Canine Islet Isolation, Cryopreservation, and Transplantation to Nude Mice

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Background: Successful human islet transplantation has led to insulin independence in type 1 diabetes. Dogs constitute an animal model for preclinical studies. We present our recent experience in canine islet isolation, cryopreservation and transplantation.

Methods: Twenty-seven pancreases from mongrel dogs, weighing 9-31 kg, were removed. Each pancreas was digested with collagenase, and then purified by density gradients. Islet number and purity were counted, and the viability of isolated islets was assessed in vitro by static incubation, perifusion study and in vivo transplantation into nondiabetic or diabetic nude mice. Additionally, freshly isolated islets were cryopreserved for 1 week, and then studied in vitro.

Results: The islet yield and purity were $121,000 \pm 135,000$ IEQ per pancreas and $81.4 \pm 1.2\%$, respectively. The stimulation index (insulin release in 300 mg/dl glucose/insulin release in 100 mg/dl glucose) of the isolated islets was 6.6 ± 1.9 (N = 7), and first and second phases of insulin secretion were demonstrated during perifusion study. After 1-week cryopreservation, the islet number decreased from 1,000 to 540 (N = 1) and insulin content decreased from 50.95 to 39.23 μg/150 islets (N = 1). These islets maintained their insulin response to high glucose. Four weeks after transplantation, the grafts showed abundant β-cells and significant insulin content. Normoglycemia was achieved in 14 of 23 diabetic recipients after transplantation with 2,000 freshly isolated islets.

Conclusion: Canine islets isolated at our laboratory were viable and maintained their physiological function both in vitro and in vivo.
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Key words: diabetes, canine islet, islet isolation, islet cryopreservation, islet transplantation.

Progressive loss of insulin secretion caused by an autoimmune process that destroys pancreatic β-cells in genetically susceptible patients heralds a lifelong dependence on insulin therapy. Despite careful treatment, half of type 1 diabetic patients suffer from chronic neurovascular complications.

Evidence indicates that the microangiopathic lesions are secondary to imperfect control of glycemia. In 1993, the Diabetes Control and Complications Trial clearly showed that intensive insulin therapy with a 2% decrease in HbA_{1c} significantly reduced the risk of microvascular complications. But this improvement in glycemic control was associated with a 3-fold increased risk of severe hypoglycemia and could not completely avoid the chronic complications.[1]

Transplantation of insulin-secreting tissue offers a physiological approach to precise restoration of euglycemia. Human islet transplantation has led to insulin independence in type 1 diabetic patients. Recently, at the University of Alberta in Canada, Shapiro and Ryan et al. reported that 100% and 83% of type 1 diabetic recipients achieved insulin independence, respectively, without fatal postoperative complications after islet transplantation.[2,3] The excellent outcome encouraged many institutes, including ours, to involve in this field.

Dog is an animal model for preclinical studies because the characteristics and isolation methods of human and canine islets are very similar. Herein, we present our recent experience in canine islet isolation, cryopreservation, and transplantation that is important for further clinical islet transplantation.

MATERIALS AND METHODS

Animals

Islets were isolated from 27 mongrel dogs of either sex, weighing 9-31 kg. Male nude mice, aged 8-12 weeks, were used as the recipients of canine islet transplantation. Diabetes was induced in the recipients by a single intravenous injection of alloxan (90 mg/kg body weight).[4] Before transplantation, diabetes was confirmed by the presence of hyperglycemia, body weight loss, and polyuria.[5] Blood was obtained from the snipped tail, and glucose was measured with a portable glucose meter (One Touch II, Lifescan,. Milpitas, CA, USA).[5,6] Only those with a blood glucose level higher than 350 mg/dl were used for the transplantation experiment.

Islet isolation

A total pancreatectomy was performed with close attention to avoid duodenal ischemia through preservation of the recurrent duodenal branches of the gastroduodenal vessels and branches of the superior mesenteric vessels.[7] We used PE-90 or PE-50 plastic catheters, 15-20 cm long, to cannulate the right and left limbs of the pancreas separately through the pancreatic ducts at the junction of duodenum and pancreas. The harvested pancreas was preserved in ice University of Wisconsin (UW) solution and was immediately transferred to our laboratory for islet isolation.[2,3,7,8] All isolation procedures were conducted under the conditions of sterile laminar air flow. The cannulated pancreas was intraductally infused with cold collagenase type V (Sigma Immunochemicals, St. Louis, MO, USA). The fully infused pancreatic tissue underwent automated digestion in a Recordi chamber at 37°C for about 15 min.[2-3,9-10] The digested materials were thoroughly washed several times with large volumes of cold Hank's balanced salt solution (HBSS) with 10% fetal bovine serum (FBS) through centrifugation. The harvested islets were further purified by centrifugation on discontinuous gradients (Ficoll, Type 400DL, Sigma, St. Louis, MO, USA).[2-3,10] Finally, the number, purity, and viability of islets were determined by dithizone staining.

Cryopreservation and thaw

Isolated islets were cyropreserved using the established protocols.[11-13] After isolation, 300-500 islets were aliquoted into a freezing tube. They were suspended at 22°C in 0.2 ml of Medium 199 (GIBCO BRL, Grand Island, NY) which was supplemented with 25 mM HEPES, 10% fetal calf serum (v/v), penicillin (100 U/ml) and streptomycin (100 μg/ml), and the concentration of dimethylsulfoxide (DMSO) was added in a stepwise manner (0.67, 1.0, and 2.0 mol/l) over a period of 30 minutes. Samples were then supercooled to -7.4°C, nucleated, and slowly cooled at 0.25°C/minute to -40°C before storage in liquid nitrogen (-196°C). After 1-week cryopreservation, these islets were thaw rapidly at a rate of 200°C/minute. The intracellular DMSO was removed by the addition of 0.75 mol/l sucrose for 30 minutes (0°C) followed by stepwise addition of supplemented Medium 199 over a 30-minute period. Finally, thawed islets were cultured at 37°C for 24 hours, and then their function was studied by static incubation and insulin content.

Static incubation of isolated and cryopreservated islets

Islet preparations were examined for their secretory responsiveness to glucose during a static incubation.[4,14,15] Thirty islets were incubated for 120 minutes in RPMI-1640 medium supplemented with 100 mg/dl glucose, and then 300 mg/dl glucose. Stimulation indices were calculated by dividing the amount of insulin release at 300 mg/dl glucose by that at 100 mg/dl glucose.[15]

Perifusion of isolated islets

Details of the perifusion procedure have been reported previously.[16] In brief, after overnight culture, 150 islets with diameters of 75-250 μm were placed into a perifusion chamber composed of the barrel of a 1-ml plastic tuberculin syringe (TERUMO, Tokyo, Japan) and 2 layers of nylon net with 10-μm pores (Pharmacia, Uppsala, Sweden) placed in the bottom. RPMI-1640 medium gassed with 95% O_2/5% CO_2 was pumped through the chamber at a flow rate of 180 μl/min. The islets were perifused first with a medium containing 100 mg/dl glucose for 2 h to equilibrate the cell conditions, and then with medium containing 300 mg/dl glucose for 30 min. The perifusate was collected at 1-min intervals throughout the experiment, and the amounts of the insulin in all samples were measured with a radioimmunoassay method. The stimulation indices were calculated by dividing the amount of insulin released during the first phase at 300 mg/dl glucose by that released at 100 mg/dl glucose.

Islet transplantation

The transplantation procedure has been described previously.[5,6,15] On the day following islet isolation, 600 and 2,000 isolated islets were transplanted under the left kidney capsules of nondiabetic and alloxan-induced diabetic mice, respectively. To accomplish this, islets were centrifuged in PE-50 tubing (Clay Adams, Parsippany, NJ, USA) connected to a 200-μl pipette tip. With the mouse under amobarbital anesthesia, the left kidney was exposed through a lumbar incision. A capsulotomy was performed in the lower pole of the kidney, and the tip of the tubing was advanced under the capsule of the upper pole, the site of final injection. The capsulotomy was left unsutured. After transplantation, the recipient's blood glucose and body weight were measured twice a week in the first 2 weeks and then once a week. Normoglycemia was defined as a non-fasting blood glucose levels < 200 mg/dl.

Immunohistochemistry

The removed grafts were fixed in formalin solution and processed for paraffin embedding, sectioning, and immunoperoxidase staining. Sections of grafts were stained for endocrine β-cells with a rabbit anti-human insulin antibody (Dako Co., Ltd, USA).
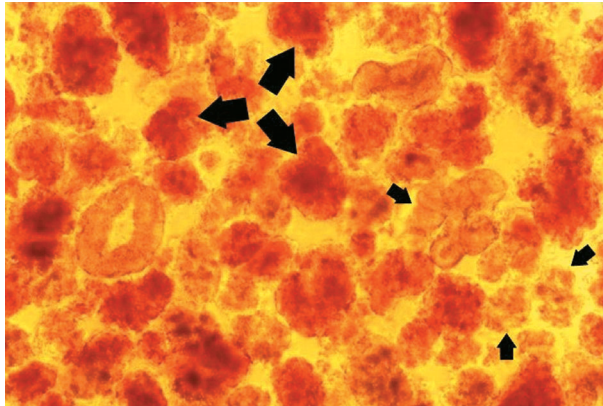
Insulin content

One hundred and fifty freshly isolated or cryopreserved islets were sonicated, and the removed graft-bearing kidneys were homogenized in acid ethanol. After sonication or homogenization, the samples were extracted overnight at 4˚C. On the following day, they were centrifuged at 2,400 rpm for 10 min (islets) or 30 min (kindeys), and the supernatant was stored at -20˚C. The pellet was again sonicated or homogenized in acid ethanol, and insulin was extracted overnight. After centrifugation, this second supernatant was added to the first extraction sample. Insulin was measured by radioimmunoassay using an INSI-PR kit (CIS US Inc., USA).
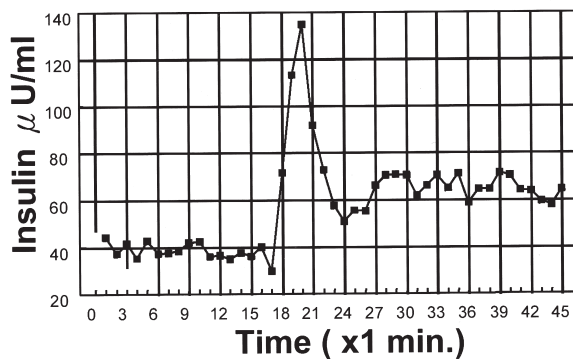
Statistical analysis

Results were expressed as mean±SD. For comparison, paired Student's t-test was employed. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The islets yield before and after purification was 250,200±341,800 and 121,000±135,000 IEQ per pancreas (i.e. 4,308±4,806 IEQ per gram of pancreatic tissue), respectively. The islets purity was 81.4±1.2%. In contrast to other non-endocrine cells, the isolated islets appeared red brown under dithizone staining (Fig. 1). The stimulation index (insulin release in 300 mg/dl glucose/insulin release in 100 mg/dl glucose) of the isolated islets assessed by static incubation was 6.6±1.9 (N = 7). In addition, the perifusion study demonstrated that insulin secretion of these islets responded well to high glucose stimulation. The insulin level at baseline was ~ 40 μU/ml with 100 mg/dl glucose perfusate. After switching perfusate to 300 mg/dl glucose at the 15th minute,

Fig. 1 Canine islet preparation after isolation. In contrast to non-endocrine cells (small arrows), the islet (large arrows) appeared red brown after dithizone staining.

Fig. 3 Islet graft at 4 weeks after transplantation with 600 islets into nondiabetic nude mice. The β-cells were stained with anti-insulin antibodies.
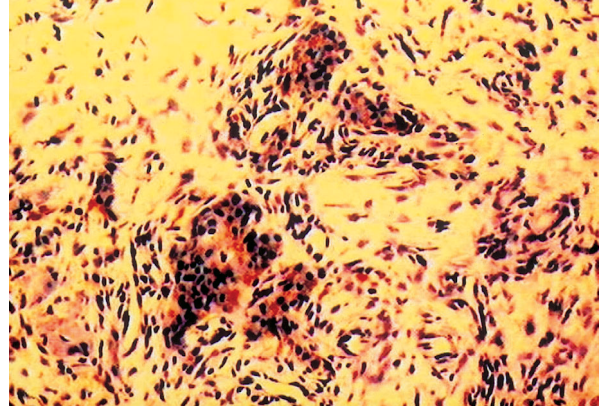

Fig. 2 Insulin secretion of isolated canine islets during the perfusion study. Baseline glucose of the perfusate was 100 mg/dl. It was switched to 300 mg/dl at 15th minute.
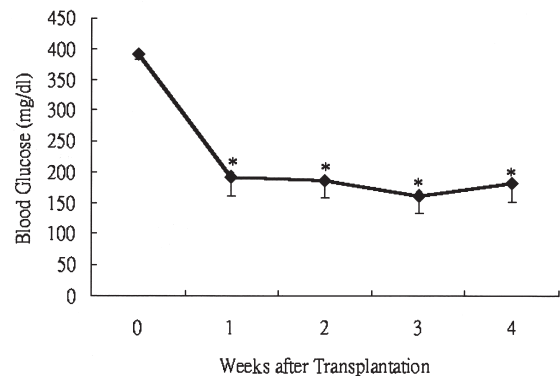

Fig. 4 Blood glucose changes of 23 diabetic nude mice after transplantation with 2,000 canine islets. Blood glucose decreased significantly throughout 4 weeks after transplantation (*$p < 0.0001$ vs. week 0).

the first phase of insulin secretion reached a peak level of ~ 140 μU/ml, followed by second phase of insulin secretion with a mean level of ~ 70 μU/ml (Fig. 2).

After 1-week cryopreservation, the islet number decreased from 1,000 to 540 (N=1) and insulin content decreased from 50.95 to 39.23 μg/150 islets (N=1), with the recovery rate of 54% and 77%, respectively. During static incubation, their insulin response to 300 mg/dl glucose was significantly higher than that to 100 mg/dl glucose (6.97 ± 0.70 vs. 3.98 ± 0.62 μg/30 islets/2h, N=4, $p < 0.02$).

Four weeks after transplantation with 600 islets

into nondiabetic nude mice, the grafts had abundant β-cells with insulin staining (Fig. 3), and their insulin contents were 4.9 and 7.7 μg (N=2). The mean blood glucose level of diabetic recipients was ~ 400 mg/dl at baseline, and it decreased significantly throughout 4 weeks after transplantation ($p < 0.0001$ vs. week 0) (Fig. 4). Moreover, normoglycemia was achieved in 14 out of 23 diabetic nude mice after transplantation with 2,000 islets.

DISCUSSION

There are 3 key elements for establishing a reli-

able islet-isolation system in large animals: 1) minimal warm and cold ischemic time; 2) minimal trauma on pancreas islets; and 3) exact control of the duration of enzyme digestion.[9] To meet these requirements, we preserved the blood supply of canine pancreas to minimize the warm ischemic time; preserved the harvested pancreas in ice UW solution[2,3,7,8] and immediately transferred to the laboratory for isolation to shorten the cold ischemia time; and used an automated method to prevent further mechanical trauma and enzymatic damage of the liberated islets. Totally, the islet preparations took 3 to 3.5 h on average after removal of the canine pancreases which is similar to other laboratory.[7]

In our study, the islet yield after purification was $4,308 \pm 4,806$ IEQ per gram of pancreatic tissue, which is slightly better than previous report of 3,638 IEQ per gram of pancreatic tissue.[9] To identify isolated islets in digested pancreatic tissue, we used dithizone staining, a zinc-chelating agent known to selectively stain pancreatic islets.[17] By using Ficoll gradients, our islet purity was 81.4 ± 1.2 %, which is between 63% reported by Kim et al.[7] and >90% reported by Chern et al.[9] The latter used Histopague as a diluent for Ficoll density gradients which has been shown to improve islet purity due to its property of low viscosity.[9,18] However, the function of islets isolated with or without Histopague was not different.[9] Islet perifusion is the most sensitive system for studying islet function in vitro.[19] The viability of our isolated canine islets was clearly demonstrated by the presence of first and second phase of insulin secretion. These islets responded well (threefold increase) to solely high glucose, which is more physiological than that stimulated by addition of 3-isobutyl-1-methylxanthine (IBMX) and/or carbachol.[9]

Cryopreservation and thaw of isolated islets provide several potential advantages: the ability to select islets based upon ABO or HLA phenotypes;[13] the permission to assess islet function and microbiological sterility;[20] and the time for the induction of recipient's unresponsiveness to the allogeneic islet graft.[21] After 1-week cryopreservation in UW solution with 2 M DMSO, there were 77% of islets recovered, consistent with previous reports.[13,21] The maintenance of the response to high glucose in these frozen-thawed islets indicates their insulin secretion remains intact following cryopreservation.[22]

Transplanting islets into nude mice is a reliable model to determine the islets viability in vivo.[10] We first transplanted 600 canine islets into nondiabetic recipients to demonstrate the viability of transplanted islets, and then 2,000 islets into diabetic recipients to further confirm their physiological function. At 4 weeks after transplantation, we observed abundant β-cells and significant insulin content in the grafts of both diabetic and nondiabetic recipients. More importantly, the physiological function of these islets was proven by the reversal of diabetes in recipients.

In summary, in this study, we demonstrated that canine islets isolated at our laboratory were viable and maintained their physiological function in vitro, after 1-week cryopreservation and at 4 weeks after being transplanting to nude mice.

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狗胰島細胞之分離、冷凍儲存及移植至裸鼠

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背 景： 人體胰島細胞移植已可使第一型糖尿病患者不需依賴胰島素。狗與人體胰島細胞的特性與分離方法類似，因此狗是人體試驗前最佳的研究模型。在此我們報告狗胰島細胞分離、冷凍儲存及移植的經驗。

方 法： 我們摘取27隻體重介於9~31公斤離種狗的胰臟，以collagenase消化後，再用比重分層來純化胰島細胞。我們計算胰島細胞數目及純度，細胞活性在離體使用葡萄糖靜態培養及周流來評估其胰島素分泌，在體內則將其移植至糖尿病及非糖尿病裸鼠作研究。此外，我們將剛分離的狗胰島細胞冷凍儲存一週後進行離體研究。

結 果： 我們得到的胰島細胞數目及純度分別是每個胰臟121,000±26,000 IEQ及81.4±1.2%。其在300 mg/dl比100 mg/dl葡萄糖所釋放胰島素量之刺激指數為6.6±1.9 (N=7)。此外，這些細胞在周流研究顯現第一相和第二相的胰島素分泌。解凍後胰島細胞數目由1,000降至540 (N=1)，胰島素含量由50.95減為39.23 μg/150個胰島細胞 (N=1)，但其在300比100 mg/dl葡萄糖靜態培養明顯表現胰島素反應 (6.97比3.98 μg/30胰島細胞/2小時，$p<0.05$)。移植四週後，移植體可見豐富的β細胞，且含胰島素。植入2,000個胰島細胞至糖尿病裸鼠，則23隻中有14隻的血糖降至正常。

結 論： 我們實驗室分離的狗胰島細胞具活性，且在離體及動物體內保有其生理功能。
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關鍵字：糖尿病，狗胰島細胞，胰島分離，冷凍儲存，胰島移植。