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Enhancing pseudoislet biofunctionality using gelatin bead technology

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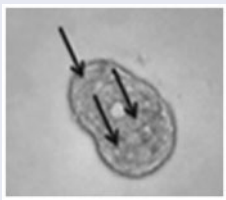
ABSTRACT

Central necrosis hampers the formation of highly biofunctional Pseudoislets (PIs), which consist of aggregates of insulin-secreting pancreatic β-cells. Necrosis arises because of a shortage of nutrient and oxygen diffusion to the core of the PIs during culture, especially when PIs exceed >200 μm. This study aimed to generate 'vents' by incorporating gelatin beads (GBs) into the center of PIs and to examine if this promotes nutrient and oxygen diffusion by blocking the center for cell residence. In addition, we examined the impact of delivering GBs loaded with anti-necrosis or anti-apoptosis drugs to the center of PIs. The BRIN-BD11 rat pancreatic β-cell line was used to generate PIs by suspension culture. PIs were generated at a seeding density of 32,000 cells/PI and cultured for up to 7 days. GBs of 40 μm diameter were produced from Gelatin A and crosslinked with 5% glutaraldehyde for 6 h. The neat GBs or GBs loaded with 100 ng/mL IL-10, or 5 μg/mL anti-IL-1β were incorporated into PIs. The cell viability of the PIs was assessed using cell counting kit-8 (CCK8) and lactate dehydrogenase (LDH) assays. Glucose-stimulated insulin release (GSIS) from PIs was evaluated after stimulation with 16.7 mM glucose for 20 min. Incorporating IL-10, or anti-IL-1β -loaded GBs to PIs synergistically enhanced cell proliferation and reduced cell death. Importantly, PIs cultured for 1 week following incorporation of cytokine-loaded GBs displayed enhanced biofunctionality in terms of higher GSIS.

GRAPHICAL ABSTRACT



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Anti-inflammation; cell viability; gelatin beads; insulin; pseudoislets

1. Introduction

Central necrosis or apoptosis hampers the function of pseudoislets (PIs) – cell aggregates formed from pancreatic islet cells – even over short culture periods. This is largely driven by the lack of nutrient and oxygen diffusion to the core of the PIs. Cellular viability and growth drop off dramatically, and insulin concentrations in the culture medium declines[1–3]. It has been reported that PIs with 150 μm are superior to larger PIs (>300 μm) in viability, *in vitro* functional assays and transplantation outcomes. MacGregor et al.[4] have shown that smaller PIs were 20% more viable, consumed twice as much oxygen and released more than double the amount of insulin. It has been confirmed for years that mammalian cells rely on oxygen and nutrients for their survival and the natural diffusion limits of these vital chemicals are approximately 100–200 μm[5]. Based on this principle, it is easy to understand that the reduction of PI size is a good strategy to increase the viability of PIs. Alternatively, if we can generate 'vents' in the center of PIs allowing nutrients and oxygen to enter, or to block the center of PIs for cell residence, or deliver anti-necrosis or anti-apoptosis drugs to the center of PIs, the cell viability of larger PIs may increase.

Gelatin is a natural polymer commonly used in the biomedical and biotechnology fields and is biocompatible. Moreover, it has been found that gelatin is a good candidate for preparation of microcapsules due to the ability to easily control drug release[6]. The highly hydrophilic properties of gelatin lend itself to drug absorption and then drug release. Drug release can be controlled via several means such as diffusion through a rate-controlling membrane, or degradation rate of gelatin[7], which can be controlled by either

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varying gelatin's molecular weight, or via the degree of crosslinking[8].

Gelatin beads have been used for sustained release of antibiotics, drugs, vaccines and hormones[9]. For instance, the incorporation of GBs into vascular tissue (human smooth muscle) achieves improvements in the delivery of growth factors[10]. The main advantages of introducing crosslinked GBs into cell aggregates is that they are biocompatible, biodegradable and have a low degree of toxicity when dissolved[9]. Large GBs enable drug delivery to a site over a long period and high loading capacity.

IL-10 is a form of cytokine that is produced primarily by monocytes. It has a pleiotropic effect in inflammation and plays a role in the regulation of immunity through down-regulation of gene expression of Th1 cytokines and co-stimulatory molecules on macrophages. Beside these, it has found that IL-10 helps in β-cell maintenance, proliferation and antibody formation[11, 12]. It also has the ability to block NF-kappa and controls JAK-STAT signaling pathway[11].

Il-1β is a cytokine that is produced by the activation of macrophages as an inactive pro-protein molecule. It is converted into an active form by the enzyme caspase 1. IL-1β acts as a mediator of the inflammatory response and plays a role in many cellular activities like apoptosis, proliferation and differentiation[13]. Hence the protein, anti-IL-1β, is likely to decrease the action of IL-1β.

Thus, IL-10 and anti-IL-1β could be effective anti-inflammatory drug candidates for large PIs fabrication in order to reduce the inflammation induced cell death in the center and may help to enhance PI biofunctionalities. We aimed to resolve the issue of central necrosis within PIs through the incorporation of micrometre scale GBs. Furthermore, the potential use of GBs as drug carriers to deliver anti-necrosis and anti-inflammation molecules, IL-10 and anti- IL-1β, has been explored in an effort to increase PI biofunctionality.

2. Material and methods

2.1. Generation and characterization of GBs

GBs were generated using W/O emulsion technique and optimized according to the method of Iwanaga et al.[14]. 40 µm diameter GBs were collected through appropriate sieves (PluriStrainer, Cambridge Bioscience Ltd, UK). Then the GBs were crosslinked with 5% (v/v) Glutaraldehyde (GA) solution (Sigma-Aldrich, UK) in the gas phase for 6 h.

2.2. Pseudoislets generation

The PIs was generated from BRIN-BD11 cells, a rat pancreatic β-cell line. The cells and PIs were cultured with Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) (Lonza, UK) and 1% Penicillin-Streptomycin (Lonza, UK). Ultra-low attachment 96-well round bottom culture plate (SARSTEDT, USA) was used to form PIs with one PI per well (32,000 cells per well). The plate was centrifuged for 6 min at 900 rpm to produce a single PI per well. The plate containing PIs were incubated up to 7 days at 37 °C and 5% CO_2.

2.3. Optimization of cytokine concentration in culture media and the impact on PI viability

In order to identify the optimum concentration of each cytokine on PI viability, a series of IL-10 concentrations (10 ng/mL to 1000 ng/mL), and anti-IL-1β concentrations (0.4 µg/mL/to 10 µg/mL) in the culture media were assessed for their impact on PI viability. The plate was incubated at 37 °C and 5% CO_2 for 7 days. The viability of PIs in the presence of the cytokines was assessed at day 7 using CCK-8 (Sigma-Aldrich; UK) assay according to the manufacturer's instruction.

2.4. Incorporation of a mock drug into GBs

The protocol of Nakase et al.[15] has been adapted to load cytokines into GBs. Alexa Fluor® 568 conjugated antibodies (Ab-dye) (Thermo-Fisher Scientific, UK) was used as a mock protein molecule to test the incorporation technique and drug release form GBs. The GBs were washed three times with acetone, then three times with deionized water and PBS for 5 min each through centrifuging at 4000 rpm. The supernatant was removed and GBs were freeze dried for 2 h before loading with 10 µL of different concentrations of the protein, 0, 0.56, 1.25, 2.5, 5, 10 µg/mL. The release of the drug from the GBs was measured by incubating the GBs in PBS at 37 °C. A 100 µL of the incubated solution was taken at various sampling time points, and fluorescence intensity was measured at excitation/emission 565/625 for the samples subjecting to 1–7 day culture.

2.5. Incorporation of cytokines into GBs

The optimum concentrations of IL-10 and anti-IL-1β were determined as 100 ng/mL of and 5 µg/mL, respectively. The 10 µL of 100 ng/mL of IL-10 or 10 µL of 5 µg/mL of anti-IL-1β solution were incorporated into 25 GBs before they were freeze dried for 2 h.

2.6. Incorporation of GBs into PI

The incorporation of GBs with and without IL-10 (100 ng/mL) or anti-IL-1β (5 µg/mL) cytokines into PIs was achieved using ULA 96-well round bottom plate. BRIN-BD11 cells (32,000 cells/well) were mixed with 25 neat or cytokine-loaded GBs in 300 µL of media and centrifuged using a plate centrifuge at 900 rpm for 6 min. The plate was incubated for 7 days at 37 °C and 5% CO_2.

2.7. PIs viability by CCK-8

The CCK-8 kit was used to measure the viability of GB-loaded PIs at different culture times. Briefly, the PIs were washed once with PBS. A 110 µL working solution was

added and then incubated at 37 °C and 5% CO_2 for 4–5 h. The samples were centrifuged for 5 min at 1000 rpm and supernatants transferred into a 96-well plate. The intensity of color was measured at 450 nm using a microplate reader (Synergy II BioTek, UK).

2.8. Cytotoxicity by lactate dehydrogenase assay

The lactate dehydrogenase (LDH) value was measured using a commmercially available LDH kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. A spontaneous LDH activity control was prepared by adding 10 µL of sterile, ultrapure water to PIs in triplicate. A maximum LDH activity control was prepared by adding 10 µL of Lysis Buffer (10×) to PIs in triplicate. 50 µL of the culture mixture of PIs at given culture time point with and without GBs was transferred to 96 well plate with 50 µL LDH working solution and mixed carefully. The samples in the plate were incubated at room temperature for 30 min in the dark. Next, 50 µL of stop solution was added to each well and mixed thoroughly. Absorbance was measured at 490 nm and correction at 689 nm using a microplate reader (Synergy II BioTek, UK).

2.9. Glucose-stimulated insulin secretion (GSIS)

The PIs were transferred to Eppendorf tubes carefully and centrifuged gently at 900 rpm for 5 min to discard medium. The PIs were washed twice with 250 µL of Hank's Buffered Saline Solution 1X (HBSS 1X) (Sigma-Aldrich, UK) supplemented with 1 mM calcium. The cells were primed with 250 µL of 1.1 mM glucose and incubated for 40 min at 37 °C and 5% CO_2. After that, the 1.1 mM glucose was replaced with 250 µL of 16.7 mM glucose. GSIS were measured after incubating the sample for a further 20 min at 37 °C. Finally, centrifugation was performed, and supernatants were transferred to a 1.5 mL Eppendorf tubes and stored at −20 °C for subsequent determination of insulin secretion by ELISA (Stratech, UK). A standard curve of insulin was calculated using the standards provided with the kit and ranged from 0 ng/mL to 5.5 ng/mL. Insulin concentration (ng/mL) released from test samples was estimated depending on the equation of the standard curve.

3. Results

3.1. Statistical analysis

Comparisons between groups were performed using unpaired Student's t-test and one-way ANOVA for multiple comparisons via GraphPad Prism 7 software. Data are presented as mean ± standard deviation (SD) from 3 to 4 independent experiments conducted in triplicate. Significance was accepted at three p value levels: $^{*}p < 0.05$; $^{**}p < 0.001$ and $^{***}p < 0.0001$.

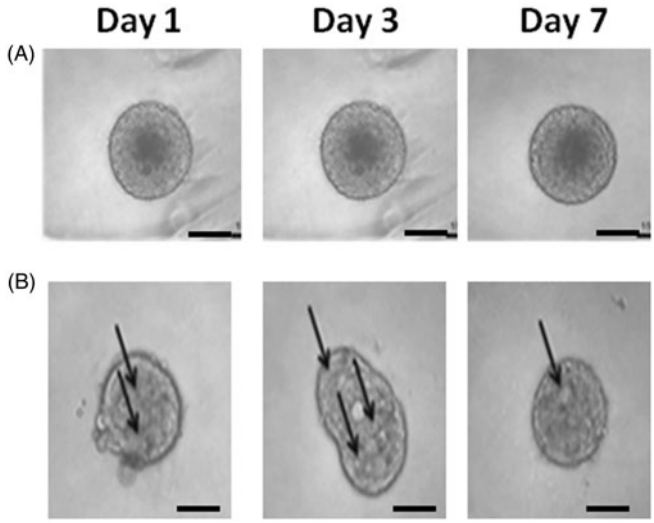

Figure 1. PI morphology and size with and without incorporating GBs. Live images of BRIN-BD11 PIs generated in ULA bottom round plates at cell seeding density of 32,000 cells/PI. (A) without GBs; (B) with GBs. Scale bar = 150 µm.

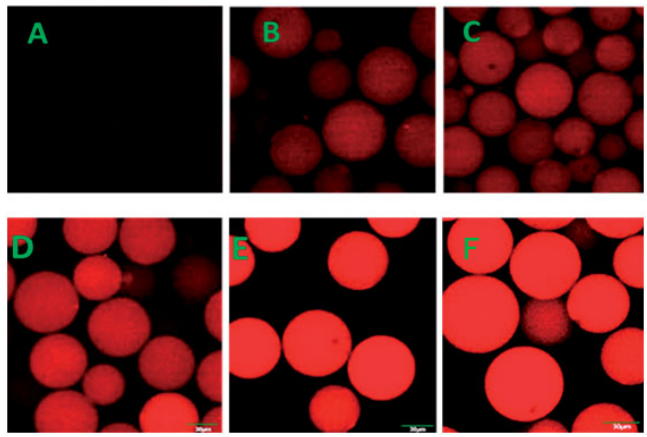

Figure 2. Confocal images of 40 µm diameter GBs incorporating different concentrations of Ab-dye 568 with culturing for 4 days. (A) 0 µg/mL; (B) 0.65 µg/mL; (C) 1.25 µg/mL; (D) 2.5 µg/mL; (E) 5 µg/mL and (F) 10 µg/mL. Scale bar = 25 µm.

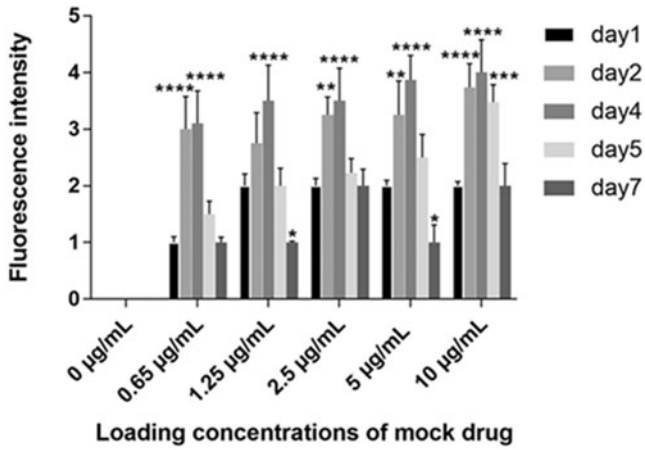

Figure 3. Effect of incubation time and loading concentration of Ab-dye 568 on the dye release from GBs. Quantitative measurement of fluorescence release from GBs to PBS from day 1 to day 7 of incubation at 37 °C. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with fluorescence of control (dye released at day 1).

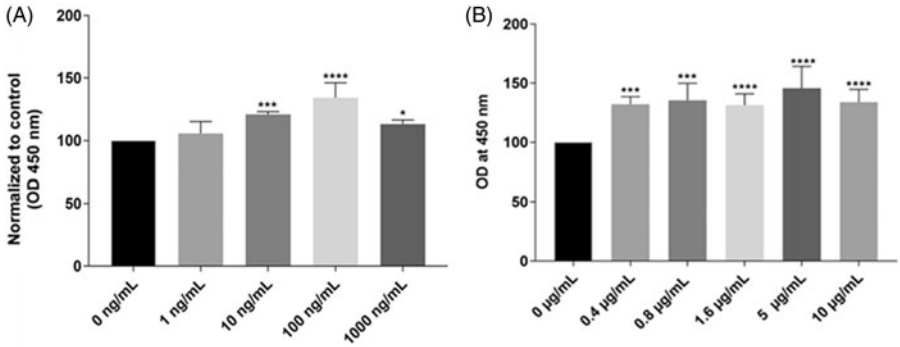

Figure 4. Effect of different concentrations of IL-10 (A) or anti-IL-1β (B) in culture media on the cell viability of PIs after 7 days culturing using CCK-8 assay. Data are presented as mean ± SD from 3 to 4 independent experiments conducted in triplicate. $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with control (PIs without cytokines).

3.2. PI morphology

Relatively symmetrical PIs have been generated using ULA round bottom 96 well plates. When using 32,000 cells per well, the average size of the formed PIs was measured and quantified at around 310 μm through optical image analysis using ImageJ. Culture of the PIs for up to 7 days did not significantly increase the size (Figure 1(a)).

3.3. Incorporation and release efficiency of the mock drug in GBs

GBs loaded with the mock cytokine (Ab-dye) and incubated in PBS for 4 days at 37 °C exhibited bright red fluorescence by confocal microscopy as shown in Figure 2. The fluorescent intensity was directly proportional to the concentration of Ab-dye loaded into GBs. The release of Ab-dye from GBs was assessed quantitatively by the fluorescence intensity at different culture time points in order to evaluate the incorporation and release efficiency (Figure 3). The release was significantly elevated from day 2, with peak fluorescence observed at day 4, regardless of initial incorporation concentration ($p < 0.01$–0.0001) compared with control (dye released at day 1). By day 7, fluorescence fell back towards control levels.

3.4. The effect of IL-10 and anti-IL-1β concentrations in culture media on PI cell viability

The effect of IL-10 and anti-IL-1β solutions at different concentrations in culture media for PIs culture on their viability were measured to identify the optimized concentration using for loading to GBs. The proliferation of PIs at day 7 culture was measured using CCK-8 assay (Figure 4). The CCK-8 assay results showed that IL-10 concentrations between 10 ng/mL and 1000 ng/mL significantly increased the proliferation of the PIs, and 100 ng/mL of IL-10 had achieved the highest viability in the culture. Similarly, anti-IL-1β concentrations between 0.4 μg/mL and 1000 μg/mL significantly increased the proliferation of the PIs with 5 μg/mL of anti-IL-1β being the concentration inducing the highest proliferation as shown in Figure 4. Hence, 100 ng/mL of IL-10 and 5 μg/mL of anti-IL-1β concentration solutions were used to load to GBs.

3.5. The effect of GBs incorporating on PIs' size and morphology

Incorporating GBs with or without cytokines into PIs slightly increased PI size to around 400 μm. Using the ULA round bottom plate to generate PIs with GBs showed that GBs have successfully been incorporated in to the center of PIs and the PIs exhibited symmetrical spheroid morphology (Figure 1(b)). Many GBs were stable in PIs after 7 day culture. The size of these PIs with and without GBs was quantified using ImageJ after day 1, 3 and 7 in culture. The sizes of PIs were slightly increased ($p < 0.01$) after 3 days and 7 days of culturing in the presence of GBs compared with PIs free of GBs (control).

3.6. The effect of the PIs' functionality with incorporating GBs

3.6.1. Cell viability using CCK-8 assay

The cell proliferation of PIs was estimated by using CCK-8 assay during 7 days culture. The results showed that PIs without the incorporation of GBs had stead cell number by day 3 culture, then have decreased cell number considerably. By day 7, 30% of original cell number has lost. The incorporation of GBs without loading cytokines into PIs showed a well maintained cell number from day 1 through days 3 and day 7 culture. Dramatically, incorporation of GBs with loading both cytokines showed a significant increase in cell viability, respectively. By day 7 culture, IL-10 and anti-IL-1β loaded GBs in PIs have achieved 40% and 30% higher cell viability than PIs alone samples (Figure 5).

3.6.2. Assessment of cellular LDH release

Cellular LDH release was used to measure the integrity of cellular membranes, which was elevated in PIs during cell death or cell apoptosis[16]. LDH values in day 1 culture were consistent among all groups. By day 7, LDH release was almost 3 times higher than on day 1. However, PIs generated significantly lower LDH values at day 7, when IL-10, or anti-IL-1β was added to the culture media, or when cultured with GBs alone or GBs loaded with IL-10 or anti-IL-1β in comparison to PIs only (control) as shown in Figure 6. The data demonstrated that incorporating GBs had

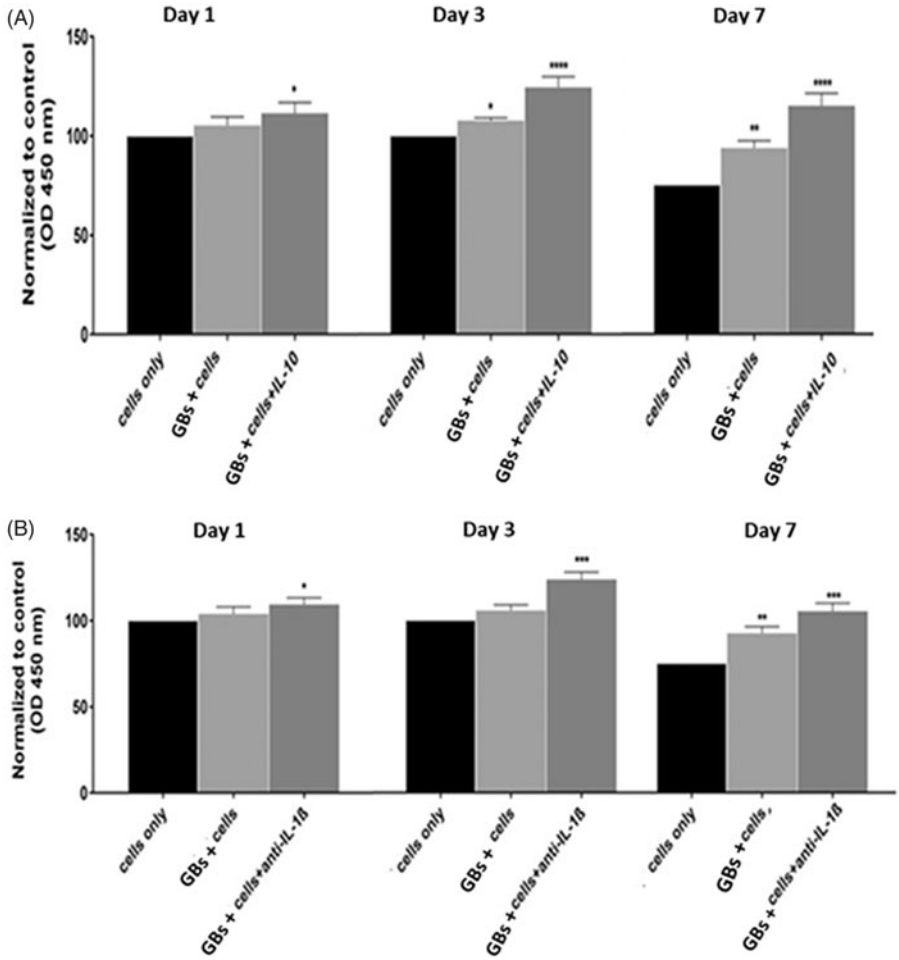

Figure 5. Effect of GBs loaded with IL-10 (A) or anti-IL-1β (B) on the proliferation of PIs during 7 days culture. Cell seeding density 32,000 cells/PI. Data are presented as mean ± SD from 4 to 5 independent experiments conducted in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$ compared with control (PIs only).

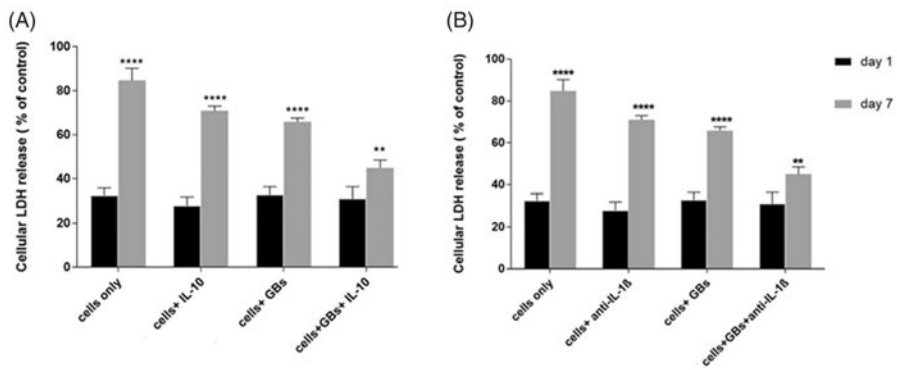

Figure 6. Cellular LDH release from PIs. (A) without GBs and with GBs loaded with IL-10; (B) without GBs and with GBs loaded with anti-IL-1β. Cell seeding density 32,000 cells/PI. Data are presented as mean ± SD from 4 independent experiments conducted in triplicate. $^{**}p < 0.01$ and $^{****}p < 0.0001$ compared with control (PIs only).

a very similar effect to adding cytokines in the culture medium in terms of LDH release. Synergistic effects were observed when GBs loaded with cytokines were incorporated into PIs.

3.6.3. Assessment of PI's GSIS

As shown in Figure 7, PIs culturing with free IL-10 or anti-IL-1β in media or PIs incorporating neat GBs or GBs loaded with cytokines, showed significant increases in GSIS compared with PIs only (control) at day 7 of culture. The incorporation of GBs loaded with IL-10 or anti-IL-1β into PIs increased the GSIS value more than two-fold.

4. Discussion

Although varied approaches have been used to generate PIs, most of these approaches involve the use of suspension surfaces[17, 18] which are often associated with central necrosis

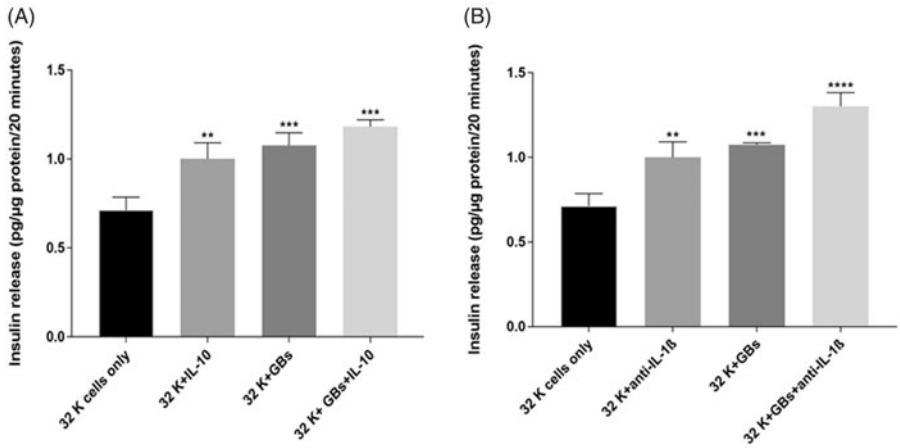

Figure 7. Effect of incorporating GBs and loading with (A) IL-10 or (B) anti-IL-1β in PIs on GSIS at day 7 culture. BRIN-BD11 cells were seeded at 32,000 cells/PI and allowed to form PIs over a 1-week period. Cells were exposed to cytokines alone, PIs incorporating with GBs alone, or GBs loaded with cytokines. Data are presented as mean ± SD from 3 independent experiments conducted in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with control (PIs only).

when PI size exceeds 200 µm[1, 3]. In this study, we have reduced central necrosis of large PIs, which manifested as high cellular viability and good biofunctionality in terms of GSIS. Two approaches were employed: Firstly, GBs were incorporated into PIs to create a 'vent' or reduce cell residence in the center of PIs; secondly, GBs were loaded with anti-inflammatory or anti-necrosis agents to enhance cell survival.

The incorporation of GBs into PIs resulted in enhanced cell viability of PIs. This may arise due to the creation of a void following the dissolution of the GBs. The resulting morphological arrangement enabled the efficient and easy exchange of gases and nutrients from the media to the cells. It was exhibited that PIs incorporating GBs formed larger structures without a considerable increase in cell numbers. This was likely due to the space created by the GBs. This arrangement of cells with 'pockets' where the GBs were present, was likely to significantly improve nutrient and oxygen diffusion to the center of the PI and may account for the improvements in viability observed here and elsewhere[19, 20]. Incorporating GBs to large PIs has benefits because this method can create a better gas and nutrient exchange environment to overcome the 200 µm diffusion limit[21].

Adding both IL-10 and anti-IL-1β to culture media increased the viability of PIs. The viability of PIs increased by only 15% when IL-10 was added to culture media comparing with PIs without GBs and free of IL-10 at day 7. Similarly, adding anti-IL-1β directly to media slightly improved the viability of PIs in terms of the CCK-8 assay (at day 7) by 14% at the same day (day 7) comparing with control (PIs free of GBs).

The sustained release of the anti-inflammatory drug from GBs enhanced PI viability. The release of IL-10 increased cell viability by 60% for PIs incorporating GBs loaded with IL-10 compared with control at day 7. Also, the viability was elevated by 35% for PIs with GBs loaded with anti-IL-1β compared with control at day 7.

Cytokines in free form placed in cell culture environment have short activity time due to degradation by proteinases released from the cultured cells. The incorporation of GBs loaded with cytokines into PIs apparently protected the cytokines from degradative processes and sustained the slow release of cytokines from GBs ensuring prolonged cell survival. Both cell viability assays and insulin production assay revealed the synergetic effect especially when the anti-inflammatory cytokines were loaded into GBs.

The improvement of the cellular activities of the PIs by GBs and further improvement by the GBs loaded with IL-10, and anti-IL-1β gave us the opportunity to study the main cause that reduced PI functionality during prolonged cultures. It is well known that IL-10 is derived from macrophages and TH2 cells and exerts anti-inflammatory effects by inhibiting IL-12 and other pro-inflammatory macrophage cytokines (such as IL-1, IL-6, IL-8 and TNFα) via increasing macrophage production of IL-1 receptor antagonist and by inhibiting the generation of oxygen and nitrogen free radicals by the macrophage[22, 23]. On the other hand, IL-1β works as a pro-inflammatory cytokine with cells displaying elevated levels of the cytokine under inflammatory challenge[18]. Importantly, IL-1β is centrally driven by beta cell apoptosis in type 1 diabetes. In the current study, anti-IL-1β was employed to inhibit the action of IL-1β as an anti-inflammatory drug. According to the observation of Rubartelli and colleagues[24], cellular IL-1β is localized in intracellular vesicles and secreted by an intracellular membrane. It is elevated under stress conditions[24, 25] but can be redirected to the extracellular space following inappropriate secretion. These studies proved that IL-1β could be blocked by using methylamine and low temperature[25]. The anti-IL-1β possibly delays or prevents cell signals from reaching to localized cellular IL-1β, and pro-inflammatory cytokine secretion would be delayed. In this study, we applied IL-10 or anti-IL-1β to PIs and improved PI activities. These observations indicate that inflammatory reactions or cell stress in PIs due to low oxygen levels might be the main cause of reductions in PI viability and functionality after prolonged culture periods. Hence, effective and sustained delivering of anti-inflammatory agents to PIs will be an effective strategy to enhance PI activities.

5. Conclusion

Adding free anti-inflammatory drugs directly to the media showed an improvement in the functionality of PIs compared with PIs free of cytokines. However, the improvement of the free anti-inflammation agents in the culture media was lower than the effect by incorporating GBs loaded with these agents into PIs. Both cell viability assays and GSIS assay showed that incorporating GBs only to PIs improve the PI performance. The sustained release of anti-inflammatory drug from GBs showed even higher enhancement of PI functionality, indicating a synergetic effect. The benefit from the release of both IL-10 and anti-IL-1β for the PIs were manifested as the rise of the cellular activities, decreasing of cellular LDH release, and increases in insulin release. The biofunctionality of larger PIs (>300 μm) over a 1-week culture was increased. The production of insulin from GBs incorporated PIs was increased by ∼1.5-fold when IL-10 was loaded onto GBs and by ∼1.6-fold when anti-1L-1β was loaded onto the GBs. Overall, the inclusion of anti-inflammatory or anti-apoptotic agents bound to GBs appears to be an effective strategy to enhance cell survival and improve PI biofunctionality.

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