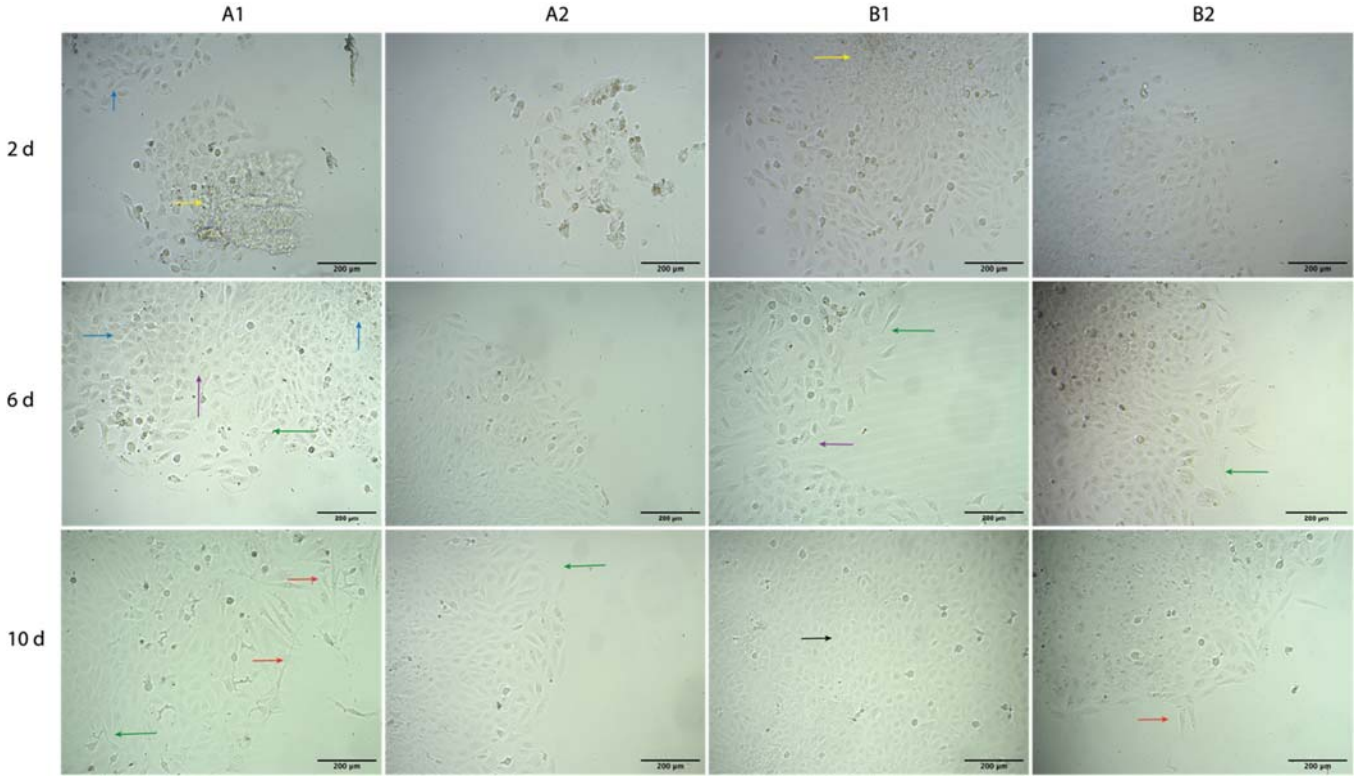

Fig. 6 Subculture microphotographs of RL-14 cardiomyocyte supernatants that were in interaction with thermosensitive hydrogels for 13 days, where A1 (Ch 2.5% w/v/β-GP 8.5% w/w/BHC 6.5% w/v); A2 (Ch 2.5% w/v/β-GP 8.5% w/w/PHC 6.5% w/v); B1 (Ch 2.5% w/v/β-GP 10.5% w/w/BHC 6.5% w/v); B2 (Ch 2.5% w/v/β-GP 10.5% w/w/PHC 6.5% w/v); the microphotograph register times were 2, 6 and 10 days. Yellow arrows are hydrogel fragments, blue arrows are ECM areas, purple arrows are cell clusters joined, black arrow is entire culture flask surface covered, red arrows are cell membranes extensions, and green arrows are cell phenotypes. t: time, d: days. Scale bar 200 µm

IV. Conclusion

Four thermosensitive hydrogels were obtained (A1, A2, B1 and B2), with a different chemical composition but with similar physicochemical characteristics, allowing cell growth and exhibiting gradual degradation over time. Furthermore, the hydrogels developed allow cell viability > 75%. These characteristics make it suitable to be considered for injectable and minimally invasive cardiac therapeutic applications in whose porous structure could be encapsulated cells to direct them to the injured site of the cardiac muscle.

Considering the gelation time and the biocompatibility results, it can be concluded that hydrogels B1 and B2 have the best physical-chemical and biological characteristics, which visualize them as suitable candidates for use in tissue engineering regenerative medicine against injuries of infarcted or ischemic myocardial tissue.

Acknowledgment

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