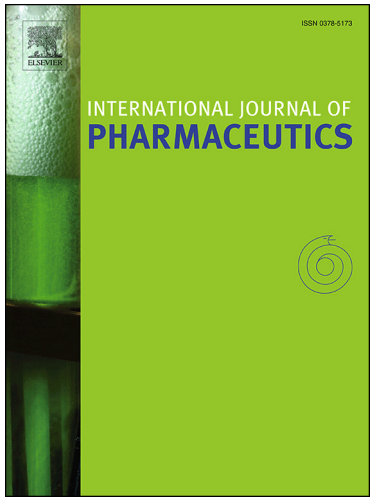

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Abstract

Pancreatic islet transplantation has proved to be a promising therapy for T1DM, in spite of the chronic immunosuppression required. Although cell microencapsulation technology represents an alternative to circumvent the immune system rejection of transplanted pancreatic islets, the environment provided by classical alginate microcapsules does not mimic the natural ECM, affecting the islet survival. Since hyaluronic acid, one of the major components of pancreatic ECM, is involved in cell adhesion and viability, we assessed the beneficial outcomes on encapsulated insulin-producing cells by the HA inclusion in alginate matrices. In this manuscript we describe how alginate-HA hybrid microcapsules enhance the viability of encapsulated cells, reducing early apoptosis percentage and decreasing membrane damage. A stable insulin production was maintained in encapsulated cells, not altering the response to a glucose stimulus. Therefore, we can conclude that the inclusion of HA within alginate microcapsules is beneficial for encapsulated insulin-producing cells, representing a step forward in the clinical translation of microcapsules technology for the treatment of T1DM.

Keywords: T1DM; alginate; hyaluronic acid; microencapsulation; insulin-producing cells

Abbreviations:

T1DM: type 1 diabetes mellitus

IPCs: insulin-producing cells

PLL: poly-L-Lysine

PDL: poly-D-lysine

PLO: poly-L-ornithine

ECM: extracellular matrix

HA: hyaluronic acid

1. Introduction

More than 350 million people in the world are currently affected by diabetes mellitus, becoming as one of the significant threats to human health. Type 1 Diabetes Mellitus (T1DM) is a disease caused by autoimmune destruction of pancreatic β-cells whose most prominent therapy is the exogenous insulin administration through daily injections. However, this treatment is associated with some complications, such as diabetic retinopathy, cardiovascular disease, diabetic nephropathy and lower limb amputations (Aghazadeh and Nostro 2017). An alternative therapy for T1DM is the transplantation of donor-derived pancreas or pancreatic islets able to restore the physiologic metabolic glucose control in T1DM patients. Nevertheless, islet transplantation entails some issues, such as the source of the islets or their reproducible standard preparation. Donors with more than 50 years old provide more pancreatic islets than younger donors, but with reduced capability to produce insulin (Lakey et al. 1996), while high body mass donors have pancreatic islets with lower insulin secretion ratios (Deng et al. 2004). Moreover, a short cold ischemic time, the time from tissue extraction from donor to isolation of pancreatic islets, can also increase the transplantation success (Lakey et al. 1995) and it should be standardized. Other issues found in islet transplantation are the low islet survival and lifelong immunosuppression to avoid immune rejection after transplantation (Yang and Yoon 2015). During the last decades, cell-based sustainable drug delivery systems for T1DM treatment, such as microcapsules or hydrogels containing insulin-producing cells (IPCs) have been developed as a strategy to overcome the pancreatic islets transplantation issues.

Microcapsules allow the exchange of nutrients, therapeutic factors, and gases through the outer layer, avoiding the entrance of immunoglobulins and the cells recognition by the immune system (Orive et al. 2014). At present, there are some biomaterials tested as microencapsulation matrices, such as agarose (Dang, et al. 2004), chitosan (Li et al. 2010), and hyaluronic acid (Gerecht et al. 2007). Among all the biomaterials, alginate is the most commonly used as a matrix for cell encapsulation due to its mechanical properties after microcapsules formation (Siti-Ismail et al. 2008). Alginate microcapsules can also be coated by

polycations, such as poly-L-Lysine (PLL), poly-D-lysine (PDL), and poly-L-ornithine (PLO) to provide higher resistance (De Castro et al. 2005), while still allowing the exchange of nutrients and therapeutic factors. Thanks to these properties, cell microencapsulation technology has been successfully applied in T1DM (Calafiore 2018a). Thus, the first T1DM patient transplanted in a clinical trial with encapsulated cadaveric human islets was able to discontinue all exogenous insulin for nine months (Soon-Shiong et al. 1994). In later studies, four T1DM patients transplanted with microencapsulated pancreatic islets significantly reduced their exogenous insulin requirements for up to seven years (Valdes-Gonzalez et al. 2005). In a separate trial, alginate microcapsules transplanted into two patients reduced their exogenous insulin requirements but, never attained complete insulin independence (Valdes-Gonzalez et al. 2010). Although alginate scaffolds have been considered inert biomaterials to entrap cells (Mallett and Korbutt 2009), they do not provide the cell anchorage required for the survival of most cell types (Genes et al. 2004).

Currently, an approach to overcome the dying cell number inside the microcapsules is the recreation of the natural extracellular matrix (ECM). The ECM is responsible of both cell-cell and cell-matrix interactions, providing mechanical and physiological support to the cells. It is composed of several molecules including laminins, collagens, elastin or hyaluronic acid among them, with a tissue-specific composition of each component. Consequently, several ECM molecules, such as laminin, collagen I or collagen IV, have been combined with alginate to obtain new encapsulation biomaterials, promoting the viability and decreasing apoptosis of microencapsulated cells (Llacua et al. 2016). Moreover, short synthetic peptides derived from natural ECM proteins, such as the arginine-glycine-aspartic acid peptide (RGD motif), are commonly used to simulate the cell-matrix interactions provided by ECM. Although RGD peptide is widely used due to its simplicity, cost-effectiveness, easy manipulation for functionalization, and low immune response (Williams 2011), it does not entirely mimic natural ECM signals by providing the required stimulus for a complete reproduction of the cell *in vivo*

environment. Hence, new biomaterials need to be studied to completely mimic the natural ECM stimulus within microcapsules environment, improving the encapsulated cell survival.

One biomaterial that could recreate pancreatic cells *in vivo* environment is hyaluronic acid (HA), a major ECM component on connective, epithelial, and neural tissues, composed by repetitions of a disaccharide unit of N-acetyl-glucosamine and β-glucuronic acid. HA has been described as a mediating molecule involved in cell signaling, regulation of cell adhesion and proliferation or manipulation of cell differentiation studies (Zhao et al. 2015). In pancreatic islets, HA is an abundant component of the mouse peri-islet ECM, synthesized by different islet endocrine cell types under regular conditions (Hull et al. 2012). HA participates in the maintenance of islets stability and integrity and anti-inflammatory properties (Li et al. 2006). However, reactive oxygen and nitrogen species generated during the inflammatory response in tissue inflammation can degrade HA macromolecules, being OH radical one of the most efficient initiators of this degradation (Valachova et al. 2016). The HA fragments accumulated after degradation of high-molar-mass HA, can initiate the induction of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (Tamer et al. 2018). Moreover, high molecular weight HA acts as a link protein-stabilized complexes with chondroitin sulfate proteoglycans, which are essential in regulating cell processes, such as proliferation (Evanko et al. 1999). HA has shown to be a promising molecule with high possibilities in cell-based therapies applied to T1DM treatment (Zamboni et al. 2017; Zamboni et al. 2018) or wound healing (Tamer et al. 2018). Thus, cultured β-cells treated with exogenous high molecular weight HA increase insulin secretion and content (Li et al. 2006), decreasing oxidative stress and neutrophil activation in a pancreatitis rat model (Campo et al. 2004). HA-based hydrogels have been extensively used in tissue engineering applications, embedding some cell types whose ECM contains HA as a major component, such as chondrocytes or cells present in connective tissue (Chung and Burdick 2009). Thus, HA provides a native ECM-like microstructure contributing to structural support and protection of embedded cells (Tan et al. 2011), while promoting cell viability (Schmidt et al. 2008). HA is also involved in immune response, reducing the immunogenicity by avoiding

adsorption of proteins which recruits immune cells (van Beek et al. 2008). The combination of HA with other biomaterials, such as gelatin (Camci-Unal et al. 2013) or heparin (Gwon et al. 2017), have also shown an enhancement of encapsulated cell viability and functionality compared to their respective single component. Moreover, the combination of HA with PLGA to obtain hybrid biomimetic scaffolds displays an improvement of cell adherence, increasing the cell viability and biocompatibility (Zamboni et al. 2017). In addition, the membranes prepared by a combination of HA, chitosan, and mitochondrial antioxidants protected and enhanced the healing of injured skin, also displaying superior healing properties in injured rabbits and rats *in vivo*. In this manuscript, we have studied for the first time the beneficial *in vitro* outcomes of Ins1E rat cells encapsulation within microcapsules composed by alginate and a high molecular weight HA commonly found in pancreatic islets ECM.

2. Material and methods

2.1. Materials

Ultrapure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio > 1.5) with MW of 75-200 kDa was purchased from FMC Biopolymer. Poly-L-Lysine hydrobromide (PLL, 15-30 kDa) was obtained from Sigma-Aldrich. Clinical grade and free endotoxins 1.1 MDa HA was purchased from Contipro.

2.2. Cell culture

Rat Ins1E cells (Merglen, et al. 2004) were cultured in complete medium consisting of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin/glutamine (Invitrogen), 1% sodium pyruvate 100 mM (Sigma), 1 M HEPES (Lonza), and 0.1% 2-mercaptoethanol (Sigma). Cells were maintained at 37 ºC in humidified 5% CO_2 atmosphere and passaged every 4-5 days.

2.3. Cell microencapsulation and pseudoislets formation

Alginate and alginate-HA were resuspended in 1% mannitol to obtain 1.5% alginate and 1% alginate 0.25% HA mixtures. Final solutions were filtered with a 0.22 µm syringe filter

(Millipore, MA, USA). Next, cells were suspended at 5x106 cells/mL in alginate and alginate-HA solutions and extruded in an electrostatic atomization generator (Nisco®). The resulting beads were completely gelled by agitation for 15 min in a 55 mM $CaCl_2$ solution. Next, the beads were incubated with 0.05% (w/v) PLL in agitation for 5 min, followed by a second coating with 0.1% alginate for 5 min. All the procedures were performed at room temperature, under aseptic conditions, and using the complete medium. Finally, microcapsules were examined under an inverted optical microscopy (Nikon TSM) to monitor the microcapsules morphology and diameter.

Pseudoislets within the microcapsules were formed by incubation of microcapsules containing Ins1E single cells with 1% sodium citrate solution (Sigma-Aldrich) in agitation for 5 min to obtain a liquid core. Next, microcapsules were washed twice in DPBS (Gibco) and incubated with complete medium. Liquefied core microcapsules were maintained at 37 ºC in a humidified 5% CO_2 atmosphere incubator for 10 days until pseudoislets were formed.

2.4. Early apoptosis quantification

The quantification of early apoptosis was assessed by Annexin-V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Early apoptosis of Ins1E encapsulated cells was analyzed at days 1, 7 and 14 after encapsulation. Thus, 200 µL of microcapsules were incubated with 1mg/ml alginate lyase (Sigma Aldrich) for 30 minutes at 37 ºC, centrifuging and rinsing twice with DPBS (Gibco). Next, cells were resuspended in a binding buffer consisting of 10 mM HEPES/NaOH, pH 7.5 containing 0.14 M NaCl and 2.5 Mm $CaCl_2$. Samples were stained with annexin V-FITC and propidium iodide for 10 min at room temperature and protected from light. Samples stained with only annexin V-FITC, only propidium iodide and unstained were used as controls. Apoptosis was quantified with a BD FACS Calibur flow cytometer and data analyzed with FlowJo LLC software. At least three independent experiments were performed for each solution.

2.5. Quantification and imaging of cell viability

Cell viability of encapsulated Ins1E cells was quantified using LIVE/DEAD viability/cytotoxicity kit (Invitrogen TM) after 1, 7 and 14 days of encapsulation. Encapsulated cells in 1.5% alginate, 1% alginate 0.25% HA were released from microcapsules by alginate lyase treatment as described above. Cell suspensions were rinsed twice in DPBS (Gibco) and suspended in culture medium with 100 nM calcein AM and 8 nM ethidium homodimer-1. After an incubation of 20 min at room temperature and protected from light, cell viability was quantified with a BD FACS Calibur flow cytometer. Unstained samples or stained only with 100 nM calcein AM or 8 nM ethidium homodimer-1 were used as controls. Data were analyzed with FlowJo LLC software. At least three independent experiments were performed for each solution.

Cell viability of Ins1E microencapsulated cells was also monitored by microscopy imaging. Thus, 25 µL of microcapsules were rinsed twice in DPBS and resuspended in 500 µL of staining solution composed of DPBS supplemented with 0.5 µM calcein AM and 0.5 µM ethidium homodimer-1. Next, the samples were incubated for 45 min at room temperature and protected from light. Finally, samples were imaged under a Nikon TMS fluorescence microscope at the wavelength of excitation 495 nm/emission 515 nm (for calcein AM staining) and excitation 495 nm/emission 635 nm (for ethidium homodimer staining). Random images were analyzed with the Eclipse Net software, version 1.20.0.

2.6. Study of membrane integrity

Membrane integrity of Ins1E microencapsulated cells was determined by the *in vitro* toxicology assay kit Lactic Dehydrogenase based (Sigma-Aldrich) at days 1, 7 and 14 after encapsulation. Thus, 100 µL of microcapsules per sample were rinsed twice with culture medium and resuspended in 1 mL of complete medium. Each sample was plated in two wells of a 24-well-plate and incubated for 90 minutes with 70 µL of lysis buffer in one well and 70 µL of culture medium in the other. Next, 50 µL of supernatant from each well was incubated with the kit cocktail mixture for 30 minutes, at room temperature and protected from light. The color development was quantified on an infinite M200 TECAN microplate reader at a wavelength of

490 nm, with absorbance reading at 690 nm as background. At least three independent experiments were analyzed for each condition.

2.7. Insulin quantification

Insulin secretion of Ins1E encapsulated cells was quantified from culture supernatants at days 1, 7 and 14 post-encapsulations. Briefly, 200 µL of microcapsules were rinsed twice with culture medium and suspended in 1mL of complete medium. After, a 24 hours incubation at 37 ºC and in a humidified 5% CO_2 atmosphere, supernatants of microcapsules cultures were collected and stored at -80 ºC. The glucose-stimulated insulin secretion (GSIS) was also tested for all the conditions. Thus, 200 µL of microcapsules were rinsed twice in DPBS with calcium and magnesium. Cells were equilibrated for 2 hours in Krebs-Ringer buffer, composed by 129 mM NaCl (Sigma-Aldrich), 5 mM $NaHCO_3$ (Sigma-Aldrich), 4.8 mM KCl (Sigma-Aldrich), 2.5 mM $CaCl_2$ (Sigma-Aldrich), 1.2 mM $MgSO_4$ (Sigma-Aldrich), 1.2 mM KH_2PO_4 (Sigma-Aldrich), 10 mM HEPES (Gibco) and 0.5% w/v bovine serum albumin (Sigma-Aldrich). Next, samples were incubated with Krebs-Ringer buffer supplemented with 3.3 mM glucose for 2 hours, collecting and storing the supernatants at -80 ºC. Then, microcapsules were placed in Krebs-Ringer buffer supplemented with 16.7 mM glucose and, after 2 hours of incubation, supernatants were collected and stored at -80 ºC. The insulin content of supernatants was quantified by Mercodia High Range Rat Insulin ELISA (Mercodia). Three independent samples and controls for each condition were assayed.

2.8. Statistics

Statistical analysis was performed with SPSS software, version 21.00.1. Data were expressed as means ± standard deviation and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$. Normality test was performed to confirm a normal distribution.

3. Results and discussion

We began encapsulating Ins1E cells within alginate and alginate-HA hybrid microcapsules using electrostatic atomization. We chose a density of $5x10^6$ cells/mL, a current density used in cell encapsulation. The microcapsules generator was not clogged with both biomaterial compositions, providing spherical microcapsules when observed under an inverted optical microscope. We were able to form beads with a mean diameter of 450 ± 10 µm and a smooth homogeneous surface (Fig 1), below the 600 µm diameter microcapsules have shown unfavorable molecular diffusion kinetics in therapeutic factors release (Calafiore 2018b). The formed capsules also showed a smooth surface, an important factor since it reduces the foreign body reaction cells recruitment (De Vos et al. 1996), providing higher biocompatibility compared to rough surface microcapsules. After confirming the size and microcapsules surface, we proceeded to monitor the viability of the encapsulated cells analyzing the influence of alginate-HA microcapsules on IPCs apoptosis and viability.

3.1. Hybrid alginate-HA microcapsules enhance encapsulated insulin-producing cell survival

After embedding Ins1E cells into alginate and alginate-HA microcapsules, we quantified the early apoptotic cells percentage inside the microcapsules at days 1, 7 and 14 after encapsulation. The percentage of apoptotic encapsulated Ins1E cells displayed a statistically significant reduction in alginate-HA compared to alginate microcapsules ($p<0.05$) at days 1 and 7 after encapsulation, with a more pronounced decrease at day 14 ($p<0.01$) (Fig 2A). Previous studies with β-cells cultured on laminin-5 enriched ECM have already shown protection against apoptosis, with a reduction of caspase-8 activity, enhancement of focal adhesion kinase, protein kinase B and extracellular signal-regulated kinase phosphorylation, suggesting that ECM plays an essential role on apoptosis of IPCs (Li et al. 2006). Since HA is an extensive component of the ECM from pancreatic islets, it is not surprising that the addition of exogenous HA within the microcapsule's matrix can decrease the apoptosis percentages of the encapsulated cells.

We also quantified the live/dead cell percentage by flow cytometry to confirm apoptosis results. The next day after encapsulation, encapsulated Ins1E cells displayed no differences of

cell death percentage between alginate and alginate-HA microencapsulated cells (Fig 2B). Similarly, no differences of cell death were detected at day 7 between alginate and alginate-HA microcapsules. However, 14 days after encapsulation, encapsulated Ins1E cell death was significantly reduced ($p<0.001$) in alginate-HA compared to alginate microcapsules, confirming the results observed in the apoptosis quantification at this time point. These results were also corroborated by micrographs obtained after the staining of microencapsulated Ins1E cells at days 1, 7 and 14 after encapsulation (Fig 2C). The improvement observed with encapsulated IPCs viability through the inclusion of HA within alginate microcapsules is closely related to previous studies describing that pancreatic islets or single β-cells exposure to whole ECM or individual ECM components improve β-cell survival (Hamamoto et al. 2003). These data suggest that specific ECM components support β-cell function and viability. In fact, pancreatic islets embedded within hybrid HA-collagen hydrogels significantly displays a viability enhancement compared to collagen embedded islets or unembedded islets, retaining their morphology and insulin secretion ability, showing also better immunoprotection than alginate hydrogels (Harrington et al. 2017). This beneficial effect of high molecular weight HA on cell viability and apoptosis is mainly mediated by CD44 receptor, since saturating concentrations of anti-CD44 antibody abolish the protective effects of hyaluronan (Lakshman et al. 2004). However, it is essential to remark that depending on the molecular weight of HA, the outcomes on cell survival can change. In spite of, under regular conditions, high molecular weight HA displays anti-inflammatory effects, under stress conditions, high molecular weight HA become fragmented, acting as proinflammatory, reducing cell viability, and enhancing cell apoptosis (Hull et al. 2012).

We also quantified the membrane integrity of encapsulated Ins1E cells in both alginate and alginate-HA microcapsules to provide more useful evidence of HA inclusion within alginate microcapsules. A progressive reduction of membrane damage was quantified from days 1 to 14 post-encapsulation in both microencapsulation matrices, with a statistically significant membrane damage reduction ($p<0.05$) at the three studied time points comparing alginate-HA

and alginate microcapsules (Fig 3). These data verified the apoptosis and viability results described above, similarly to previous studies showing better viability and morphological integrity of neonatal rat islets within cuprophane hollow fibers containing HA (Velten et al. 1999). In fact, the reduction of membrane damage is widely influenced by the immobilization efficiency provided by HA and its similar dynamic viscosity to natural soft tissues, leading to an enhancement of cell membrane integrity in HA-containing solutions (Bothner and Wik 1987). Therefore, we can conclude that HA-containing microcapsules protect encapsulated Ins1E from the high stress derived from the encapsulation process, enhancing cell viability and decreasing cell apoptosis and membrane damage.

3.2. HA does not affect the insulin secretion ability of encapsulated insulin-producing cells

We also tested the ability of encapsulated Ins1E cells to secrete insulin during the same periods studied above when embedded within both biomaterials. Encapsulated Ins1E cells progressively upregulated the secretion of insulin during the considered time points, without statistically significant insulin secretion differences between both biomaterials (Fig 4). It was surprising the lack of differences in insulin release after the different viabilities quantified at both matrices along the studied timepoints, but the presence of HA in the matrices could influence, not only the cell viability but also the release of insulin, may be exerting a compensative effect. Currently, there are controversial data regarding the role of HA in the insulin secretion by pancreatic islets. On the one hand, some authors describe that high molecular weight HA increases the insulin secretion. For example, coating culture well-plates with high molecular weight HA increases insulin secretion from HIT-T15 cells through the enhancement of connexin 43-mediated gap-junctional intercellular communications (Li et al. 2006). On the other hand, other authors describe that the accumulation of HA in autoimmune diabetes leads to fewer compact islets than healthy islets, affecting the insulin production of the mechanosensitive islets (Nagy et al. 2018). In fact, some ECM component interactions, such as integrin-laminin, have shown to be important in regulating insulin release from β-cells, underscoring the importance of ECM components in regulating β-cell function (Parnaud et al.

2006), while the role in islet structure and function of other components, such as proteoglycans and hyaluronan, remains unknown. We consider that more exhaustive studies should be performed to clarify the role of HA in the functionality of IPCs, considering factors such as HA concentration or molecular weight.

Finally, we quantified and compared the insulin secretion responding to glucose concentration from Ins1E encapsulated cells in alginate and HA-alginate matrices. Encapsulated cells responded to glucose stimulus by secreting insulin at all the studied time points, without statistically significant differences between both microencapsulation matrices (Fig 5), reflecting again that the inclusion of HA does not affect the functionality of IPCs. Other hybrid biomaterials with HA, such as HA-collagen hydrogels, have also shown to be able to improve *in vitro* viability of embedded rat islets retaining their glucose sensitivity for 28 days. These encapsulated rat islets administered to the omentum of outbred rats reversed long-term diabetes and prevented graft rejection in all animals for more than 80 weeks without fibrotic overgrowth or cellular rejection (Harrington et al. 2017).

3.3. Cell viability of encapsulated cell clusters is also enhanced by HA

To confirm the results obtained with single cells on cell cultures with a similar environment than pancreatic islets, we prepared a new batch of microcapsules with a liquefied core to test encapsulated Ins1E cell clusters within alginate-HA microcapsules (Fig 6A). Similar to non-liquefied microcapsules, Ins1E alginate and alginate-HA liquefied microcapsules displayed no statistically significant differences at day 1 and 7 after encapsulation (Fig 6B). However, at day 14, we quantified a statistically significant reduction ($p<0.01$) of alginate-HA encapsulated Ins1E cells compared to alginate microcapsules, showing HA as a specific ECM component to support β-cell viability. Likewise, other ECM molecules, such as collagen, have shown beneficial effects on islet cells survival, while reducing necrosis and apoptosis. In fact, the combination of alginate with some proteins, such as collagen IV, fibronectin, and laminin, reestablishes cell-matrix interactions lost during cell isolation, resulting in a cell viability enhancement and postulating those combinations of alginate and ECM molecules as an

encapsulation platform for islet cell delivery (Beenken-Rothkopf et al. 2013). Moreover, the encapsulation of immunoisolated pancreatic islets with collagen type IV and the laminin sequences RGD and PDSGR, reduces the release of danger-associated molecules and nitric oxide from islets, enhancing the survival of pancreatic islets embedded within alginate-collagen-laminin biomaterials, compared to alginate microcapsules without ECM molecules (Llacua et al. 2018). Poly-lactide-co-glycolide scaffolds modified with collagen IV also improve mouse islet survival, decreasing early-stage apoptosis in islet cells while reducing the restoring time to euglycemia from 17 to 3 days after transplantation in a syngeneic mouse model (Yap et al. 2013).

4. Conclusions

Based on the results described in the present manuscript, we can conclude that the inclusion of HA in alginate matrices forming microcapsules provides beneficial effects regarding viability increment, apoptosis reduction, and lower membrane damage to encapsulated insulin-producing cells, while maintaining their insulin secretion ability and glucose responsiveness. Since HA represents one of the major components of the pancreatic ECM, we conclude that mimicking the natural pancreatic ECM can improve islets survival, one of the main current bottlenecks in the cell replenishment therapies proposed for the treatment of T1DM. However, more detailed studies with other pancreatic ECM molecules, such as collagen IV or laminin, should be performed to approach closer bio-artificial matrices to the *in vivo* islet microenvironment.

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AUTHOR CONTRIBUTIONS

Conceptualization: JC; LS and JP; Methodology: AC and AE; Validation: JC, LS, AC, AE and JP; Formal Analysis: JC, AC and JP; Investigation: AC and AE; Resources: RH, JC, GO and JP; Data Curation: AC and JC; Writing – Original Draft Preparation: AC and JC; Writing – Review & Editing: AC, JC, AE, LS and JP; Supervision: JC, LS and JP; Project Administration: JC, LS and JP; Funding Acquisition: RH, JC, GO and JP.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

FIGURE LEGENDS

Figure 1.- Microcapsules morphology. Brightfield microscopy micrographs of Ins1E encapsulated cells. Scale bar: 100 µm.

Figure 2.- Viability of Ins1E encapsulated cells. (**A**) Early apoptotic cell percentage quantification of Ins1E cells using flow cytometry after annexin/PI staining. (**B**) Cell death percentage quantification of Ins1E cells using flow cytometry after calcein/ethidium staining. (**C**) Fluorescence microscopy micrographs of encapsulated cells after calcein/ethidium staining. Note: Values represent mean ± SD. *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Scale bar: 100 µm.

Figure 3.- Membrane integrity of Ins1E encapsulated cells. Cell damage percentage quantification of Ins1E cells using the Lactic Dehydrogenase *in vitro* toxicology kit. Note: Values represent mean ± SD. *: $p<0.05$.

Figure 4.- Insulin release of Ins1E encapsulated cells. Insulin release of Ins1E cells determined by ELISA after 24 hours of complete medium incubation. Note: Values represent mean ± SD.

Figure 5.- Insulin release after glucose stimulation of Ins1E encapsulated cells. Insulin release of Ins1E cells determined by ELISA after 2 hours of incubation with 3.3 mM glucose Krebs-Ringer Bicarbonate Buffer and 2 hours of incubation with 16.7 mM glucose Krebs-Ringer Bicarbonate Buffer after 1, 7 and 14 days of encapsulation. Values represent mean ± SD. **: $p<0.01$ and ***: $p < 0.001$.

Figure 6.- Viability of Ins1E pseudo-islets formed within liquefied microcapsules. (**A**) Brightfield micrographs of pseudo-islets formed with Ins1E cells after 14 days. (**B**) Cell death percentage quantification of liquefied microencapsulated Ins1E cells using flow cytometry after calcein/ethidium staining. Note: Values represent mean ± SD. **: $p<0.01$ and ***: $p<0.001$. Scale bar: 100 µm.