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## Encapsulation of human islets in novel inhomogeneous alginate-Ca<sup>2+</sup>/Ba<sup>2+</sup> microbeads: *in vitro* and *in vivo* function

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### Abstract

Microencapsulation may allow for immunosuppression free islet transplantation. Herein we investigated whether human islets can be shipped safely to a remote encapsulation core facility and maintain *in vitro* and *in vivo* functionality. In non-encapsulated islets before and encapsulated islets after shipment, viability was 88.3±2.5 and 87.5±2.7% (n=6, p=0.30). Stimulation index after static glucose incubation was 5.4±0.5 and 6.3±0.4 (n=6, p=0.18), respectively. After intraperitoneal transplantation, long-term normoglycemia was consistently achieved with 3,000, 5,000, and 10,000 IEQ encapsulated human islets. When transplanting 1,000 IEQ, mice returned to hyperglycemia after 30–55 (n=4/7) and 160 days (n=3/7). Transplanted mice showed human oral glucose tolerance with lower glucose levels than non-diabetic control mice. Capsules retrieved after transplantation were intact, with only minimal overgrowth. This study shows that human islets maintained the viability and *in vitro* function after encapsulation and the inhomogeneous alginate-Ca<sup>2+</sup>/Ba<sup>2+</sup> microbeads allows for long-term *in vivo* human islet graft function, despite long-distance shipment.

### Introduction

Obstacles to the widespread use of islet transplantation for the treatment of juvenile diabetes include an insufficient number of appropriate donors, toxic immunosuppression protocols, recurrence of the underlying autoimmune disease, and sensitization of the recipient (1–5). Although new sources of cells and tissue may be developed in the future (6–8), the destruction of the graft by the immune system may remain difficult to prevent without systemic immunosuppression. The side effects associated with long-term immunosuppression have been well documented. Enveloping the islets in semi-permeable capsules may enable transplantation of islets without immunosuppression. Lim and Sun first described this approach of embedding the pancreatic islets in alginate-PLL-alginate microcapsules and provided evidence that encapsulated islets could regulate blood glucose in rats (9). After this ground breaking study, many investigations into encapsulated islet transplantation have been performed with documented immunosuppression-free graft

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survival in mice (10–12), rats (13–15), dogs (16, 17), monkeys (18, 19), and humans (20, 21).

A variety of semi-permeable membranes have been evaluated as immunoprotective barriers. Alginate has been the most frequently used material, as it is non-toxic and the encapsulation can be carried out under physiological conditions, conserving islet viability and function (22). Alginate composition, molecular weight, and concentration determine the microcapsule structure and properties, together with the composition of gelling and anti-gelling ions. Stability, permeability, size, and biocompatibility are all considered important capsule properties. Stability and permeability can be further improved by adding a polycation layer to the surface of the alginate gel core, as coating was used in the original procedure of Lim and Sun. However, the addition of a polycation has been shown to provoke inflammation (23, 24) and is, quite likely, the major cause of capsule toxicity. Recently, Calafiore *et al* initiated a phase I trial with proof of concept of preserving islet function without immunosuppression by using an alginate-PLO microcapsule (21). However, the low number of islets transplanted per patient has limited the success of this pilot trial and insulin independence has not been achieved.

In this study, the current knowledge of alginate structure – function relationship was optimized in a novel alginate capsule design. An alginate with a high molecular weight and a high content of G was used in combination with  $\text{Ca}^{2+}$  and a minimal concentration of  $\text{Ba}^{2+}$  as this allows for very strong and stable microcapsules (25). In addition, sodium was exchanged with mannitol as osmolyte in both the alginate and in the gelling solution resulting in a higher concentration of alginate at the capsule surface than in the center (26) that further improves the capsule stability. Hence, stable microcapsules were obtained without a proinflammatory polycation (23). The microcapsules were permeable to IgG (25), but microcapsules of similar permeability has previously been shown to protect both allo- and xenografts in mice (27, 28).

Since the field of immunoisolation involves expertise from different fields such as surgery, chemistry, engineering, and cell biology, an international approach was taken in the current study. The goal was to combine experience on islet isolation, microcapsule design, chemistry, engineering, and transplantation to improve the outcome of encapsulated islet transplantation. Human islets were isolated at the University of Illinois at Chicago (UIC), shipped to the Norwegian University of Science and Technology (NTNU) for microencapsulation, and then returned to Chicago. In this study, we assessed the viability and function of human islets *in vitro* and *in vivo* after encapsulated in this novel inhomogeneous alginate- $\text{Ca}^{2+}/\text{Ba}^{2+}$  beads and shipped trans-Atlantically in order to investigate the feasibility of a large international collaboration to advance encapsulated islet transplantation more rapidly into clinical trials.

## Research design and methods

### Human islet isolation

Human pancreata were procured from heart-beating deceased multi-organ donors. The pancreata were sent in cold preservation solutions (UW, University of Wisconsin or HTK, Histidine-Tryptophan Ketoglutarate) (29–31) to the Cell Isolation Laboratory at UIC for islet isolation. Human pancreata from 6 donors were used in this experiment. Pancreatic islets were isolated using the method described by Ricordi *et al* (32). Briefly, after cleaning the pancreas from the surrounding tissue, it was perfused with Liberase HI (Roche, Indianapolis, IN) with a concentration of 1.7 mg/ml in cold perfusion solution. The distended pancreas was then transferred to the Ricordi digestion chamber, connected to a modified, closed circulation tubing system, and warmed up to 37°C. During the digestion,

the chamber was shaken gently. Samples were continuously taken to determine digestion progress. Once free islets were detected under the microscope, the digestion was stopped by flushing cold (4°C) Hank's Buffer Salt Solution (HBSS, Mediatech, Herndon, VA) into the circulation system to dilute the enzyme. After being collected and washed in M199, the tissue was incubated with UW solution before purification on a cell separator (COBE 2991, Cobe, Lakewood, CO). Following purification, islet yield (expressed as islet equivalents, IEQ), tissue volume, and purity were determined according to standard methods (33). Isolated islets were cultured in CMRL-1066 media (Mediatech, Herndon, VA), supplemented with 1.5% human albumin, 0.1% insulin-transferrin-selenium (ITS), and Pen/Strep in T175 flasks at 37°C overnight culture before islets were shipped in the same media at a maximal density of 1,000 IEQ per ml at room temperature.

### Islet shipment and microencapsulation

After culturing overnight, the isolated islets were shipped by World Courier in supplemented CMRL-1066 medium at room temperature in a Styrofoam box to the NTNU in Trondheim, where they were microencapsulated in alginate, prepared from stipes of *Laminaria hyperborea* (Pronova UP-LVG from Nova Matrix, Oslo, Norway). The alginate had a content of 63% guluronic acid (G) units and the average length of G-blocks consisting of two or more continuous G units  $N_{G>1} = 12.6$  was measured by <sup>1</sup>H-NMR. The weight average molecular mass of 243 kDa and the polydispersity ( $M_w/M_n$ ) of 1.80 were measured by SEC-MALLS. The encapsulation method was previously described (34). Briefly, the islet suspension was centrifuged (1,000 rpm, 2 min, RT), and the islets were resuspended in a small volume of culture media (CMRL-1066 supplemented with 300 mg/L glutamine, 16.7 mM HEPES, Pen/Strep, and 15 % heat inactivated A+ human serum). The islet suspension was mixed with a 2.0 % sterile filtered alginate solution (in 0.3M mannitol, pH 7.2–7.4) to a final suspension of 10,000 IEQ per ml alginate in a 2.0% alginate solution. Gel beads with an alginate gradient were formed by dripping the alginate/cell suspension in a gelling solution of 50 mM CaCl<sub>2</sub> and 1 mM BaCl<sub>2</sub> (in 10 mM MOPS, 0.14 M mannitol and 0.05% Tween20, pH 7.2–7.4). Small droplet production was obtained using an electrostatic bead generator (7 kV, flow: 10 ml/hr per 0.4 mm needle used)(34), resulting in alginate capsules of  $509 \pm 30 \mu\text{m}$  in diameter (mean  $\pm$  SEM, n = 3 sets of experiments of 10 beads each). The alginate microcapsules were collected on a filter and washed three times with approximately 10 ml culture media before being transferred to Petri dishes for culturing at 37°C and 5% CO<sub>2</sub>. All encapsulation and culturing procedures were performed under sterile conditions. After being encapsulated, islets were shipped back to UIC for assessment. The encapsulated islets were shipped in culture medium CMRL-1066 supplemented with 300 mg/L glutamine, 16.7 mM HEPES, Pen/Strep, and 15 % heat inactivated A+ human serum.

### In vitro assessment

The encapsulated islets were assessed *in vitro* for viability and functionality after arrival in Chicago (after they were shipped from Chicago to Trondheim, encapsulated in Trondheim, and shipped back from Trondheim to Chicago). This corresponded to a period of 5–7 days after islet isolation. Non-encapsulated islets were assessed 1–2 days after islet isolation without any shipment.

**Dithizone (DTZ) staining and viability**—Encapsulated and non-encapsulated islets were assessed using DTZ for insulin granule zinc staining. Islet viability was assessed by fluorescent staining with Syto-Green/Ethidium Bromide (35, 36). For the Syto-Green/Ethidium Bromide staining, double fluorescence was performed to assess the amount of live (green) versus dead (red) islet cells in a representative sample, where a minimum of 100 islets were counted per sample.

**Static glucose incubation**—A stimulation index (SI) was calculated by dividing insulin secretion during high glucose exposure by the insulin secreted during low glucose exposure (37). Non-encapsulated and encapsulated islets were challenged with Krebs-Ringer bicarbonate buffer (KRBB, pH 7.35) containing 0.5% BSA (Sigma) under low glucose concentration conditions (1.6 mM) and with high glucose concentration (16.7 mM). Groups of five handpicked, non-encapsulated or encapsulated islets were placed in five different wells of a 12-well plate and were then incubated with 1 ml of KRBB low glucose concentration (1.6 mM glucose final concentration) for 30 min, allowing them to stabilize insulin secretion before the supernatant was collected and discarded. Islets were then incubated for 60 min in KRBB low glucose at 37°C and 5% CO<sub>2</sub>, and supernatant was collected under a microscope taking care not to remove any islets from the well. The same step was repeated by addition of KRBB high glucose solution (16.7 mM glucose final concentration) and incubation for 90 min. Supernatant was collected and frozen at –20°C for later measurement using an ELISA kit for human insulin (Merckodia, Uppsala, Sweden).

**Dynamic insulin secretion**—This was performed with non-encapsulated and encapsulated islets based on the method described by Roe *et al* (38, 39). Islets were transferred to a perfusion chamber and equilibrated by perfusing with KRBB containing 1.6 mM glucose for 15 min before glucose stimulation. After stimulation with high glucose solution (16.7 mM) for 15 min, 1.6 mM low glucose solution was infused in the chamber for 5 min. Aliquots from each perfusion chamber were collected at 1 min intervals throughout the experiment and frozen at –20°C for later insulin measurement.

**Intracellular Ca<sup>2+</sup> measurement**—Intracellular Ca<sup>2+</sup> was measured during glucose stimulation for functional evaluation of non-encapsulated and encapsulated islets using wide-field fluorescence imaging with dual-wavelength excitation fluorescent microscopy (39, 40). The islets were loaded with Fura-2/AM, a ratio-metric Ca<sup>2+</sup> indicator (5 μM; Molecular Probes Inc., Eugene, OR), and incubated for 30 min at 37°C in KRBB (1.6 mM glucose). The coverslip was mounted in a microperfusion chamber (Medical System Inc, Paola, KA) on the apex stage of an inverted microscope equipped for epifluorescence (TE-2000 U, Nikon Inc, Melville, NY) and perfused by a continuous flow (rate 2.5 ml/min of 5% CO<sub>2</sub>-bubbled KRBB at 37°C (pH 7.4). KRBB containing high glucose and KCl concentration (16.7 mM and 30 mM) were administered to the islets and followed for 15 min. Single encapsulated and free islets were visualized with 10x objectives. Intracellular-free Ca<sup>2+</sup> concentration after glucose stimulation was measured using a fluorescent video charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan) and expressed as a ratio of fluorescence intensity of Fura-2 dual-wavelength excitation at 340 and 380 nm. Fluorescence emission at 519 nm was detected using Metafluor software (Universal Imaging, Downingtown, PA). The basal level of Ca<sup>2+</sup> was estimated by measuring pre-stimulation levels.

## In vivo assessment

**Diabetic nude mice**—Male nude mice (Harlan Industries, Indianapolis, Indiana) weighing 22–25 g were used as recipients for encapsulated human islets transplantation. Animals were housed and surgery was performed under a laminar flow hood located in “barrier” rooms at the Biologic Research Laboratory at UIC. Diabetes was induced by a single intraperitoneal (IP) injection of Streptozotocin (STZ, 220 mg/kg body weight; Sigma, St. Louis, MO) freshly dissolved in citrate buffer (pH, 4.5) at the day of human islet isolation. Animals were considered diabetic after two or more non-fasting blood glucose levels from the tail vein exceeded 350 mg/dl, which generally occurred after a maximum of 72 hours post injection. All procedures were performed under the guidelines of the National Institutes of Health and approval by the Animal Care Committee (ACC) at UIC.

**Encapsulated human islet transplantation**—In order to evaluate the *in vivo* function, we transplanted various amounts of encapsulated human islets intraperitoneally into diabetic nude mice, 10,000 (n=3), 5,000 (n=10), 3,000 (n=9), and 1,000 IEQ (n=7). Diabetic mice were placed under gas anesthesia (isoflurane) using a mini-mask (Viking Medical). A short midline abdominal incision (approximately 0.5 cm) was made and the encapsulated islets were slowly infused into the peritoneal cavity. Blood glucose and body weight were monitored every other day in the first week and twice a week afterwards.

**Oral Glucose Tolerance Test (OGTT)**—The OGTT was performed at week 30 after transplantation in the mice transplanted with 3,000 IEQ encapsulated islets, non-transplanted diabetic, and normal controls. The OGTT was performed by the method described by Korbitt *et al* (41). Briefly, after 4–6 hours fast, D-glucose (3 g/kg body weight) was administered as a 20% solution by gastric gavage into anaesthetized mice (isoflurane). Blood glucose levels were determined at 0, 15, 30, 60, 90, and 120 min by tail puncture.

**Recovery of the capsules**—For testing of the post-recovery microcapsule properties and histological analysis, encapsulated islets were removed at day 102 (n=1), day 130 (n=1), and day 200 (n=2) post-transplantation. At the end of the study (at 260 and 329 days of post transplant) capsules were again removed from 13 mice transplanted with 1,000 to 10,000 IEQ of encapsulated human islets. Briefly, under gas anesthesia (isoflurane), a midline abdominal incision (approximately 0.5 cm) was made and 3 ml of warmed sterile HBSS was infused into peritoneal cavity to wash out the capsules. This procedure was repeated five times. Following the removal of the capsules, blood glucose was continuously monitored to confirm the achieved normoglycemia was due to the capsule transplant and not spontaneous recovery of the native islets. Islet function after explantation was tested by dynamic insulin secretion study using the encapsulated islets removed after 130 days, following the method described above.

**Immunohistochemical study of the recovered capsules**—Following the removal, the encapsulated islets were prepared for histological study using the method described by Kobayashi *et al* (42). Briefly, the capsules were fixed in Bouin's solution for 2 hrs, dehydrated in graduated alcohol (70–100%), and embedded in paraffin. 5  $\mu$ m sections were stained with H&E. In order to confirm the function of encapsulated islets, expression of insulin was assessed by immunohistochemical staining using polyclonal Guinea Pig anti-insulin antibody (1:100, Zymed Labs, San Francisco, CA) as the primary antibody. For detection of the primary antibody, FITC-conjugated Goat anti-mouse IgG (1:100, Sigma, Saint Louis, Missouri, USA) secondary antibody was used according to the methods recommended by manufacturers. A Carl Zeiss LSM 510 confocal microscopy was used to visualize the antibody.

## Statistical methods

Statistical analysis was performed by Student's t-test. p values <0.05 were considered statistically significant. Results are expressed as mean  $\pm$  SEM when not indicated differently.

## Results

### Viability and *in vitro* static glucose incubation test

The encapsulation and shipment did not seem to have a measurable deleterious effect on the viability and insulin secretion in the static glucose stimulation assay of isolated human islets. These tests did not show any significant difference (Figure 1 and Table). Transport time of

free islets before encapsulation was  $32.7 \pm 4.8$  hours and re-shipment duration after encapsulation was  $38.4 \pm 7.7$  hours.

### Dynamic insulin secretion and intracellular $\text{Ca}^{2+}$ measurement

Following incubation with low and high glucose KRBB solution, we observed a discrete time lag of the acute release of insulin in encapsulated islets. However, apart from a lag-time of approximately five minutes, the results showed that the encapsulated islets retained a normal insulin secretion pattern and magnitude in response to glucose stimulation (Figure 2A). The preserved function of the encapsulated islets was supported by similar intracellular  $\text{Ca}^{2+}$  level dynamics in response to glucose as compared to non-encapsulated islets (Figure 2B).

### *In vivo* assessment of the encapsulated human islet

After transplantation, the blood glucose of diabetic nude mice reversed to normal values in all animals (Figure 3). In three groups of mice transplanted with 3,000, 5,000, and 10,000 IEQ encapsulated human islets, 11 mice maintained normoglycemia until the end point of the study, which was from 260 to 329 days post transplantation. Seven mice died without hyperglycemia presumably the end of their natural life span observed in athymic nude mice, and 4 mice were sacrificed after confirming hyperglycemia due to the removal of capsules. For animals transplanted with 1,000 IEQ, 4 mice returned to hyperglycemia 30–55 days after transplantation, and the remaining 3 mice also returned to hyperglycemia after 160 days of transplantation. OGTT showed that the encapsulated human islets regulated glycemia similar to human levels, and non-treated and non-diabetic mice had higher fasting and post-challenge glucose levels (Figure 4A).

At the end of the study, capsules were retrieved from 13 mice transplanted with 1,000 to 10,000 IEQ encapsulated human islets by peritoneal lavage. In 12 out of 13 mice, the encapsulated islets were found to be free floating. The viability of retrieved encapsulated islets was  $77 \pm 7\%$  ( $n=7$ ). The retrieved capsules remained intact with a continuous smooth surface, and H&E staining showed the viable islet in the capsule. Moreover, fluorescent insulin staining for the retrieved capsules confirmed healthy insulin containing islets (Figure 5). By testing the retrieved islets' response to glucose in a static incubation assay, the encapsulated islets from all groups of mice were highly responsive, with a mean stimulation index of  $9.5 \pm 3.7$ . From the perfusion study, we observed that the retrieved encapsulated human islets responded remarkably well with 5-fold increase in insulin secretion (Figure 4B).

In general, between the 4 groups differing by the number of encapsulated and transplanted IEQ, no differences were seen on the retrieved capsules in regard to overgrowth, viability, and the function of islets in a static incubation assay.

## Discussion

Our study demonstrated that human islets encapsulated in inhomogeneous alginate- $\text{Ca}^{2+}/\text{Ba}^{2+}$  microcapsules were fully functional *in vitro* and *in vivo* even after exposure to long shipments and an encapsulation process. The viability and excellent insulin secretion function after encapsulation and trans-Atlantic shipments have been demonstrated *in vitro*. Encapsulated human islets were then transplanted to diabetic nude mice to determine metabolic function and minimal mass required to achieve normoglycemia. For accurately evaluating the metabolic function, an immuno-incompetent nude mouse model was chosen to exclude the effects of rejection.

Human islets retained good viability (85%) after encapsulation. Good viability and function was expected, as omitting the polycation layer in the capsule simplifies the encapsulation process and is believed to cause minimal stress on the encapsulated islets. The maintenance of islet quality was also confirmed by both static and dynamic insulin secretion tests showing similar insulin response for islets that underwent the two trans-Atlantic shipments and encapsulation as those of control islets. Good human islet function *in vitro* after several shorter shipment times and encapsulation has previously been described by Sandler *et al* (20). Hence, our study confirms these findings, also for longer shipment times. In our *in vivo* study, diabetic nude mice were transplanted with different quantities of encapsulated islets. When using healthy islets, lower numbers are believed to be needed for achieving insulin independence than for non-healthy islets. There was no difference within the 3 groups (3,000, 5,000, and 10,000 IEQ) in terms of obtaining normoglycemia. Nevertheless, in the group of mice transplanted with 1,000 IEQ encapsulated human islets, 57% and 43% of the recipients returned to hyperglycemia after 55 and 160 days of transplantation, respectively. This indicates that 1,000 IEQ encapsulated human islets (=50,000 IEQ/kg) represent a minimal islet mass model when transplanting encapsulated human islets to STZ induced diabetic mice. The transplanted mice were able to control their blood glucose after a glucose challenge. However, the human islets responded as in humans and thus with lower glucose concentrations than for the control mice. No differences were seen between the groups on recovered islets or capsules from sacrificed mice. All capsules were found free of overgrowth with viable islets that responded in a static incubation assay.

It is important to note that in our previous study, transplantation of 1,000 IEQ of non-encapsulated human islets under the kidney capsule reverse diabetes in 50% of diabetic nude mice, and 2,000 IEQ usually renders all the transplanted mice normoglycemic. This indicates that despite the intraperitoneal location and the presence of the microcapsule, the encapsulated islets perform similarly to free islets. Other studies on encapsulated islets in mice have shown that a varying number of islets may be needed to normalize the blood glucose, depending on the source of the islets and whether the islets are syn-, allo-, or xenografts (27, 28). For human islets, 1,800 IEQ encapsulated islets have been shown to restore normoglycemia in one out of three Balb/c mice for 40 weeks in a study performed by Schneider *et al* (43).

In conclusion, this study shows that human pancreatic islets preserve the viability and function through encapsulation in inhomogeneous alginate-Ca<sup>2+</sup>/Ba<sup>2+</sup> beads and distant transportation. Furthermore, the shipment of human islets is not a limitation for overseas collaboration and combined efforts to provide a functional cure for diabetes through encapsulated islet transplantation.

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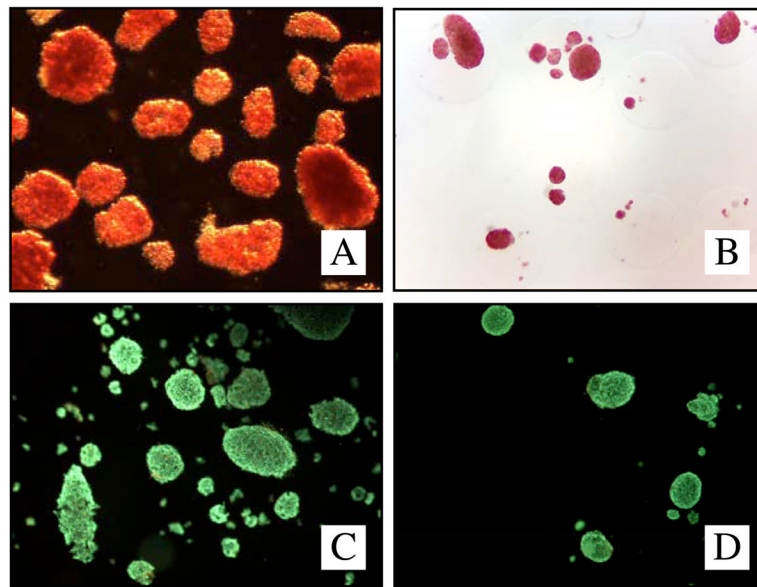
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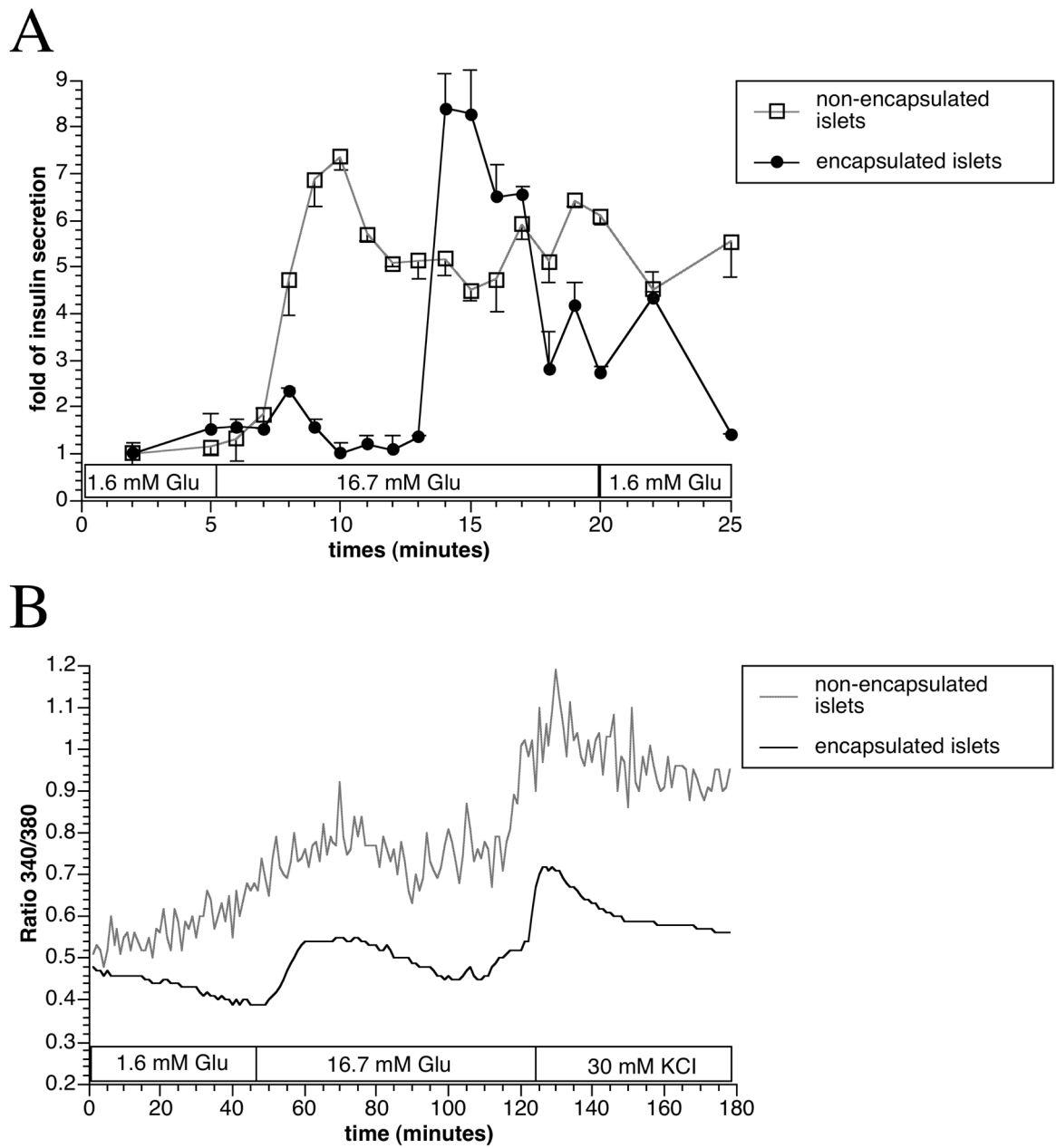


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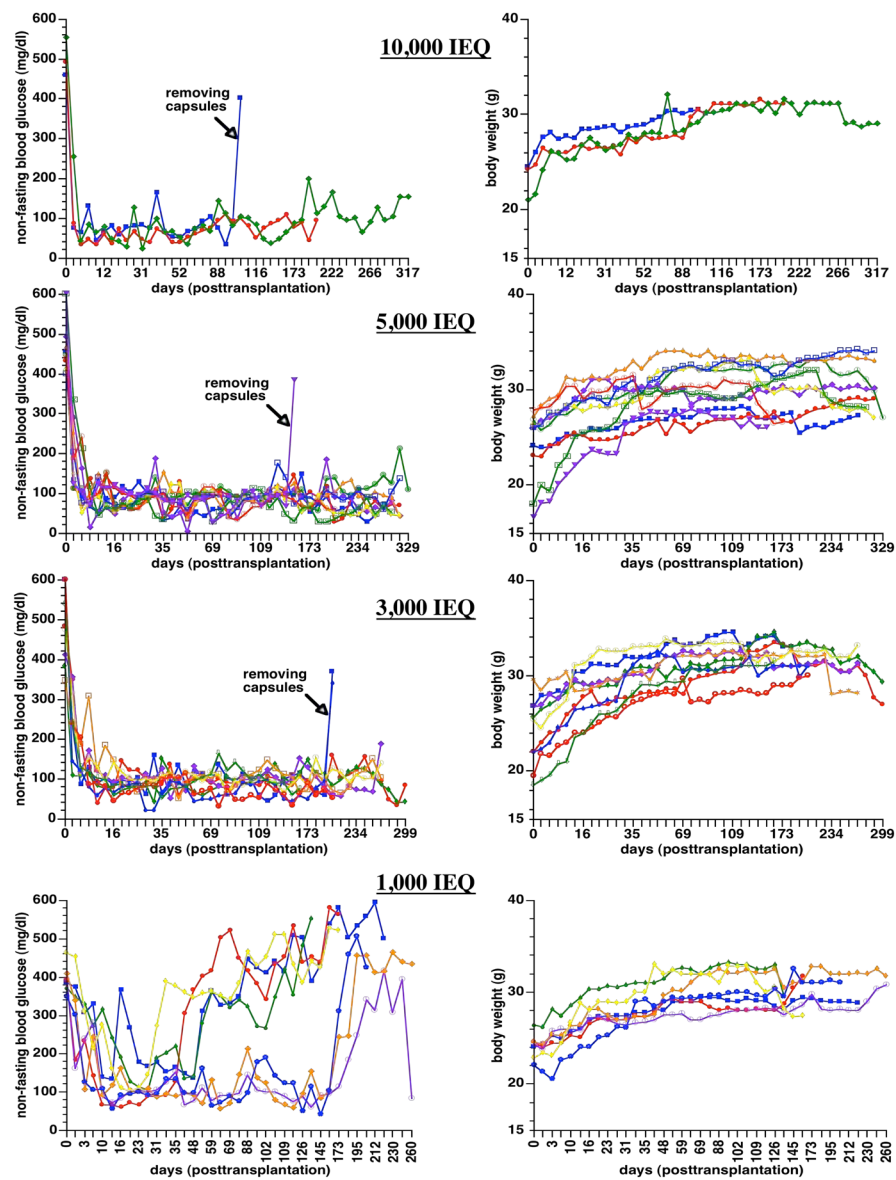
**Figure 1.** DTZ-staining non-encapsulated (A) and encapsulated human islets (B) (light-microscope, 40x). Viability staining (Sytogreen, green for alive cells; Ethidium bromide, red for dead cells) non-encapsulated (C) and encapsulated islets (D).



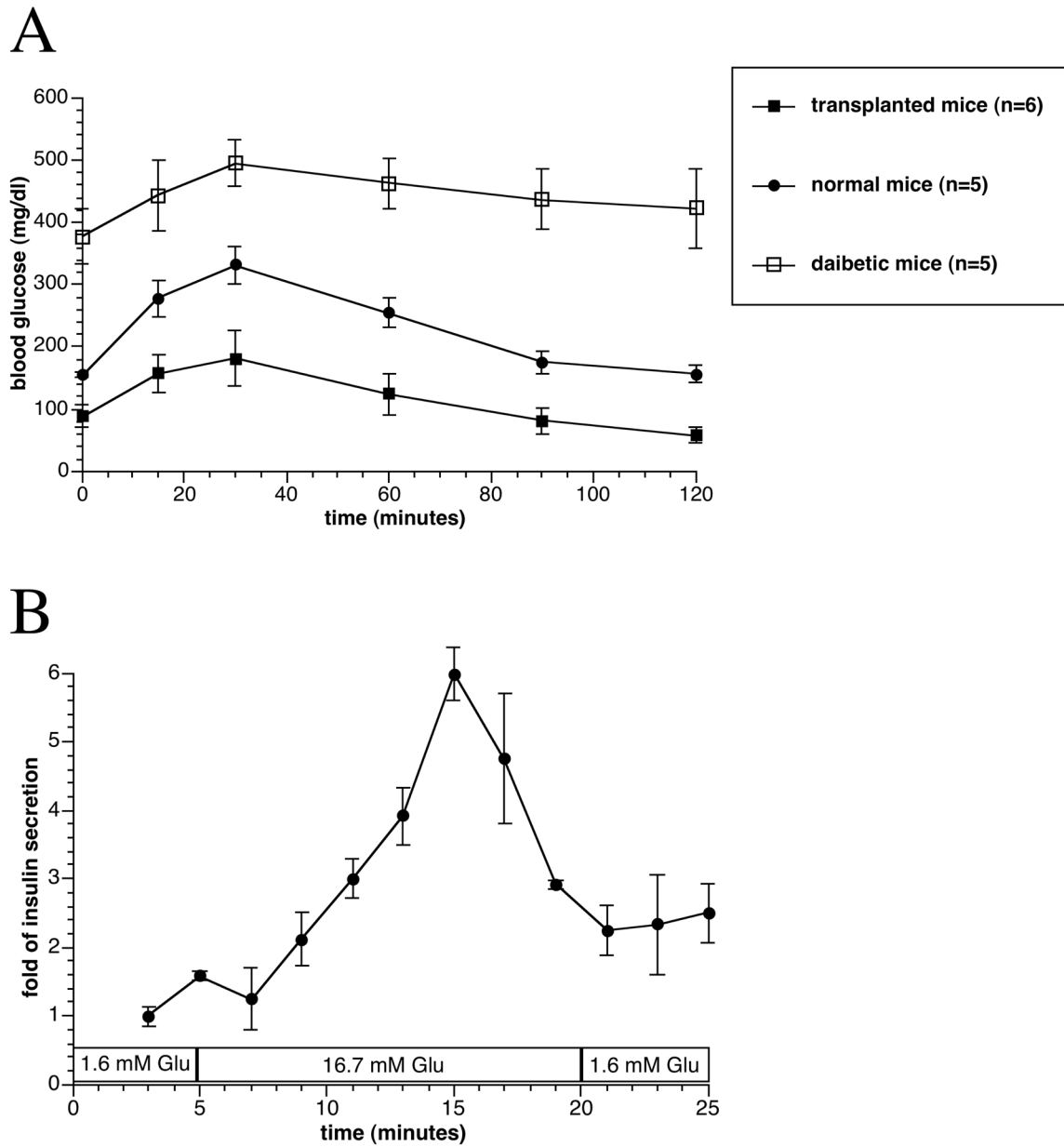
**Figure 2.** *In vitro* dynamic insulin secretion (A) and intracellular Ca<sup>2+</sup> measurement (B) with non-encapsulated control and encapsulated islets. Values were presented as mean ± SEM from three independent experiments.

**Blood glucose**

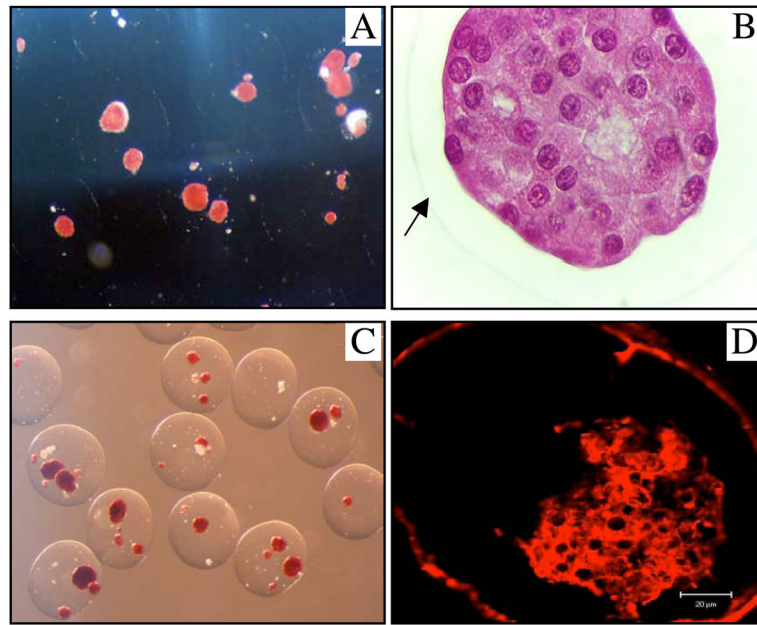
**Body weight**



**Figure 3.** Non-fasting blood glucose levels and body weights change in the STZ-induced diabetic nude mice transplanted with different amounts of encapsulated human islets (10,000, 5,000, 3,000, and 1,000 IEQ).



**Figure 4.** Oral glucose tolerance tests performed at 30 weeks after transplantation in the 3,000 IEQ encapsulated islets transplanted, diabetic non-transplanted, and normal control mice. Values were presented as mean  $\pm$  SEM (A). *In vitro* dynamic insulin secretion test of the retrieved capsules at 130 days after transplantation. Values were presented as mean  $\pm$  SEM from three different experiments (B).



**Figure 5.** Direct light microscopy photos with DTZ (A, 40 $\times$ ) and H&E (B, 400 $\times$ ) staining of the retrieved capsules at 130 days after transplantation. The arrow indicates the border of the capsule. DTZ (C, 40 $\times$ ) and insulin fluorescent stained (D, Confocal Microscopy) pictures of the retrieved capsules at day 329 after transplantation.

**Table**

Viability and stimulation index of the non-encapsulated control and encapsulated islets after shipment, encapsulation, and reshipment.

Group	Non-encapsulated islets	Encapsulated islets	P-value
Viability	88.3±2.5	87.5±2.7	P=0.30
Stimulation index	5.4±0.5	6.3±0.4	P=0.18