Design and characterization of microspheres for a 3D mesenchymal stem cell culture

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Abstract

Recent studies have shown the relevance of growing mesenchymal stem cells (MSCs) in three-dimensional environments with respect to the monolayer cell culture on an adherent substrate. In this sense, macroporous scaffolds and hydrogels have been used as three-dimensional (3D) supports. In this work, we explored the culture of MSCs in a 3D environment created by microspheres, prepared with a fumarate-vinyl acetate copolymer and chitosan. In this system, the environment that the cells feel has similarities to that found by the cells encapsulated in a hydrogel, but the cells have the ability to reorganize their environment since the microspheres are mobile. We evaluated their biocompatibility *in vitro* using RAW 264.7 macrophages and bone marrow mesenchymal stem cells (BMSCs). The results with RAW 264.7 cells showed good cell viability, without evident signs of cytotoxicity. BMSCs not only proliferate, but also rearrange to grow in clusters, thus highlighting the advantages of microspheres as 3D environments.

Keywords: microspheres, chitosan, 3D culture environment, mesenchymal stem cells, regenerative medicine

1. Introduction

Regenerative medicine utilizes different strategies to stimulate and support tissue regeneration after an injury, due to the limited ability of self-healing of certain tissues. Mesenchymal stem cells (MSCs) are used to accelerate wound healing and several methods allow their expansion using large-scale cell culture [Satija et al., 2009]. The success of MSCs therapy depends on a prolonged *ex vivo* culture, which eventually undergoes an aging process [Shojaei et al., 2019; Khademi-Shirvan et al., 2020]. Traditionally, MSCs are cultured in a two-dimensional (2D) system of standard tissue culture flask; however, in monolayer culture the microenvironment is quite different from that *in vivo*. This difference will influence signal transduction of stem cells, and causes 2D cultured stem cells lose their biological feature and functions [Zhang et al., 2018]. Hence, the application of three-dimensional (3D) cell culture techniques in stem cell research has received increased interest. The 3D cell organization provides greater cell–cell contacts and

interactions of cells with the extracellular matrix (ECM), and mimics better the natural microenvironment of a tissue, compared with traditional 2D monolayer cultures [Huang et al., 2020]. On the other hand, strategies based on the use of stem cells for the regeneration of damaged tissues involve either direct injection of cells into the body or the tissue-engineering approach, where cells are combined with a scaffold to aid delivery and promote tissue formation. Due to the issues of cell retention and efficiency of stem cell differentiation, tissue-engineering approaches surpass the cell-alone strategy significantly [Li et al., 2018].

Previously, it has been emphasized that the aggregation of MSCs into multicellular spheroid results in improvement of antiinflammatory and angiogenic properties, and facilitates differentiation into different cell lineages, as well as their survival after transplantation [Petrenko et al., 2017]. Spheroids favor cell to cell interaction but they lack the initial adhesion to a natural or artificial extracellular matrix [Ferreira et al., 2018] that can be crucial to the fate of the pluripotent cell favoring preservation of pluripotency or even inducing spontaneous differentiation to one of the possible lineages [McMurray et al., 2011]. Several biomaterial-assisted methods have been shown to promote the generation of spheroids, and the application of CH membranes is probably the most widely reported technique [Petrenko et al., 2017].

In this work, we explored the use of microgels to create a three-dimensional environment for the culture of MSCs. By microgel we mean a suspension of microspheres, each with the consistency of a gel, among which are the cells. In this way, the cell finds an artificial extracellular matrix in its environment that has similarities to what the cell would find encapsulated in a hydrogel, but with the advantage that the cell can reorganize its environment because the microspheres are mobile. Moreover, the cells acquire mobility and may or may not aggregate or open spaces to generate their own extracellular matrix in their environment. The system is quite different from microcarriers in which cells grow on its surface, forming a monolayer on the biomaterial [Leong & Wang, 2015]. The expansion of cells in microcarriers seeks to take advantage of the large specific surface area of the microcarriers to increase the efficiency of the culture, but actually it is not so far from the 2D culture system.

The field of microgels has demonstrated their versatility, modularity and tunability for an abundance of applications ranging from tissue engineering to therapeutic delivery [Newsom et al., 2019]. Microgels are effective as cell and molecule transporters due to their properties of hydrophilicity, tunability and biomimicry. In addition, microgels provide a 3D environment mimicking the ECM characteristic of high-water content, which allows cell migration and nutrient/waste exchange due to the micro-scaled size. Plus the added benefit of being easily injected at the site of application while maintaining biocompatibility of the starting material. Different biomaterials have been used to develop microgels or microspheres [Guan et al., 2017]. Biopolymers, mainly including natural polysaccharides, proteins and a few biodegradable synthetic polymers, have been researched to develop 3D supports, providing a versatile platform for cell culture and microtissue formation [Huang et al., 2020]. Hydrogels based on chitosan (CH) and CH derivatives have raised significant interest among researchers and have been studied into a broad range of biomedical applications, since it is a biodegradable, nontoxic and biocompatible naturally derived polymer [Croisier & Jérôme, 2013; Rufato et al., 2018]. CH has many functional groups and can be hydrated by water molecules, an advantage for its use as microgel, but resulting in the weakening of its mechanical strength. Due to this characteristic, different approaches to improve the physicochemical properties of CH were developed, such as the combination with natural or synthetic polymers [Croisier & Jérôme, 2013]. Furthermore, polyfumarates have shown good mechanical properties and an adjustable degradation rate based on the

appropriate selection of the comonomer composition [Pasqualone et al., 2013]. Then, the combination of CH with a polyfumarates is a way to balance its mechanical properties and degradation, as we have previously shown [Lastra et al., 2017].

Chitosan-based microspheres were also proposed for cell culture [Garcia Cruz et al., 2008; Zhang et al., 2018; Huang et al., 2018; Wang et al., 2019]. For example, Garcia Cruz et al. developed a 3D construct that was obtained by combining MSCs and genipin-crosslinked chitosan microparticles [Garcia Cruz et al., 2008]. Moreover, Zhang et al. developed hybrid microspheres from chitosan and graphene oxide, which supported stem cell expansion, growth, and proliferation [Zhang et al., 2018]. Furthermore, chitosan-based microspheres were prepared for hepatocyte and macrophage cultures and better cell activity was found compared to 2D cell culture in both studies [Huang et al., 2018; Wang et al., 2019].

Additionally, it was demonstrated that the combination of a close packing of microspheres and cells promotes integrin expression of cells and the protein mediated adhesion to the 3D substrate [Garcia Cruz et al., 2008b], allows modulating the mechanical properties of the cell-material construction [Baraniak et al., 2012] or drug delivery or growth factors [Garcia Cruz et al., 2013]. In a nutshell, microspheres are a promising cell culture tool with great potential in fields such as tissue engineering and cell therapy. Recently, our groups reported the development of a system for cartilage regeneration based on microspheres chitosan/poly(L-lactic acid) which create an adequate biomechanical environment for the differentiation of the MSCs migrating to the region of cartilage defect, after injuring subchondral bone in a rabbit knee model [Zurriaga Carda et al., 2019]. Three months after surgery, the histological characteristics of the regenerated tissue were found to be those of hyaline cartilage. These promising results inspired us to continue studying these types of systems and their possible use in regenerative medicine.

The aim of this work is to explore a new biomaterial in the form of microspheres to create the 3D environment for tissue development *in vitro* or *in vivo*. Previously, 2D culture supports were obtained from a mixture of CH and a fumaric polymer for bone and cartilage tissue engineering applications, presenting very good properties in terms of biocompatibility. [Lastra et al., 2017; Lastra et al., 2018]. In the present study, we developed and characterized polymeric microspheres made of this mixture using a water/oil emulsion technique for their potential use to create 3D culture environments. We evaluated their biocompatibility *in vitro*, using RAW 264.7 macrophages and bone marrow stem cells.

2. Experimental

2.1. Materials

Vinyl acetate (VA, 99%), isopropyl alcohol, and benzoyl peroxide (BP, recrystallized from methanol) were purchased from Merck (Buenos Aires, Argentina). Fumaric acid was given by Maleic S.A., Argentina. Diisopropyl fumarate (DIPF) monomer was prepared and purified as previously described [Cortizo et al., 2007]. Chitosan (CH, Sigma-Aldrich, medium molecular weight), Borax ($Na_2B_4O_7.10H_2O$, Timper Laboratorios, 99.9%), extra virgin olive oil (Terra nova, La Riojana Cooperativa Ltda.), acetic acid (Scharlau) and other solvents were provided by Merck or Sintorgan. The Dulbecco's modified Eagle's medium (DMEM) were acquired from Invitrogen (Buenos Aires, Argentina) and fetal bovine serum (FBS) from Natacor (Argentina).

2.2. Fumarate Copolymer Synthesis

Radical copolymerization of VA with DIPF, was carried out in bulk, initiated by BP under microwave energy, hydrolyzed and characterized following the methodology

previously reported [Lastra et al., 2017]. Materials were characterized by infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H-NMR) and size exclusion chromatography (SEC) as described previously. This resulting copolymer was designed as diisopropyl fumarate-vinyl acetate copolymer hydrolyzed (PFVH).

2.3. Preparation of microspheres

To obtain the microspheres, a mixture of 50% w/w chitosan (CH) and the previously synthesized copolymer (PFVH) were cross-linked in situ with borax. Briefly, on a 1% w/v solution of CH in dilute acetic acid (3% v/v) was added to the same volume a PFVH solution 1% w/v in acetic acid with continue agitation. Subsequently, borax (4% w/w of the blend of PFVH/CH) was added in order to carry out the cross-link. Then, the aqueous polymeric mixture was sonicated for 5 min and was dripped above commercial olive oil (water/oil 1:3) with a flow of 10 ml/hs under 1500 rpm magnetic agitation at 30 °C. The emulsion obtained was left under stirring for 24 hours. Finally, the microspheres were isolated by centrifugation for 10 min, at 1500 rpm. The oil phase was discarded and successive washes were performed with distilled water until neutrality, recovering the microspheres by filtration with a 40 µm pore nylon filter. The polymeric microspheres (µsp) were sterilized with ethanol (70% v/v) overnight at 4 °C. Then, they were washed three times with phosphate-buffered saline sterile (PBS, pH 7.4) and were exposed UV light 20 min before its use.

2.4. Microspheres characterization

The microspheres were coated with gold and their morphology was examined using scanning electron microscopy (SEM; Phillips 505, The Netherlands), with an accelerating voltage of 20 kV. The images were analyzed using Soft Imaging System ADDAII. The mean diameter and size distribution of microspheres were studied using a Nikon Eclipse TS100 inverted optical microscope and pictures were taken with a Nikon Coolpix 4500 digital camera. Images were analyzed using the Image J program (www.macbiophotonics.ca/imagej) with a Microscope scale plugin and the results are expressed as the average of one hundred measurements.

2.5. Biocompatibility Studies

2.5.1. Cell Cultures and Incubations

Murine macrophage RAW 264.7 cells are derived from a cell line established from murine tumors induced with Abelson leukemia virus in Mus musculus, [Raschke et al., 1978]. They can express different markers of cytotoxicity in response to toxic substances, such as interleukins synthesis, nitric oxide production (NO) and expression of nitric oxide synthases (NOS), in addition to morphological changes [Denlinger et al., 1996]. Because of these characteristics, they are considered an excellent model for *in vitro* studies of cytotoxicity of different substances.

Bone marrow stem cells (BMSCs) were obtained according to standard procedures in our laboratory [Torres et al., 2019]. Briefly, male Sprague-Dawley rats were slaughtered under anesthesia by rapid neck dislocation and BMSCs were collected by flushing medullary canal of the dissected femur with DMEM, under sterile conditions. All experiments with animals were carried out according to the Guide for the Management and Use of Laboratory Animals [Guidelines on Handling and Training of Laboratory Animals, 1992], under the conditions established in the national bioethical standards - Provision ANMAT 6677/10 - e international - Nuremberg Code, Helsinski Declaration and its amendments.

BMSCs and RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100

U/ml penicillin and 100 g/ml streptomycin) (basal media) at 37 °C, 5% CO_2 and in a humidified atmosphere of 95% air.

2.5.2 Cell viability

The biocompatibility of microspheres was assessed using RAW 264.7 cells or BMSCs under 2D (in monolayer) or 3D (in suspension) culture condition by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) bioassay [Lastra et al., 2018]. This assay measured the reduction of the MTT to formazan by intact mitochondria in living cells. To do this, microsphere suspension was placed in an eppendorf tube for stabilization for at least 24 hs in DMEM without phenol red, 10% FBS and antibiotics in static conditions, at 37 °C and 5% CO_2, as previously published [Clara-Trujillo et al., 2019]. Then, $1x10^4$ cells/ml were co-cultivated in basal media with the sterile microspheres ($1x10^4$ µsp/ml) on culture plates of 48 wells (2D) or eppendorf tubes of 500 µl (3D). Tissue culture polystyrene (TCPS) wells or eppendorf tube spheres-free were used as controls for 2D or 3D culture conditions, respectively. To optimize adhesion in all cases, the cells were first harvested for 1 hour in the incubator (37 °C, 5% CO_2) with the microspheres but with only 50 µl of cell culture media, after which 250 µl of basal media was added. After each evaluation time, the conditioned media was replaced with a solution of 0.1 mg / ml MTT and incubated for an additional 1 hour. Then, the formazan precipitate was dissolved in dimethylsulfoxide (DMSO, Merck) and the absorbance was read at 570 nm using an automatic ELISA plate reader (Infinite® F50, Tecan Trading AG, Switzerland).

2.5.3 Evaluation of Cytotoxicity

To determine the potential cytotoxicity of the polymeric microspheres, we evaluated the cell viability and the production of nitric oxide (NO) and interleukin-1β (IL1β) with RAW 264.7 cells. The macrophages were cultured with the sterile microspheres under 2D or 3D culture condition and were compared to spheres-free culture (control condition). After 24 and 48 hours of incubation, the cell viability by MTT assay was measured and the supernatants were collected to evaluate NO and IL1β production by the Griess' assay or ELISA kit (BD OptEIATM mouse IL-1β ELISA), respectively [Lastra et al., 2018].

2.5.4 Cell morphology

In order to study the cellular interaction with the microspheres, BMSCs morphology and distribution was examined with SEM and fluorescence microscopy. Cells were seeded with the microspheres in a 2D or 3D system and cultured for 7 days, as it was described for cell viability assay. After this culture period, samples to be observed by SEM were washed with PBS and fixed with 10% p-formaldehyde for 10 min, dehydrated with methanol for 5 min and dried at room temperature. Finally, the samples were coated with gold and SEM images were obtained (SEM, Philips 505). For fluorescence microscopy, cells were washed twice with PBS, fixed with 4% paraformaldehyde/PBS for 10 min at room temperature and permeabilized with absolute ethanol for 4 min at -20 °C. Alexa Fluor 488 Phalloidin stock solution in methanol (495/518 nm Life technology) was diluted in PBS (1:100) and incubated for 2 hs at room temperatures in the dark, to stain F-actin fibers [Bravi-Costantino et al., 2020]. Nuclei were visualized adding Propidium Iodide (1:300, Molecular Probes, Buenos Aires, Argentina). Finally, the samples were mounted between microscope slides and coverslips using glycerin: PBS (9: 1) and visualized in a confocal laser scanning microscopy (CLSM) Leica TCS SP5 connected to a digital color camera. The photographs were taken with an objective of 10X and 63X. Images were recorded with the cell Sens Software (Olympus, Tokyo, Japan) and were analyzed with the free software ImageJ. For all these studies, the cells growing in 3D conditions were placed on glass coverslips before fixing them.

2.6. Statistical Analysis

Results are expressed as the mean ± standard error of mean and, unless indicated otherwise, were obtained from two separate experiments performed in triplicate. Differences between groups were assessed by one-way ANOVA with Tukey post hoc test. For non-normal distributed data nonparametrical Kruskal – Wallis with Dunn's post hoc test was performed, using Graph Pad InStat v. 3.00 (Graph Pad Software, San Diego, CA, USA). $p < 0.05$ was considered significant for all statistical analyses.

3. Results and discussion

3.1. Polymer microsphere production and characterization

The polymeric microspheres were produced by an oil/water (o/w) emulsion using borax as crosslinker of the two copolymers, the previously synthesized (PFVH) and CH. Borax was chosen as it is considered a low cytotoxic crosslinking agent. In addition, our previous studies showed that its incorporation into this polymer mixture allows good compatibility of the polymers and no cytotoxic effects were observed under these conditions [Lastra et al., 2017; Lastra et al., 2018]. Microparticles produced with a rough surface and spherical shape were evidenced by SEM (Figure 1A).

The size distribution was studied with wet microspheres, incubated at least 24 hours in DMEM medium, and were observed by optical microscopy finding an average size of 70.4 ± 2.0 µm (Figure 1B).

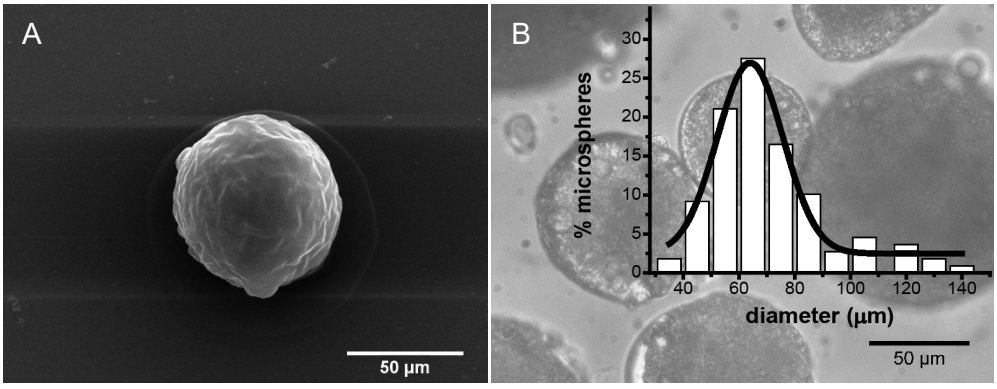

Figure 1. SEM image (A) and size distribution (B) of the microspheres

Compared to existing commercial microcarriers, such as polystyrene, gelatin and cellulose microsphere, the fabrication process of PFVH-CH microspheres in this study is simpler and more efficient because it needs no polyreaction with biologically incompatible materials [Healthcare & Biosciences, 2005]. Another advantage is that CH and fumaric polymers are also low cost because they are easily obtained from fishing industry and oil industry waste, respectively.

3.2. Biocompatibility studies

3.2.1 Macrophage studies

In a first study, the biological response against the designed microspheres was evaluated *in vitro* with murine macrophages RAW 264.7. Macrophage viability in the presence of the microspheres (μsp) were studied after 24 and 48 hours of incubation under 2D (in monolayer) or 3D (in suspension) culture conditions (Figure 2). Data for these assays are expressed as percentage with respect to the basal, considering the cell viability at 24 hours in the control condition (without μsp) as basal condition.

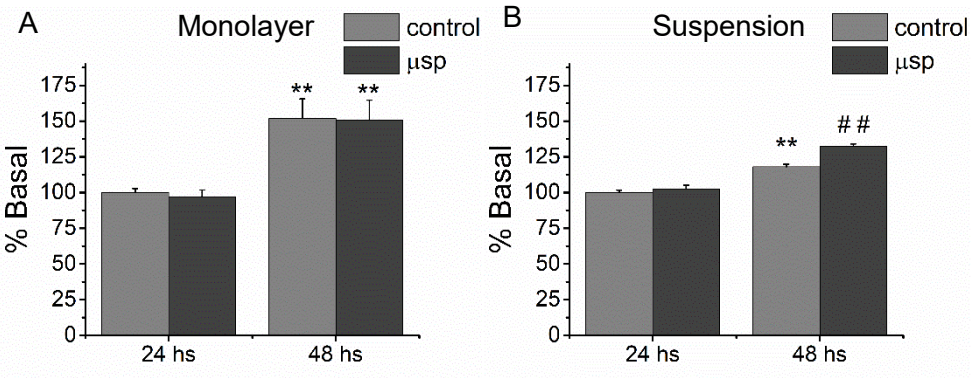

Figure 2. MTT assay with RAW 264.7 cells in (A) monolayer and (B) suspension, **p<0.01 vs 24 hs, ## p<0.01 vs control 48 hs

The results displayed in Figure 2A show that there are no differences in cell viability in the presence or absence of the microspheres in the monolayer culture. Such outcomes suggest that the designed microspheres do not affect the viability of RAW 264.7 cells under 2D *in vitro* culture conditions. In addition, under 3D culture conditions (Figure 2B), it can be seen that after 24 hours there was no difference in cell viability between control and culture in the presence of the microspheres. However, after 48 hours of culture a significant increase in cells viability was found in the presence of the microspheres with respect to the control cells. This could be because RAW 264.7 cells in the presence of the microspheres find a more convenient surface to adhere and proliferate than in the tube (non-adherent surface). This idea is supported by the observation (Figure 3) that RAW cells seem to grow on the surface of the microspheres after 48 hours of suspension culture. The image was recorded by optical microscopy of RAW 264.7 cells co-incubated with the microspheres for 48 hours in suspension.

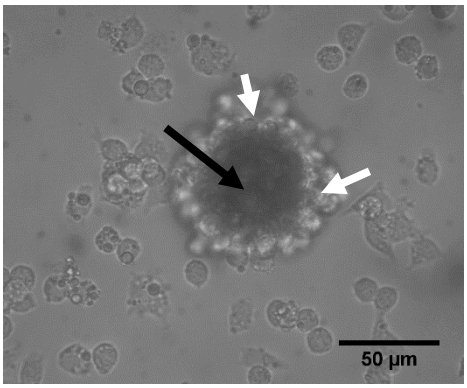

Figure 3. RAW 264.7 cells co-incubated with the microspheres 48 hs, the white arrows point to the cells and the black arrows to microspheres.

Additionally, we have assessed whether the microspheres can generate a cytotoxic effect in monolayer or suspension system. Thus, we evaluated the levels of NO and IL1β released to the culture medium by RAW 264.7 cells after 24 and 48 hours. Table 1 shows the results for NO levels, where no significant differences were found between the presence or absence of microspheres in the macrophages culture, both for the 2D and 3D culture systems. Similar results were obtained for IL1β levels released in the medium for the different culture conditions after 24 and 48 hours (table 2).

	NO (nmol/ml)			
	monolayer		suspension	
	24 hs	48 hs	24 hs	48 hs
Control	0.48 ± 0.11	0.80 ± 0.10	1.08 ± 0.20	1.22 ± 0.34
Microspheres	0.69 ± 0.06	1.01 ± 0.06	1.20 ± 0.17	1.31 ± 0.35

Table 1. NO production (Results expressed as the media ± standard error of mean, n= 8).

	IL1β (pg/ml)			
	monolayer		suspension	
	24 hs	48 hs	24 hs	48 hs
Control	4.22 ± 0.63	3.53 ± 0.28	2.27 ± 0.21	2.67 ± 0.48
Microspheres	4.78 ± 0.69	4.06 ± 0.34	2.37 ± 0.16	2.57 ± 0.36

Table 2. IL1β production (Results expressed as the media ± standard error of mean, n= 8).

The low cytotoxicity of chitosan generates great interest for its use in the development of biomaterials [Croisier & Jérôme, 2013]. Nevertheless, its combination with synthetic materials in order to improve its physicochemical properties can introduce undesired cytotoxic effects. It is also known that surface topography can influence inflammatory cell response [Padmanabhan & Kyriakides, 2015]. Therefore, the evaluation of these properties is important when developing a new material for biomedical applications. In this context, macrophages represent a good model to research the cytotoxicity of biomaterials, because they have the ability to react against foreign bodies, producing and releasing inflammation mediators such as interleukin-1β (IL1β) and nitric oxide (NO) [Denlinger et al., 1996]. For our study material, the outcomes of these cells showed good cell viability and absence of a cytotoxic response against these materials at the times studied.

3.2.2 BMSCs studies

Since we were interested in the development of biomaterials for culture and expansion of stem cells, we also studied the biocompatibility of microspheres designed with bone marrow stem cells (BMSCs). We assessed cell viability, actin-cytoskeleton development and cell morphology in these systems.

The results of the MTT assay in 24 and 48 hours showed good viability of the BMSCs growing in the presence of the microspheres (μsp) in monolayer (Figure 4A) and in suspension (Figure 4B). Results are expressed as percentage with respect to the cell viability at 24 hours in the control (basal condition). The data found in both cases for the

BMSCs incubated with the microspheres did not show significant differences as regards to the condition of cells growing in the absence of the microspheres (control).

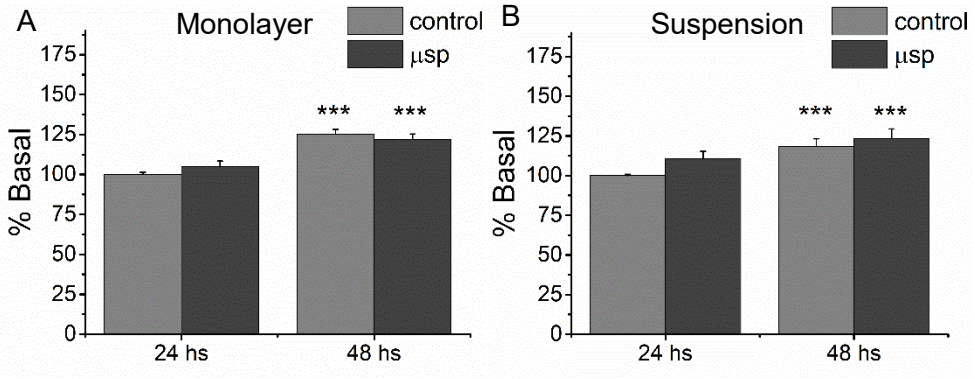

Figure 4. MTT assay with BMSCs in (A) monolayer and (B) suspension, ***p<0.001 vs 24 hs

Regenerative medicine has shown that seeding scaffolds with MSCs has greater regeneration ability because it increases in situ reparation processes by supplying progenitors as well as stimulatory factors [Satija et al., 2009]. The use of microgels in the culture of BMSCs *in vitro*, in addition to promoting cell development in a more biomimetic environment, allows cells, along with the supporting biomaterial, to be injected directly into damaged tissue, after the process of expansion and / or differentiation *in vitro*. In this sense, it is important to adapt the cell-biomaterial construct to the properties of the tissue *in vivo*, in particular tuning its mechanical properties and its permeation to water and to substances soluble in aqueous medium. The required properties differ greatly from one application to another (cartilage or bone regeneration, wound healing, heart muscle and others). We have seen in this work that with the composition used the cells proliferated adequately, but differences in the weight ratio between the two polymers that form the gel or the variation in the degree of crosslinking would allow their behaviour to be adapted in terms of mechanical properties and degree of water absorption to the requirements of each therapy.

To obtain further information about our system, we observed the BMSCs grew in the presence of the microspheres after 7 days with SEM (figure 5). For comparison, Figure 5A shows these cells growing in control condition on a standard two-dimensional culture. Figures 5B and 5C display the condition where the BMSCs were co-incubated with the microspheres in the 2D culture system. Since the well surface was not completely covered by microspheres, part of the cells grew forming a monolayer on the well with no contact to the microspheres, (figure 5B) but, at the same time, other microspheres seem to be covered by a monolayer of cells (figure 5C). In the 3D culture system with BMSCs, we observed that the microspheres were forming clusters (Figure 5D). While in suspension, these microspheres were not found to form clusters neither in the absence of cells (figure 1) nor when incubated with RAW 264.7 cells (figure 3). Therefore, we believe that this clustering could be attributed to an interaction between the microspheres and the BMSCs in the 3D system, as it was also described for other similar systems [Garcia Cruz et al., 2008; Levato et al., 2015]. For example, Garcia Cruz et al. developed microspheres of chitosan where the stem cells could adhere and proliferate, forming a 3D construct [Garcia Cruz et al., 2008]. In the work of Levato et al., they studied microcarriers derived from polylactic acid for cell therapy and found formations of large cell-microcarrier aggregates in static culture conditions where cell proliferation tended to occur in individual or small groups of microspheres [Levato et al., 2015].

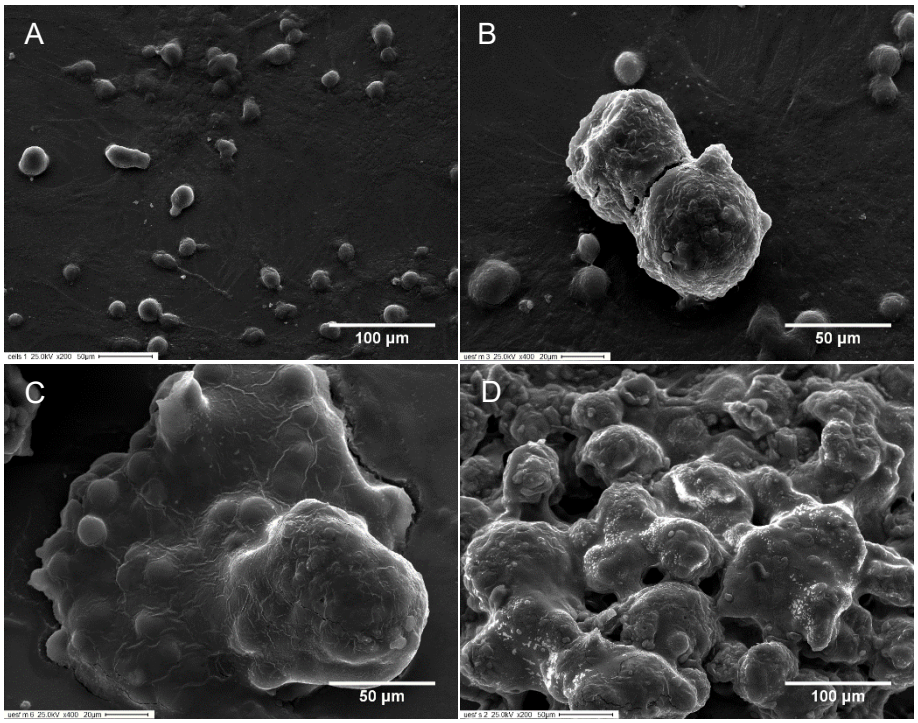

Figure 5. SEM images of BMSCs growing in monolayer alone (A), with microspheres (B, C) and in suspension (D)

Additionally, the BMSCs and the microspheres were observed through fluorescence microscopy after 7 days of culture in the 2D and 3D systems (Figure 6). Actin fibers staining of BMSCs show the interaction of cells with the microspheres. The microspheres were also visualized taking advantage of the chitosan characteristic fluorescence [Garcia Cruz et al., 2008b]. Figure 6A and 6B show the BMSCs growing in the 2D system in the absence and presence of the microspheres, respectively. As seen in figure 6B, many cells are located surrounding the surfaces of the microspheres. Figures 6C and 6D display images of the BMSCs growing in the 3D system in the presence of the microspheres, demonstrating the presence of clusters. Because of the high fluorescence intensity of the microspheres in Figure 6C, it is not possible to see cells clearly between the microspheres. However, under higher magnification, the presence of the BMSCs between the microspheres became evident (Figure 6D), allowing us to confirm the presence of cells and microspheres in these clusters. Under all experimental conditions, we observed mostly flattened and elongated cells with prominent stress fibres of the actin cytoskeleton.

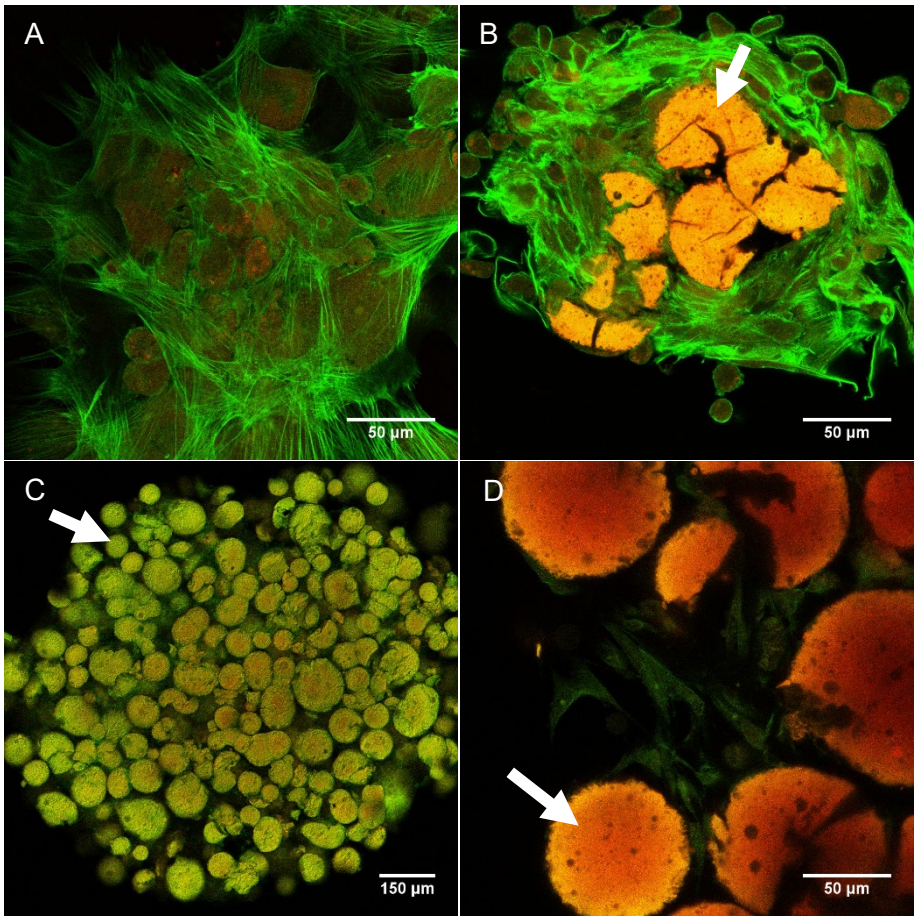

Figure 6. Confocal microscopy images of BMSCs growing in monolayer alone (A), with microspheres (B) and in suspension (C, D), the white arrows point microspheres.

Altogether, these results suggest that in this 3D environment the BMSCs proliferate forming aggregates, where the cells seem to fill the spaces among the microspheres. These results are in accordance with those reported by Clara-Trujillo [Clara-Trujillo et al., 2019]. In that work, they studied magnetic microspheres prepared with acrylates and acrylic acid copolymers functionalized with fibronectin or hyaluronic acid. They reported that mesenchymal stem cells tend to proliferate forming aggregates with the microspheres, where cells are not directly adhered to the microspheres, but they produce their own extracellular matrix.

It is worth noting that the real 3D cell culture should not only allow cell attachment and growth on outermost surface of the microspheres, but also enable sufficient and multidirectional cell-cell interactions. This is important for the cultured cells to maintain normal morphology and function as *in vivo* [Huang et al., 2020]. Our microspheres appear to promote the formation of aggregates where there are multidirectional cell-cell interactions, similar to spheroids. Recent reports provide evidence that the aggregation of MSCs into three-dimensional (3D) multicellular spheroids results in the enhancement of its therapeutic potential, by improving the anti-inflammatory and angiogenic properties, stemness and survival of MSCs after transplantation [Petrenko et al., 2017]. Our results demonstrate the positive impact of this system in order to use it as a model for 3D culture of MSCs, although we should continue studying this system with respect to the pluripotential capacity of MSCs in this model.

4. Conclusions

In this work, progress was made in the development of an innovative three-dimensional support, such as microspheres, from the combination of the natural CH polymer and a fumaric synthetic polymer. Although this material has been previously studied to create 2D matrices for the repair of osteochondral lesions, its use in the production of microgels for BMSCs culture in 3D environments open up the range of possible applications in regenerative medicine.

When we study these microspheres *in vitro* with RAW 264.7 macrophages, we found good cell viability without evidence of cytotoxic response. Our microspheres also allowed the growth of stem cells in a 3D environment, with a proliferation rate similar to the 2D culture. Finally, SEM and actin staining showed how BMSCs grow in biomimetic microenvironments, promoting the formation of aggregates with multidirectional cell-cell interactions. Our results showed as a main advantage the fact that the microspheres constitute a more flexible and biomimetic 3D environment that can be freely remodeled and restructured by the cells without the restrictions imposed by a rigid material. In addition, microgels provide a microenvironment whose initial cell-material interactions are favored, which plays a vital role in cell adhesion, cell interactions and spheroid formation. This study is a proof-of-concept to show that the proposed environment carries out cell proliferation of MSCs under biosimilar conditions and promises to be a useful cell culture tool, whose possibilities deserve further exploration.

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