Viability and Functions of Alginate-microencapsulated Islets Isolated from Neonatal Pigs

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ABSTRACT : Patients with Type I diabetes mellitus have been treated with porcine insulin for several decades and pigs have recently been deemed an ideal source of microencapsulated islet cells for clinical xenotransplantation. In this study, neonatal pigs were anesthetized and sacrificed prior to a pancreatectomy. Islet cells were isolated from pancreas via collagenase digestion. Islet cells were separated and collected by hand under microscopic guidance. These cells were suspended in 1.4% sodium alginate solution and encapsulated by dropping them into 1.1% calcium chloride solution and in which the round gel in size was 250-400 μm in diameter. Viability of the microencapsulated islet cells cultured in medium at 37°C was assessed by MTT assay. Furthermore, insulin released in response to glucose challenge was investigated using an enzyme-linked immunosorbent assay. Secretion of insulin was low in response to the basal glucose solution (4.4 mM) in medium and was significantly higher in response to the high glucose solution (16.7 mM). The viability of microencapsulated islet cells did not differ significantly over a period of 7 days; that is, the increasing pattern of insulin concentration in the culture medium after glucose stimulation interval day was similar throughout the 7 days cultivation. In summary, experimental evidences indicated that the effects of alginate-microencapsulation prolonged survival of the neonatal porcine islets *in vitro* cultures and the insulin response to glucose of the islets was maintained. (**Key Words :** Neonatal Pig, Islet, Microencapsulation, Insulin, Insulin Secretory Responsiveness)

INTRODUCTION

Type I diabetes mellitus (IDDM) is characterized by the autoimmune destruction of insulin-producing cells in the pancreas. Present methods for treating IDDM, including insulin administration and antidiabetic drugs, are not effective in preventing adverse complications, especially long-term complications (Diabetes Control and Complications Trial Research Group, 1993). Replacing pancreatic endocrine tissue via islet transplantation is a physiological approach to particular restoration of hyperglycemia in IDDM patients (Narang and Mahato, 2006). Segmental pancreatic grafts obviate the need for exogenous insulin and postpone development of glomerular lesions in diabetic patients. However, the supply of donor organs is clearly a significant limitation of allotransplantation. To overcome this supply problem, microencapsulated islet cells from pigs are currently under

consideration for xenotransplantation (Shapiro et al., 2000; Ryan et al., 2001, 2002). The shortage of pancreases from human donors remains a primary obstacle to routine use of islet transplants as a treatment option (Brunicardi and Mullen, 1994). Furthermore, the desire to transplant microencapsulated islet tissue without need for immunosuppression has resulted in the development of immunoisolation devices (Lim and Sun, 1980; Rayat et al., 1999). Islets encapsulation is a frequently explored immunoisolation approach. For decades, IDDM patients have been using porcine insulin (Krickhahn et al., 2002). The pig is currently regarded as an ideal source for microencapsulated islet cells for clinical xenotransplantation (Rayat et al., 1999). Therefore, isolating viable and functional porcine islet cells is a critical issue.

Porcine pancreatic islets may be a potential source for islet xenografts for diabetic patients because of following reasons. Firstly, pig insulin has been used in humans for patients with diabetes mellitus for a long time (Krickhahn et al., 2002). Secondly, pigs breed rapidly, have large litter sizes, and have many morphological and physiological

similarities to humans (Ye et al., 1994). Thirdly, ethical problems may be smaller than any other species, because pigs are bred worldwide for meat. Finally, the risk of transmission of xenotic pathogens to human recipients may be reduced by breeding under specific pathogens free (SPF) conditions (Heald et al., 1996).

Adult porcine islets, however, have been difficult and expensive to obtain and with additional problem that, once isolated, adult porcine islets are fragile and difficult to maintain in culture (Marchetti et al., 1991; Kin et al., 2002). A more attractive source of tissue that does not have the technical problems is neonatal pigs. Neonatal porcine islets constitute a potential source of cells for clinical transplantation because of their inherent ability for proliferation and differentiation (Korbutt, 1996; Kin et al., 2005). Neonatal porcine islets were shown to consist of fully differentiated endocrine cells (35%) and endocrine precursor cells (57%) (Korbutt et al., 1996). Studies have demonstrated that neonatal porcine islets grafts grow and are able to reverse hyperglycemia after transplantation in immunoincompetent diabetic mice (Yoon et al., 1999; Rayat et al., 2000) and large experimental animals (Elliott et al., 2005a; 2005b; Kin et al., 2005; Cardona et al., 2006). Isolated islet cells derived from neonatal porcine pancreas are as a possible xenogeneic source for human transplantation. Although to date no one has succeeded in providing a significant alternative source of transplantable islets, progress in these areas has provided hope that islet transplantation will be the treatment of choice for people with IDDM in the future (MacKenzie et al., 2003; Cardona et al., 2006; Narang and Mahato, 2006). Therefore, the techniques and understands of isolation, encapsulation, and functions of neonatal porcine islets remain to be largely explored. Although, it has been demonstrated that porcine neonatal pancreatic islets are capable of secreting significant quantities of insulin in response to an *in vitro* glucose challenge (Brit et al., 1981; Archer et al., 1983), less studies have examined their survival and maintained potential or functional ability of encapsulated islets *in vitro* culture. Herein, effect of microencapsulation on the survival of neonatal porcine islets and insulin secretory activity in long-term and *in vitro* culture experiments were performed and discussed.

MATERIALS AND METHODS

Isolation of islet cells from neonatal pigs

Islets from neonatal pigs were isolated using protocols developed by Korbutt et al. (1996) and modified by Rayat et al. (2000). Briefly, neonatal Landrace×Yorkshire pigs (1.4-1.8 kg) were anesthetized and sacrificed prior to a pancreatectomy. The pancreas was sectioned into pieces (roughly 1-2 mm³) and placed in Hank's balanced salt

solution (HBSS) free of Ca^{2+} and Mg^{2+} (Gibco BRL, Grand Island, NY, USA), comprising 1.5 mg/ml collagenase type P (Boehringer Mannheim, Indianapolis, IN, USA), 10,000 U DNase I (Boehringer Mannheim), and 1 μmole/ml vitamin E (Sigma, St. Louis, MO, USA), for tissue digestion at 30°C for 20-40 min. The digested tissue was filtered using a mesh with a pore size of 2,000 μm and then washed several times in chilled HBSS. The filtered cells were stained by dithizone (Sigma) and islet cells were collected by hand-picked under a dissecting microscope. Mean number of islets in each of six diameter ranges of 60-350 μm was determined to convert the total yield into 150-μm diameter islet equivalents (IE) (Ricordi et al., 1990). At minimum 400 IE/experiment were purified by being handpicked and placed in chilled HBSS until microencapsulation. This study was approved by the institutional Animal Care and Use Committee of the Animal Technology Institute Taiwan.

### Microencapsulation of islet cells

Islet cells were suspended in 1.4% sodium alginate solution and droplets containing islet cells were produced (Garfinkel et al., 1998). The droplets of cells-containing alginate were poured into 1.1% calcium chloride solution to form a calcium alginate gel. The resulting microcapsules were washed with normal saline (NS), incubated with 0.05% poly-L-lysine (Sigma), washed again with NS, incubated with alginate, and washed a final time with NS. Alginate microcapsules (250-400 μm in diameter) were formed via this technique.

Viability determination

Viability of islet cells was determined by a colorimetric assessment of mitochondrial activity using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) as part of the CellTiter 96™ Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) (Han et al., 2005). Non-encapsulated (control) and alginate-encapsulated islets were cultured in basal cultured medium containing M199 (Gibco BRL) supplemented with 4.4 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 10% (v/v) fetal calf serum (FBS; Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured overnight in humidified air (5% CO_2, 95% air) at 37°C. During the test, samples of 20 IE/well were incubated in 24-well culture plates (Becton Dickson Labware, Franklin Lakes, NJ, USA).

Glucose stimulation of insulin secretion

To determine whether control and microencapsulated islets cells were glucose-responsive *in vitro* (i.e., able to respond to glucose by producing C-peptide), a static incubation assay was performed according to the procedure previously reported (Korbutt et al., 1996). The islets (20 IE
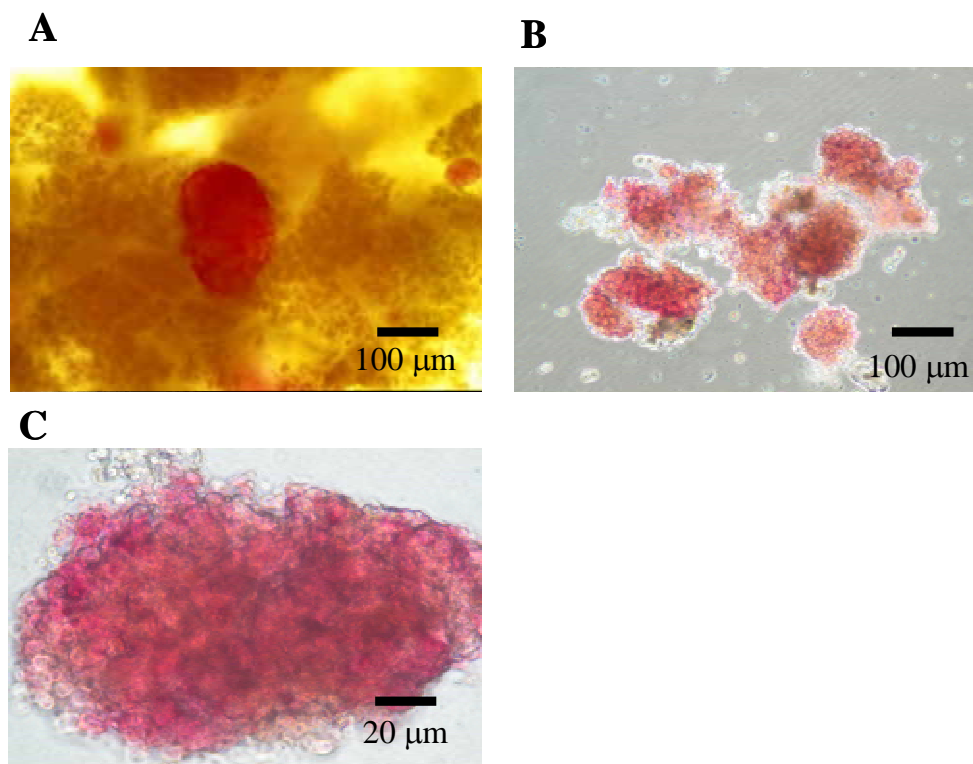
Figure 1. Identification of pancreatic islets by dithizone staining. Islets within the pancreas of neonatal pigs (A) and isolated pancreatic islets (B and C) are stained with dithizone.

in each treatment in a 24-well culture plate) were kept under basal culture condition (M-199 medium with 4.4 mM glucose, 37°C, and 5% CO_2). A glucose challenge with 16.7 mM glucose was performed at interval days and the culture medium was changed to basal cultured medium following the glucose challenge. The glucose challenge was performed as follows: (1) the basal cultivated medium of islets was changed to be modified Krebs-Ringer-bicarbonate (KRB) solution (pH 7.4) (Opara et al., 1992) containing 1% BSA, 10 mM nicotinamide and a basal 4.4 mM (80 mg/dl) glucose; (2) 1 h after treatment, this solution was replaced with the KRB solution containing 16.7 mM (300 mg/dl) for 30 min with sample collection at interval 10 min; and (3) following 16.7 mM glucose stimulation, the solution was changed to the basal cultivated medium.

Insulin levels in solution samples were measured as described previously (Zhang et al., 2001) using an enzyme immunoassay (Rat Insulin ELISA Kit; Crystal Chem Inc., Chicago, IL, USA) that identifies mouse insulin at 156 pg/ml-10,000 pg/ml. Stimulation indices of insulin secretory activity were calculated by dividing the amount of insulin released after 16.7 mM glucose treatment by that released at 4.4 mM glucose.

Statistical analysis

Results are expressed as means±standard deviation (SD)

of n independent observations. All statistical analyses were performed using SigmaStat version 2.0 for Windows. Statistical significance was determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The principal goals of this study were (i) to isolate and microencapsulate neonatal porcine islets and (ii) to determine whether these microencapsulated islets behaved in a glucose-responsive manner *in vitro*.

The islets existed in porcine pancreas were identified by dithizone staining (Figure 1A). Islet cells were isolated by collagenase digestion. Viable islets containing β-cells were stained deep red on dithizone staining (Figure 1B and 1C). These isolated islets separated from islet cells were encapsulated in the calcium-alginate gel. The diameter of microencapsules was 250-400 μm (Figure 2). These non-encapsulated and alginate-microencapsulated islets were applied to test *in vitro* glucose-insulin kinetics over a period of 9 days. Viability and glucose challenge (16.7 mM) tests were performed on days 1, 3, 5, 7 and 9 (Figure 3).

Figure 4 presents MTT assay results on islets in culture during the 9-day experimental period. Islet survival decreased significantly (0.82±0.10%; $p < 0.05$ compared
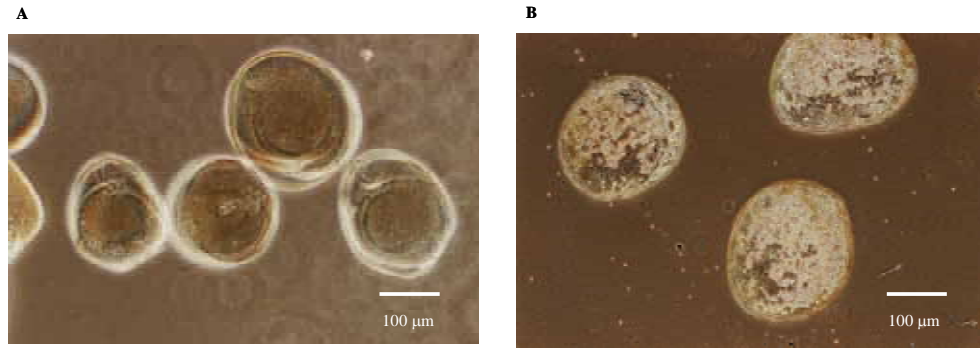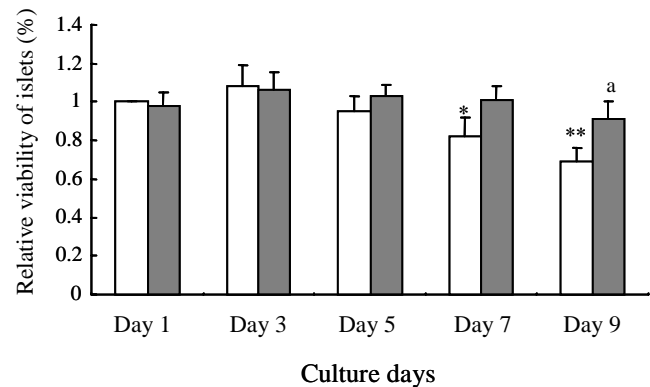
Figure 2. Light microscopic examination of calcium-alginate microencapsulated gel. A: without encapsulated cells; B: islet cells were alginate-microencapsulated. The round gel was 250-400 μm in diameter.
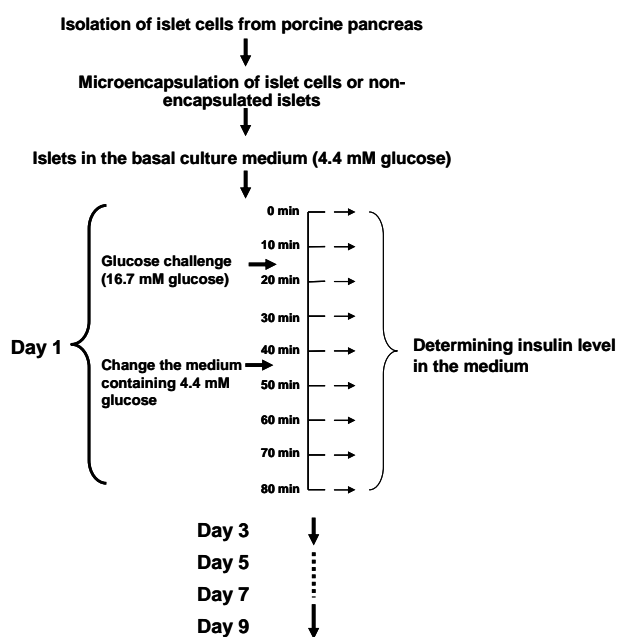
Figure 3. Scheme for determining *in vitro* glucose-insulin kinetics of isolated islets.

Figure 4. Results of the MTT assay performed on non-encapsulated islets (□) and alginate-microencapsulated islets (■) in the culture during the 9-day experimental period. Cell viability was determined on days 1, 3, 5, 7 and 9 of culturing with six independent experiments per each condition. Ratios to the mean in the group of non-encapsulated (control) islets at day 1 are shown. * $p<0.05$ and ** $p<0.01$ vs. the group at day 1, and [a] $p<0.01$ vs. the group of non-encapsulated islets.
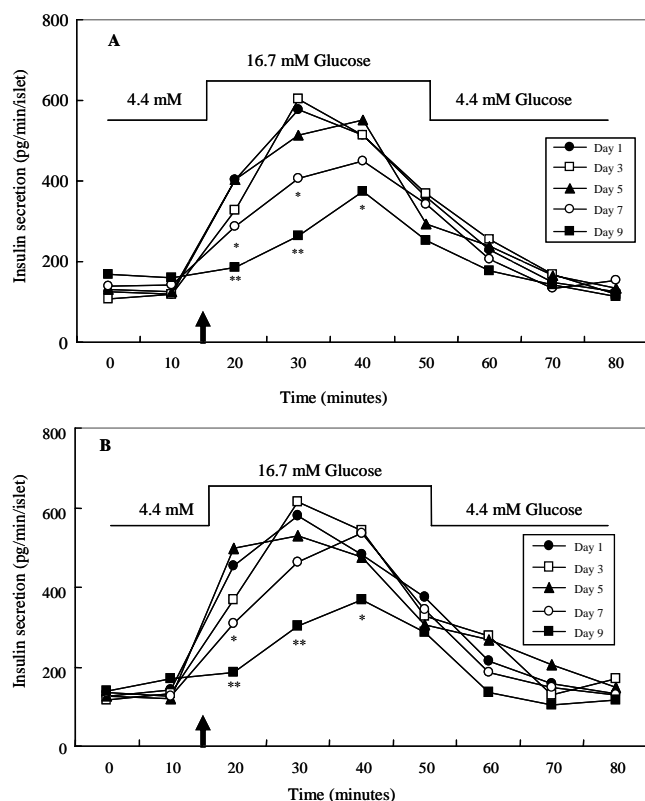
with that on day 1) when non-encapsulated neonatal porcine islets were cultured at 37°C for 7 days; when the culture period was extended to 9 days, survival of non-encapsulated islets was further reduced compared with that on day 1 ($0.69\pm0.07\%$; $p<0.01$). When islets were alginate-microencapsulated, however, islets loss during culturing was markedly lower and with survival exceeding 90% after 9 days of culturing. At culture day 9, viability of the alginate-microencapsulated islets was significant better ($p<0.01$) than that of non-encapsulated islets (Figure 4).

When islets were maintained in the basal culture medium containing 4.4 mM glucose, the medium insulin level was 106-168 pg/min/islet for non-encapsulated islets and 115-138 pg/min/islet for alginate-microencapsulated islets over a period of 9 days in the *in vitro* study. The insulin levels for non-encapsulated and alginate-microencapsulated islets were similar. Patterns of insulin secretory responsiveness to 16.7 mM glucose challenge were also similar for non-encapsulated islets (Figure 5A) and alginate-microencapsulated islets (Figure 5B) at days 1, 3, and 5. In the alginate-microencapsulated islets, glucose challenge resulted in a similar insulin secretion from day 1 to day 7 treatments. During the period, insulin secretory response of encapsulated islets was continuous for 10-15 min stimulation and returned to basal levels after perifusion with the low-glucose solution (Figure 5B). An approximate 4 to 5-fold increase from basal insulin secretion was noted. Glucose stimulation (30 min) of microencapsulated islet cells rose increased from a mean basal rate to a peak of 536-614 pg/min/islet and returned to the basal rate when glucose was returned to the basal level (Figure 4). Conversely, insulin secretion (stimulation indices of insulin secretory activity = 3.26 ± 0.33, $p<0.01$) was significantly lower on day 9 than that after glucose stimulation on days 1, 3, 5, and 7 (Figure 6). In the non-encapsulated islets, significantly

Figure 6. Stimulation indices of insulin secretory activity of non-encapsulated islets (□) and alginate-microencapsulated islets (■). Stimulation indices were derived by dividing the amount of insulin released at 16.7 mM glucose by that released at 4.4 mM glucose. * $p < 0.05$ and ** $p < 0.01$ vs. the parallel groups at day 1, and [b] $p < 0.01$ vs. the parallel groups of non-encapsulated islets.
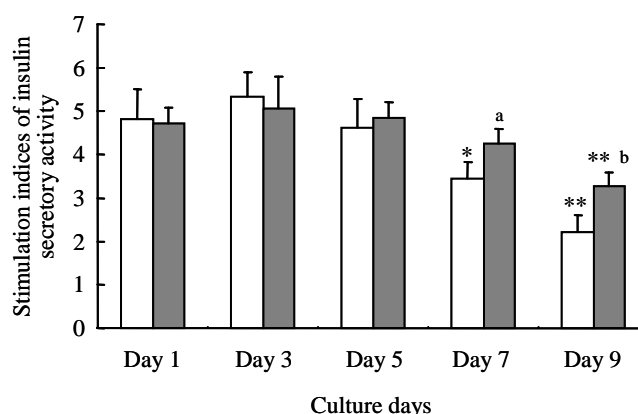
Figure 5. Insulin secretory activity of non-encapsulated islets (A) and alginate-microencapsulated islets (B) in response to 16.7 mM glucose challenge. Following a basal culture (4.4 mM glucose in the medium), the glucose concentration in the tested buffer was increased to 16.7 mM and after 30 minutes of glucose challenge, the glucose concentration was reduced back to basal level (the treatment in Figure 3). Insulin concentration in the tested buffer was measured by ELISA over a period of 9 days, with 16.7 mM glucose stimulation on days 1, 3, 5, 7 and 9. An increase in insulin concentration following glucose stimulation (arrow) is shown. Insulin release experiments were performed with four independent experiments for each condition. A significant difference (* $p < 0.05$, ** $p < 0.01$) compared with parallel values at day 1.

lower insulin secretory activity was observed after 7 days of culture than that on day 1. The stimulation indices of insulin secretory activity in the non-encapsulated islets were significantly lower at 3.42±0.41 ($p < 0.05$) at day 7 and 2.23±0.39 ($p < 0.01$) at day 9 compared with that on day 1. The stimulation indices in the alginate-microencapsulated islets were significant larger than those in the non-encapsulated islets at day 7 ($p < 0.01$) and day 9 ($p < 0.01$) (Figure 6).

DISCUSSION

In this study, we have followed the method developed by Garfinkel et al. (1998) to microencapsulate islets which were isolated from neonatal porcine pancreas. These

chelated alginate-microencapsulated islets could be cultured in the medium for 7 days without decreasing viability and maintained the function insulin recreation response to glucose challenge. The experimental result showed that the isolated islet cells could not growth, but maintained survival and ability of insulin production in the culture with nutrient solution at 37°C. This growth phenomenon was different from an insulin-producing cell line HIT-T15 (Schaffellner et al., 2005); however, similar to the islet cells isolated from dog (Korbutt et al., 2004), rat (Panza et al., 2000) and pig (Korbutt et al., 1996) pancreas.

The stimulation indices of glucose-stimulated insulin release of the microencapsulated porcine neonatal islets after exposure to 16.7 mM glucose were similar to the results reported by Korbutt et al. (1996) in porcine neonatal islets, higher than those reported in rat (Garfinkel et al., 1998) and porcine (Monroy et al., 1997) islets, and less than those reported in canine (Korbutt et al., 2004) as well as monkey (Balamurugan et al., 2004) islets. In fact, different strategies were preceded in these studies and a variety of factors concerning glucose-stimulated insulin release include species, cultural temperature, glucose concentration, method of islets isolation, etc. It was known that islets can release a very few insulin at 4.4 mM glucose *in vitro* perifusion assay (Panza et al., 2000). However, the released insulin in the porcine neonatal islets at 4.4 mM glucose was from 106 to 168 pg/min/islet in this study. We assumed that this discrepancy may lie in the differences between the procedures. In the present study, the islet cells were kept and cultured in a static incubation after the islets isolated from pancreas.

In the experiment of glucose responsiveness, the alginate-microencapsulated islets showed a slight delay of

insulin release in response to the change in 16.7 mM glucose solution as previous studies have demonstrated (Lim and Sun, 1980; Lacy et al., 1991). This delay response became more significant when the culture period was extended to 7 and 9 days. Moreover, the maximum insulin secretion was also significantly decreased in the day 7 and day 9 cultures. It could be proposed the extended culture induces islet cells injury causing passive discharge of insulin and ultimately an abnormal secretory capacity (Korbutt et al., 2004).

Experimental results in this study confirmed that collagenase digestion of neonatal porcine pancreas can recover an abundant yield of viable islet cells (Korbutt et al., 1996). Experimental data demonstrated that alginate-microencapsulated islets isolated from neonatal porcine pigs have a higher viability and insulin secretory responsiveness than non-encapsulated islets. These *in vitro* results were confirmed by a previous report that demonstrated improved survival of microencapsulated islets during *in vitro* long-term culturing (Korbutt et al., 2004). The effects of, and high survival and insulin secretory activity in, the microencapsulated islets may be due to microencapsulation providing an environment that mimics the anatomical three-dimensional pancreatic matrix structure, preventing islet deterioration and preserving survival and functional viability (Korbutt et al., 2004). Other studies have similarly determined that extracellular matrices have a significant role in inducing islet growth and differentiation (Beattie et al., 1996; Bonner-Weir et al., 2000).

CONCLUSION

This study isolated and microencapsulated pancreatic islet cells from neonatal pigs; these cells survived and remained functional for prolonged periods for 7 days by *in vitro* culturing. Alginate-microencapsulation prolonged survival of neonatal porcine islets in *in vitro* culturing and maintained islet insulin secretory responsiveness to glucose. The pancreatic islet cells isolated from neonatal pigs had an available and inherent capacity to be maintained *in vitro*. These cells can be considered for application in clinical transplantation for IDDM; however, many questions remain to be addressed.

ACKNOWLEDGEMENT

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