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The Effects of Alginate Encapsulation on NIT-1 Insulinoma Cells: Viability, Growth and Insulin Secretion

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Abstract. *Transplantation of microencapsulated insulin-secreting cells is proposed as a promising therapy for the treatment of type I diabetes mellitus. In recent years, important advances have been made in the field of immunoisolation and many studies have shown that alginate provides some major advantages for encapsulation over other systems. Since it is known that the extracellular matrix influences the behaviour of encapsulated cells, the aim of the present work has been to study the consequences of encapsulation on some cell functions. For this purpose, cell growth and dynamics of insulin release of NIT-1 cells entrapped in alginate capsules compared with those exhibited by free NIT-1 cells were investigated by means of growth curves, assays, Trypan blue staining and ELISA test. All investigations performed allowed us to conclude that alginate-entrapped NIT-1 cells maintain their growth features and secretory functions although with some important differences. In particular, alginate encapsulation affects the cellular growth profile and causes the lost of time dependence of insulin secretion profile.*

Transplantation of pancreatic islet cells has been shown to be a potential modality of treating insulin-dependent diabetes mellitus (1). However transplantated cells are rapidly destroyed by immune rejection and immunosuppressive drugs to protect islets grafts are required, resulting in a strong limitation for the clinical application of this therapy (2). Consequently, many efforts have been directed towards the development of new strategies to avoid immunosuppression. One such strategy is immunoisolation (3). Indeed, over the last three decades, the encapsulation of insulin-secreting cells within semipermeable membranes has emerged as a promising approach, with the purpose of protection from the host's immune system for long-term treatment of type I diabetes (4, 5).

The previous attempts to implantation of microencapsulated islets were unsuccessful since the early semipermeable membranes were not able to supply sufficient oxygenation to the enclosed insulin-secreting cells nor to provide adequate *in vivo* kinetics of insulin diffusion (6). Therefore, the proper choice of biopolymeric materials for the construction of the capsules should necessarily ensure selective permeability together with islet viability and functionality (7). Nowadays, many different immobilizing materials have been proposed and encapsulation in alginate hydrogel beads so far represents the most versatile and well-established method for cell protection from the host immune system/immunoisolation (8-11).

Alginate is a family of polysaccharides produced by brown algae and bacteria (12-14). In molecular terms, they are unbranched binary copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) arranged in a pattern of blocks along the chain with homopolymeric regions of M (M-blocks) and G (G-blocks) residues interspersed with regions of alternating structure (MG-blocks). Biomedical applications of alginate exploit its capability to form stable hydrogels in the presence of low concentrations of calcium or other divalent cations and thus the possibility to immobilize cells in a single-step process, under cell-compatible conditions (9, 15).

There are two important and strictly related issues concerning implantation of alginate-entrapped insulin-secreting cells: the biocompatibility of alginate and the influence of the capsule microenvironment on immobilized cells growth and functionality (11). In terms of

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biocompatibility there are several studies in the literature reporting that alginate biocompatibility is dependent on the ratio of two different acid monomers and the purity of the material used (16-18). It has been reported that alginates with a high content of M units can trigger an inflammatory response by stimulating innate immune cells to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 and interleukin-6 (19). In addition, crude alginate from seaweed contains polyphenols, proteins and endotoxins (20). Polyphenols are known to be harmful to immobilized cells, while endotoxins are potent immunogenic molecules. Therefore, polymer purification is required to increase the biocompatibility of the implantation material and to improve the survival and metabolic function of encapsulated islets (21).

Moreover, cellular growth and secretory activity are fundamental requirements for judging the functionality of implanted microfabricated biocapsules and their therapeutic applications. Thus, the role of the alginate 3D-matrix in supporting cell growth and functionality is crucial and it might influence the overall biocompatibility of the system (11).

In this short-term study, we investigated the effects of alginate encapsulation on the *in vitro* functions of NIT-1 mouse insulinoma cells. Indeed, rodent insulinoma cell lines have became useful tools in cell biology studies (22) because they have been shown to retain the functional attributes of normal islets with the advantages that they are easily available, stable and continuously proliferate in culture (4, 23, 24). In particular, our aim was to evaluate the effects of alginate encapsulation on cell viability, growth pattern and metabolic activity of alginate-entrapped NIT-1 cells in comparison with free cells.

Materials and Methods

Cell culture. Mouse insulinoma NIT-1 cells were obtained from the laboratory of Dr. A. Pileggi (Diabetes Research Institute, Leonardo M. Miller School of Medicine, University of Miami, FL, USA).

Cells were cultured as colonies in T25-flasks and media were replenished three times per week. The medium consisted of Kaighn's modification of Ham's F-12 medium (F-12K) with 2 mM L-glutamine and 1.5 g/ml sodium bicarbonate (ATCC, LGC Promochem, Italy) and supplemented with 10% heat-inactivated dialysed foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37˚C in an atmosphere of 95% humidified air and 5% CO_2. They were subcultured every 3-4 days and then split 1 to 3. Where not otherwise specified, all cell culture reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Cell encapsulation in alginate beads. Sodium alginate samples isolated from *Laminaria hyperborea* stipe were provided by FMC Biopolymer (Norway) (Mw=1.3×10^5, fraction of guluronic acid residues (F_G)=0.69; fraction of GG dyads (F_{GG})=0.56). Cell-containing beads were made by mixing a sterile sodium alginate solution (1.5% w/v; 0.15 M NaCl, 0.01 M Hepes, pH 7.4) with NIT-1 cell pellets [previously washed with phosphate buffered saline (PBS)] to a final concentration of 1.5×10^6 cells/ml. Calcium beads were obtained by dripping the cell/polymer blend into a calcium-based gelling solution. The droplet size was controlled by using a high-voltage electrostatic bead generator (25). Bead generator parameters were: 5 kV, 10 ml/h, steel needle with 0.7 mm outer diameter, 2.5 cm distance from the needle to the gelling solution (0.05 M $CaCl_2$, mannitol 0.15M, 0.01 M Hepes, pH 7.4). The gel beads were then stirred for 10 minutes in the gelling solution and rinsed with saline solution (NaCl 0.9%) and F-12K medium prior to use.

Growth curve determination. Free NIT-1 cells were plated into 100 mm culture dishes at 5×10^5 cells per plate in F-12K complete cell culture medium. At regular time intervals, cells were washed with PBS, trypsinized and counted with a Burker chamber. The same number of entrapped NIT-1 cells were plated and grown under the same culture conditions. At regular time intervals alginate capsules were dissolved with 10 mM EDTA solution and the total cells counted with a Burker chamber.

Cell viability. MTS assay: The MTS assay is based on the bioreduction of a tetrazolium compound into a coloured, aqueous soluble formazan product by mitochondrial activity of viable cell. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture and can be measured at 490 nm. Cell viability was evaluated by means of MTS assay with a plate reader (GDV, Italy) to determine optical density.

The assay was performed in 96-well plates where 5×10^4 free or entrapped NIT-1 cells per well were cultured with 200 μl of F-12K complete culture medium. In order to study the viability shortly after encapsulation, at regular time intervals (24, 48, 72 and 96 hours) 20 μl of the MTS solution (Promega Italia, Italy) were added to each well and the plates were then incubated at 37˚C in 5% CO_2 and 95% air for 3 hours. After incubation, the resulting optical density was read at 490 nm with a 96-well plate reader. All the experiments were performed fivefold and the results expressed as mean±standard deviation.

Trypan blue staining: Cell viability was also determined by the vital dye exclusion method. Free NIT-1 cells were plated into 100 mm culture dishes at 5×10^5 cells per plate and grown in F-12K complete cell culture medium. At regular time intervals (daily for 11 days of incubation), the cells were washed with PBS, trypsinized, added to the medium previously collected and pelletted down at room temperature for 8 minutes. The pellet was mixed 1:1 with 0.4% trypan blue solution for 5 minutes and the cells counted with a Burker chamber. Entrapped NIT-1 cells were plated and grown under the same culture conditions. At the same time intervals, alginate capsules were first dissolved with 10 mM EDTA solution for 5 minutes and then the cells were treated according to the experimental protocol described above. The number of cells staining blue (nonviable cells) was expressed in relation to the total number of cells (stained plus unstained). All the experiments were performed in triplicate and the results expressed as mean±standard deviation.

Insulin measures. Insulin release in cell culture medium was used to assess the functionality of insulinoma cells and was assayed by ELISA. The protein levels were determined with a commercial immunoassay kit (Linco research, St. Charles, MO, USA) and were calibrated on a microplate reader at 450 nm corrected from

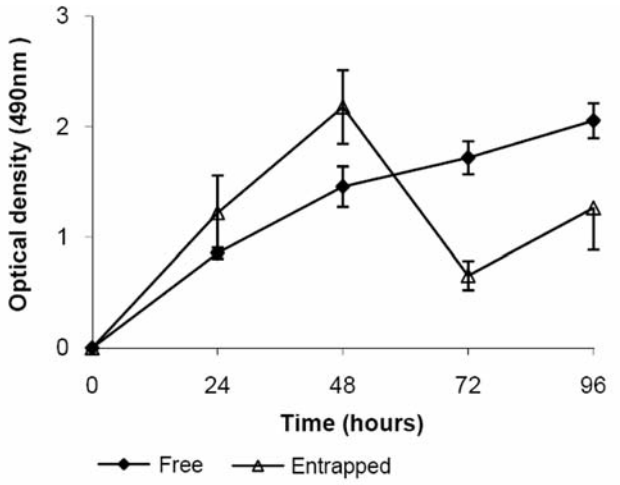

Figure 1. *Cell viability of free and alginate-entrapped NIT-1 cells obtained by MTS assay. Each point represents the mean of five different experiments. The error bars represent the standard deviations of the calculated means.*

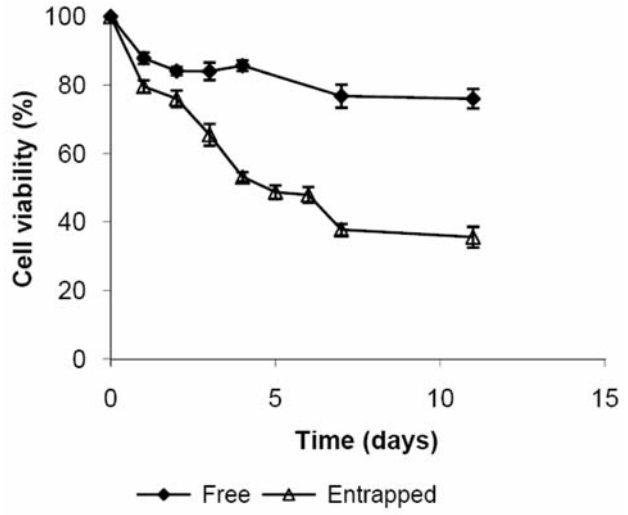

Figure 2. *Cellular survival of free and alginate-entrapped NIT-1 cells determined by trypan blue staining. Each point represents the mean of three different experiments. The error bars represent the standard deviations of the calculated means.*

540 nm. All standards, controls and samples were run in duplicate. The immunoassay utilized a monoclonal mouse anti-rat insulin antibody with 100% specificity and a sensitivity of 0.2 ng/ml. The insulin concentrations were determined with dedicated software (DV990win6; GDV Italy) plotting the absorbance of the standards against the standard concentrations to derive the unknown sample concentrations.

ELISA tests were performed on filtered cell culture media collected at regular intervals times from free and alginate-entrapped NIT-1 cells up to 10 days of incubation.

The experiment was performed twice and the results, normalized to the total number of cells in the culture, were expressed as mean±standard deviation.

Results

Cell viability. The MTS measurements performed on the free insulinoma cells within 96 hours from seeding (Figure 1) showed that, under our experimental conditions, NIT-1 cells were able to proliferate according to a linear trend. Trypan blue staining within 11 days from seeding also demonstrated that cell survival ranged from 77% to 88% (Figure 2).

The MTS assays performed on the entrapped cells showed that for the first two days after entrapment NIT-1 cells were still able to proliferate but the cell viability then decreased until 72 hours, when only 38% of cells were viable. Finally, in the last 24 hours of culture, the surviving entrapped cells continued to proliferate and the cell viability increased to 61.5% (Figure 1). The subsequent trypan blue staining demonstrated a persistent decline in entrapped cell viability (Figure 2). In fact, the cell survival dropped to 35% in the first 7 days of culture and remained constant until the 11th day, indicating that encapsulation affected cell survival but did not completely nullified.

Cellular growth and insulin secretion. NIT-1 cells were plated into culture dishes and every day, for 10 days of incubation, cells were counted with a Burker chamber. The resulting growth curve (Figure 3A) showed an increase in the total cells number during the first 4 days of culture and allowed the doubling time of this cell line to be estimated in approximately 24 hours.

The ELISA measurements performed on NIT-1 cells showed an insulin release into the culture medium, indicating the basal metabolic and secretory activity of these cells. Figure 3B illustrates the temporal change in the insulin secretion over the duration of the experiment. The dynamics of hormone release was time dependent: a rapid increase was observed in the first 24 hours, followed by a continuous decrease in the amount of insulin release in the following 3 days; finally, in the remaining culture period (4 days) the insulin secretion increased again exceeding the initial peak value.

A comparison between the insulin release kinetics and the growth curve over the same time period highlighted that insulin was secreted by NIT cells and that together with a time dependence, there is also cell dependence. Indeed, Figure 3C shows that an increase in the total cell number corresponded to a decrease of metabolic and secretory activity of single cells, although the overall amount of insulin in the culture medium remained at a constant level (Figure 3D).

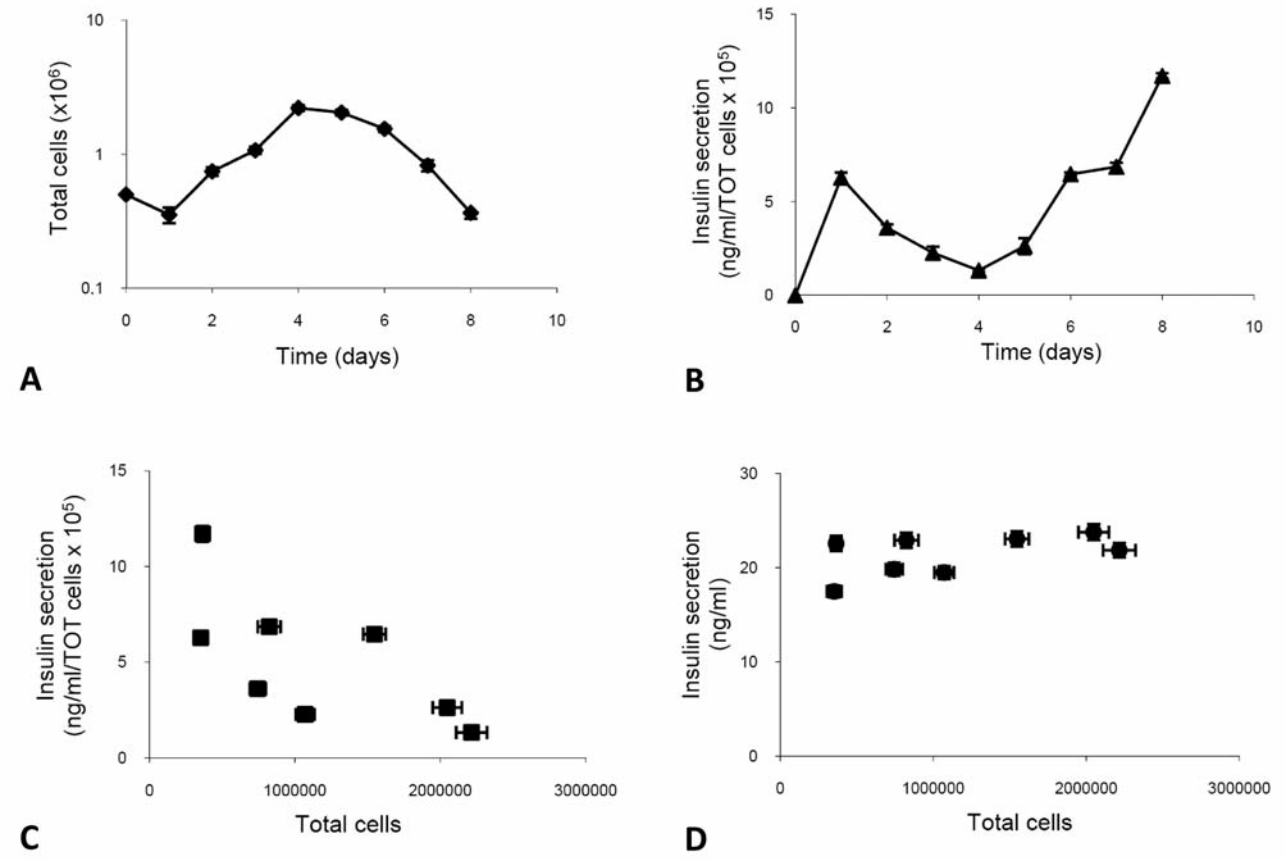

Figure 3. *Characteristics of free NIT-1 cells. A, NIT-1 growth curve; B, temporal change in the rate of insulin secretion normalized to the total number of cells; C, cell dependence of the insulin secretory profile; D, level of insulin in the culture medium.*

The same measurements were performed with alginate-entrapped NIT-1 cells to evaluate the effects of alginate incapsulation on their secretory and growth features. The resulting growth curve (Figure 4A) showed that under these experimental conditions, the overall number of cells in culture fluctuated continuously. The study of the temporal change in the insulin secretion (Figure 4B) demonstrated that on encapsulation, the dynamics of hormone release lost its time dependence: insulin secretion fluctuated with cellular metabolic activity. In contrast, a comparison between the kinetics of insulin release and the growth curve revealed that the cell dependence was preserved. In fact, in the presence of constant levels of insulin in the medium, the increase of the total cell number corresponded to a decrease of metabolic and secretory activity of the single cell, similary to free NIT-1 cells (Figure 4C, D).

Discussion

The protection of pancreatic islets from immune attack is one of the features currently under study to improve the taking of islets and their functional survival after cell transplantation. Encapsulation of islets in biocompatible microcapsules characterized by a selective immune-permeability is currently considered as an efficient and attractive method of immunoprotection. Along this line, some years ago, the microencapsulation of Langerhans islets with semipermeable biological membrane in order to prevent rejection in the absence of pharmacological immune suppression was proposed (8, 26). Extensive research has been carried out on encapsulation technology and these studies (2, 7, 27) demonstrated that the proper choice of biopolymeric materials for the construction of the capsules is essential in determining their selective permeability and in maintaining islet vitality and functionality for a few months, with the consequence of partial independence from exogenous insulin.

In the present short-term study, we firstly investigated cellular viability, growth and metabolic activity of free NIT-1 cells. Our results showed that insulinoma cells in the basal state are viable and metabolically active. In addition, this study demonstrated that the insulin secretory profile was time dependent, in accordance with a biphasic secretion pattern already described. Indeed several researchers (28-31) observed

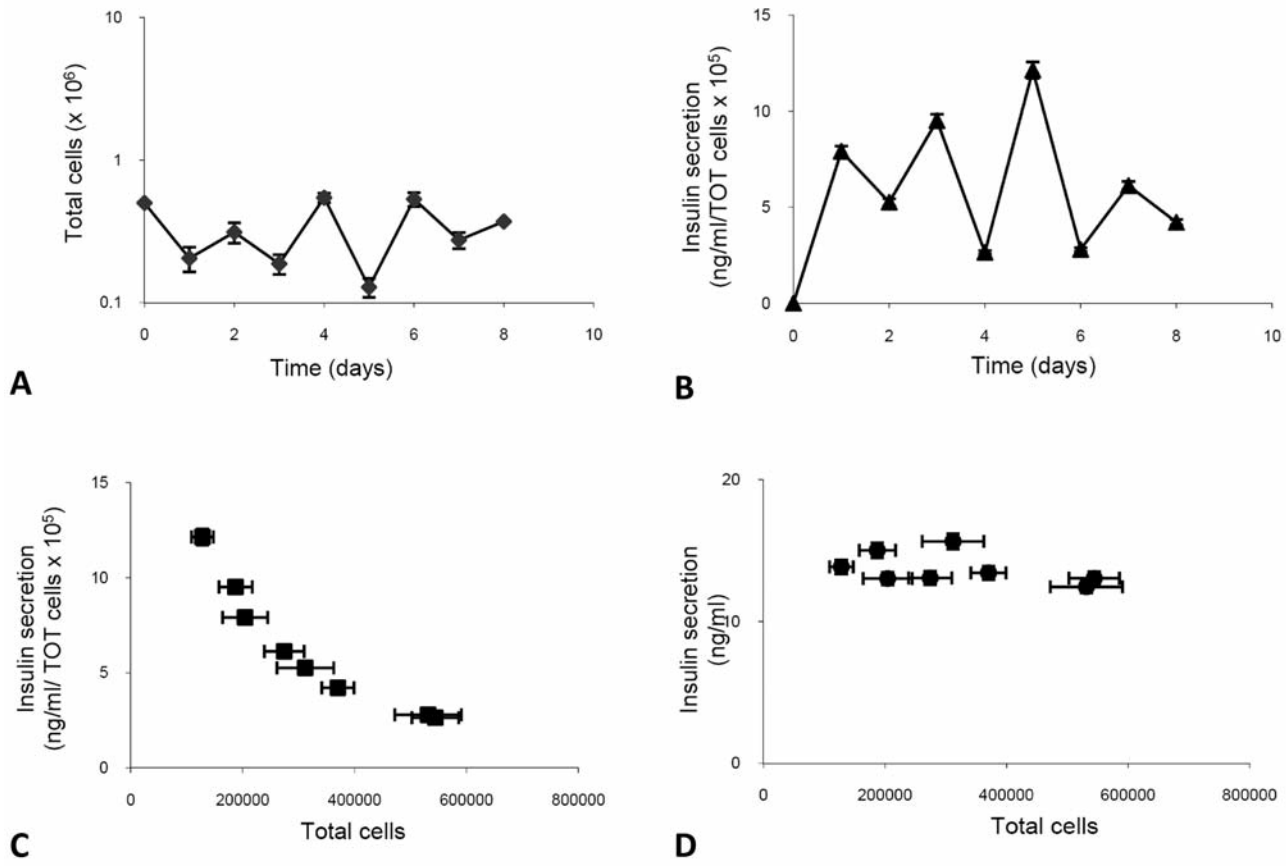

Figure 4. *Characteristics of alginate-entrapped NIT-1 cells. A, the growth curve of alginate- entrapped NIT-1 cells; B, temporal change in the rate of insulin secretion normalized to the total number of cells; C, cell dependence of the insulin secretory profile; D, level of insulin in the culture medium.*

that the initial acute phase of insulin release is due to the discharge of insulin stored in readily releasable granules and is followed by a second prolonged phase attributed to elevated insulin biosynthesis and to the preparation of other groups of granules for release. These secretion dynamics might be explained by the hypothesis that autocrine insulin signalling can itself regulate secretory activity of pancreatic β-cells. Accordingly, in recent studies (32, 33) it has been stated that during the first phase of secretion, a positive insulin feedback contributes to the rapid increase of insulin release, while negative feedback may eventually take over to suppress insulin secretion when the concentration reaches high levels in the microenvironment of the β-cells. In our study, the analysis of the growth curve in comparison with the kinetics of insulin release pointed out a cell dependence for the hormone release. Our hypothesis that cell-to-cell interactions play an important role in insulin secretion is also supported by a recent observation that cell density influences the secretory capacity of the cells (34) and is in accordance with the study of Halban *et al*. (35) that showed that basal insulin release was elevated in isolated cells, whereas cultures with cells in close contact had a lower basal insulin release.

We performed the same analysis on NIT-1 entrapped cells, demonstrating that encapsulation affects many aspects of cell behaviour, including viability and survival. Indeed, the present study pointed out that the basal linear trend of proliferation is lost after alginate encapsulation, proving that the treatment interferes with cell viability. However, although alginate encapsulation affects cellular survival, our data also showed that 35% of cells were viable and retained their proliferative ability. This gives support to the studies demonstrating that the islets can adequately survive when enveloped in alginate capsules (36, 37). The effect of the polysaccharidic matrix on the metabolic activity of entrapped NIT-1 cells was also investigated. We observed that insulinoma cells display differences in the growth as well as in the secretory profiles when they are encapsualted in hydrogel. In particular, a loss of time dependence in the insulin secretory profile was highlighted. It has been hypothesized that the entrapment interferes with the diffusion

of insulin and, supporting this hypothesis, a recent study demonstrated that this difference is due to the change in the cellular microenvironment and to the different cell-cell and cell-matrix interactions caused by cell growth in alginate beads (31). Our results have also demonstrated that despite the differences in the cellular growth profiles between free and encapsulated NIT-1 cells, the insulin secretion cell dependence is still maintained, proving the importance of cell-to-cell interactions under both of the experimental conditions considered.

In conclusion, the data presented in this study indicate that NIT-1 cells that survive encapsulation maintain their ability to proliferate and their metabolic functionality, although with significant differences, providing important preliminary data for clinical application of pancreatic cell encapsulation in the therapy for diabetes.

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