

Original Article

Caprine pancreatic islet xenotransplantation into diabetic immunosuppressed BALB/c mice

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Abstract: Background: Type 1 diabetes mellitus is a devastating disease for which there is currently no cure, but only lifetime management. Islet xenotransplantation is a promising technique for the restoration of blood glucose control in patients with diabetes mellitus. The purpose of this study was to explore the potential use of caprine (goat) islet cells as xenogeneic grafts in the treatment for diabetes in a mouse model.

Methods: Caprine pancreases were harvested and transported to the laboratory under conditions optimized to prevent ischemia. Islets were isolated, purified, and tested for functionality. Caprine islets (2000 islet equivalent) were transplanted beneath the kidney capsules of diabetic BALB/c mice under thalidomide-induced immunosuppression. Blood glucose and insulin levels of grafted mice were evaluated by glucometer and enzyme-linked immunosorbent assay kit, respectively. The functionality and quality of caprine pancreatic islet grafts were assessed by intraperitoneal glucose tolerance tests.

Results: The viability of purified islet cells exceeded 90%. Recipient mice exhibited normoglycemia (<11 mM glucose) for 30 days. In addition, weight gain negatively correlated with blood glucose level. The findings verified diabetes reversal in caprine islet recipient mice. A significant drop in non-fasting blood glucose level (from 23.3 ± 5.4 to 8.04 ± 0.44 mM) and simultaneous increase in serum insulin level (from 0.01 ± 0.001 to 0.56 ± 0.17 µg/l) and body weights (from 23.64 ± 0.31 to 25.85 ± 0.34 g) were observed ($P < 0.05$). Immunohistochemical analysis verified insulin production in the transplanted islets.

Conclusions: Purified caprine islets were demonstrated to successfully sustain viability and functionality for controlling blood glucose levels in an immunosuppressed mouse model of diabetes. These results suggest the use of caprine islets as an addition to the supply of xenogeneic islets for diabetes research.

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Key words: caprine islets – streptozotocin-injected mice – type 1 diabetes – xenotransplantation

Abbreviations: DAB, 3,3'-diaminobenzidine; DTZ, dithizone; FDA-PI, fluorescein diacetate hydrolysis-propidium iodide; HRP, horseradish peroxidase; IEQ, islet equivalent; IPGTT, intraperitoneal glucose tolerance test; PBS, phosphate-buffered saline; STZ, streptozotocin

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Introduction

In type 1 and some cases of type 2 diabetes, hyperglycemia is a consequence of β -cell mass deficiency in the pancreas. Type 1 diabetes is a T-cell-mediated autoimmune disease, which results from the selective destruction of insulin-producing pancre-

atic islet β -cells. The β -cell destruction is believed to be mediated through the actions of CD4 and CD8 T lymphocytes, B lymphocytes, macrophages, and dendritic cells [1]. Pancreas transplantation is a way to reverse diabetes in patients with insulin-dependent diabetes. However, transplantation of isolated islets offers advantages over whole

pancreas transplantation, such as surgical simplicity and the ability to reduce the immunogenicity of islets by immunoalteration or immunoisolation protocols [2–4]. Pancreatic islet replacement could potentially compensate for the lack of β -cells and reverse the metabolic problems caused by insulin-dependent diabetes mellitus in human and animals [5]. In addition, islet transplantation could prevent early damage caused by hyperglycemia such as microangiopathic expansion [6].

Thalidomide (α -N-phthalimido glutarimide) is a glutamic acid derivative that is used for the treatment for various inflammatory and autoimmune diseases such as multiple myeloma, leprosy, systemic lupus erythematosus, and rheumatoid arthritis [7,8]. Thalidomide inhibits tumor necrosis factor- α in monocytes and macrophages and has an immunomodulatory function [8–10]. New analogs of thalidomide have been developed, which exhibit low toxicity and enhanced potency in blocking cytokine production [11]. Thalidomide has also been shown to suppress I κ B kinase activity in T lymphocytes, which results in reduced nuclear factor- κ B activation [7]. Chen et al. [12] used thalidomide as an alternative immunomodulatory drug in islet xenotransplantation into immunocompetent mice to protect the islet grafts from microvascular injury and improve their survival and function.

A known obstacle to islet transplantation therapy is the shortage of human donor pancreases. The use of non-human islets can be a solution to this, and some research teams have developed porcine islet preparation protocols [13,14]. However, the fragility of adult porcine islets is a concern; they can be easily fragmented during isolation and purification, a less noticeable occurrence in islets from other species [15–18]. The fragility of porcine islets can also lead to loss during culture, immunoalteration procedures, cryopreservation, and banking [19,20].

The pancreatic gland of goats is readily obtainable, and islets isolated from caprine pancreases were less fragmented as determined by comparison of islet size in intact pancreas sections and isolated islets [21]. To our knowledge, this is the first study to evaluate the potential use of caprine islets for islet transplantation therapy in diabetes. This study assessed the in vivo function of caprine pancreatic islet grafts in streptozotocin (STZ)-induced diabetic immunosuppressed mice, following the in vitro evaluation and characterization of the viability and function of the isolated islets. The in vivo findings supported the use of caprine islets for diabetes reversal in STZ-induced diabetic murine models.

Materials and methods

Preparation of islets

Islets from five male Kajang goats pancreata were used. The means of the donor characteristics were as follows: age 12 ± 2 months, weight 16 ± 3 kg, pancreas weight 22.6 ± 1.3 g. Caprine pancreases were collected from a slaughterhouse and transferred to the laboratory with care to minimize ischemic time (10 min if warm and 90 min if cold). Isolation and purification of islets were carried out as described previously [21]. Briefly, the caprine islets were isolated by collagenase XI-S then being purified/selected by Ficoll density gradients and wire mesh. Viability and functionality of the isolated islets were assessed by dithizone (DTZ) staining, fluorescein diacetate and propidium iodide (FDA-PI) staining, and the glucose-stimulated insulin secretion test. In vitro DTZ and FDA-PI staining were conducted on purified caprine islets to evaluate purity, viability, and apoptosis using the scoring system devised by Karaoz et al. [22]. In brief, purified islets were placed into 1 of 5 categories according to the percentage of viable cells (estimated from green/red staining). Isolated islets with a purity of $>90\%$ and a viability of $\geq 95\%$ were considered as qualified for xenotransplantation. The glucose-stimulated insulin secretion test was used to assess the functionality of islets exposed to different concentrations of glucose. The islet equivalent (IEQ) of islet preparations was determined with the Ricordi Algorithm, and 2000 IEQ were placed into separate petri dishes for transfer to recipients.

The animal care and use committee of the faculty of veterinary medicine at the Universiti Putra, Malaysia, approved the animal research proposal, under the AUP number 10R102/June 2010–May 2011.

Diabetes induction with streptozotocin

Diabetes was induced in BALB/c mice with an STZ (Sigma-Aldrich, St. Louis, MO, USA) solution prepared immediately prior to injection. One high-dose STZ injection (200 mg/kg) was administered intraperitoneally (i.p.) to each mouse, which had been fasted for at least 4 h prior to injection [23,24]. The blood glucose level of each mouse was measured with Accu-Chek Performa test strips and meter (Roche, Indianapolis, IN, USA) twice a day. Hyperglycemic mice (>18 mm glucose) were targeted for urine glucose evaluation using Accu-Chek Diabur-Test 5000 strips (Roche, Mannheim, Germany). A urinary glucose-positive test (glucose

level \approx 278 mM) was considered as the verification of diabetes in STZ-induced mice.

Male BALB/c mice were divided into five experimental groups: normal control (NC, n = 5), STZ-induced diabetic (DC, n = 5) mice, diabetic graft recipients that were immunosuppressed with thalidomide (GDT, n = 10), diabetic graft recipients that were not immunosuppressed (GD, n = 5), and diabetic mice with sham grafts (phosphate-buffered saline [PBS] injected) that were immunosuppressed (SDT, n = 5). Mice were housed for over 1 month in clean and sterile individual cages with sterile bedding and unlimited access to sterile water and food.

Diabetes induction assessment

Blood glucose levels were measured daily in STZ-induced diabetic mice. Mice with non-fasting blood glucose levels higher than 18 mM for five consecutive days and a positive urinary glucose test were considered diabetic (Table 1).

Immunosuppressive drug treatment of mice prior and after islet xenotransplantation

Thalidomide is immunosuppressive at a dose of 200 mg/kg body weight [25]. Thalidomide was dissolved in dimethylsulfoxide (DMSO) and injected i.p. in treated mice (islet recipients, sham controls) daily. Thalidomide treatment was begun at least 3–5 days before islet transplantation and continued until the end of the experiments.

Islet xenotransplantation procedure

Recipient mice (body weight, 23–25 g) were anesthetized with a cocktail of ketamine and diazepam (100 and 5 mg/kg, respectively) [26]. The left sides of the mice were shaved, and a small incision was made to access the kidneys. The kidney was located by touch through the skin of the anesthetized mouse. Caprine islets were transferred beneath the kidney capsule through tubing, and the incision at the kidney capsule was cauterized and cleaned with an antibiotic-soaked cotton swab. The peritoneal wall incision was sutured and wound clips were applied at the dermal layer [27,28].

Table 1. Experimental mice biodata

Number	Sex	Age (week)	Weight (g)	STZ (mg/kg)	Blood glucose (mM/l) before STZ	Blood glucose (mM/l) after STZ
25	Male	7	24.14 \pm 0.94	200	7.1 \pm 0.83	23.3 \pm 5.4

Body weight, STZ induction dosage, and blood glucose level before STZ injection at the beginning of experiment and after STZ injection before islets transplantation. All data are showed in mean \pm SE. STZ, streptozotocin.

Islet graft monitoring

Non-fasting blood glucose levels post-transplantation were measured four times a week; body weights and urinary glucose were measured twice a week. The blood glucose level of each mouse was measured using an Accu-Chek glucometer (Accu-Chek Performa; Roche) as described above, starting 1 day after transplant for 4 weeks. Urine glucose was determined using Accu-Chek Diabur-Test 5000 strips (Roche).

Intraperitoneal glucose tolerance test

Two weeks after transplantation, recipient mice and control groups were subjected to intraperitoneal glucose tolerance tests (IPGTT). One bolus of glucose (2 g/kg body weight) was injected i.p. after a 16-h fast. Tail vein blood samples were collected at 0, 15, 30, 60, and 120 min post-glucose loading. Blood glucose levels were measured as described. Blood samples with glucose levels \geq 30 mM were diluted with blood from non-diabetic mice, remeasured, and the blood glucose recalculated [29,30].

Histological procedures on islet grafts

Thirty days after transplantation, the islet-grafted kidneys were retrieved and rinsed with PBS. After fixing in Bouin's solution and embedding in paraffin, serial sections of 4 μ m thickness were cut by microtome and spread on glass slides. Tissue sections were deparaffinized, washed twice with PBS for 3 min each and then transferred to 0.1% trypsin in PBS to retrieve antigens for 15 min at 37 °C. Slides were then washed in PBS twice, for 3 min each. Slides were blocked with a dual endogenous enzyme block for 10 min, washed twice with PBS, and incubated with rabbit monoclonal anti-sheep insulin antibody (1 : 2000 in blocking buffer; Abcam, Cambridge, UK) at 4 °C in a humidified atmosphere overnight. The slides were washed twice in PBS. Peroxidase-conjugated goat anti-rabbit antibody (Envision™ system + horseradish peroxidase (HRP) DAB + Rb/Mo Kit; DAKO, Glostrup, Denmark) was added and allowed to react for 30 min at room temperature. The slides were washed twice and 3-3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution was

added and incubated for 10 min. The reaction was stopped by washing the slides with distilled water. The slides were counterstained with hematoxylin.

Statistical analysis

All data are presented as means \pm SEM and analyzed using SPSS version 20.0 (IBM Corporation Armonk, NY, USA). The Student's *t*-test, one-way ANOVA, and least significant difference tests were used for statistical comparison between experimental and control groups. The significance level was set at $P < 0.05$.

Results

In vitro viability and functionality of caprine islets

Under FDA/PI staining, viable islet cells appeared green when examined under a fluorescent microscope, while dead and non-viable islets appeared red. When using this method to assess the quality of islets after the isolation procedure, the viability of caprine islets was approximately 95% (Fig. 1). Caprine islets isolated from different pancreas samples had a mean IEQ of $11\,9073 \pm 17\,814$.

The purified islets responded to variations in glucose concentration with a proportionate release of insulin. High glucose (25 mM) stimulated greater insulin secretion into the medium ($0.90 \pm 0.21 \mu\text{g/l}$); lower glucose (1.67 mM) stimulated less insulin secretion ($0.54 \pm 0.15 \mu\text{g/l}$; Fig. 2). This was considered evidence of functional β -cells.

In vivo functionality of caprine islet grafts

Before islet transplantation and at the end of the experiment, the serum insulin levels of the grafted

mice were measured using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). The insulin level of the grafted mice ($0.56 \pm 0.17 \mu\text{g/l}$) was significantly higher than the insulin level of the thalidomide-injected mice before islet transplantation and that of the diabetic ungrafted groups ($0.01 \pm 0.001 \mu\text{g/l}$; Fig. 3). This observation demonstrated that caprine islet grafts were functional and able to secrete insulin in response to glucose in STZ-induced diabetic immunosuppressed BALB/c mice.

A rapid rejection was noted in the grafted mice that were not treated with thalidomide, and blood glucose values increased dramatically in 2–4 days (Fig. 4). Some died of severe diabetes with blood glucose values exceeding 30 mM. In contrast, the thalidomide-treated grafted mice exhibited euglycemia for the 30 days of observation.

Blood glucose levels (non-fasting) were monitored four times per week in experimental and control groups until the end of the experiment. As shown in Fig. 4, mean blood glucose levels were significantly different between groups: grafted diabetic thalidomide-treated mice (GDT) and normal control mice (NC) versus sham-grafted diabetic thalidomide-treated mice (SDT) and diabetic control mice (DC), and also within the grafted diabetic group (GD). Figure 4 shows the mean blood glucose levels of all groups (GDT, $n = 10$), GD ($n = 5$), SDT ($n = 5$), DC ($n = 5$), and NC ($n = 5$) over 1 month. Normoglycemia (≤ 10 mM glucose) was achieved at 3–5 days after transplantation and was maintained for the observation period of 30 days in the GDT group. Mean blood glucose levels were 6.8 and 9.7 mM for the NC and GDT groups, respectively, and 28.1 and 29.3 mM for the SDT and DC groups, respectively. However, in the GD group, the mean blood glucose level decreased

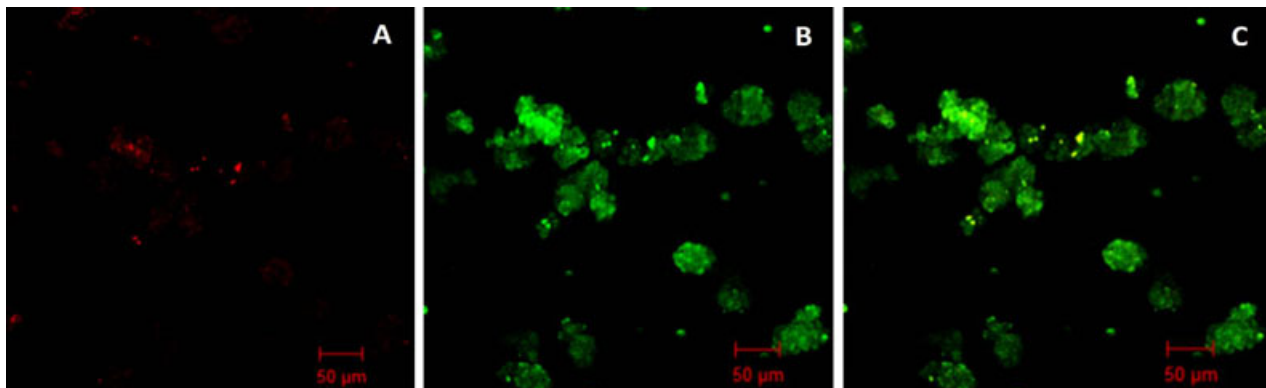


Fig. 1. Cell viability assessment. Fluorescein diacetate and propidium iodide (FDA/PI) staining was performed for determination of cell viability in normal pancreatic islet. The small bright spots in the fluorescent micrographs correspond to the intense red staining of PI-positive (dead) cells (A). The widely stained green regions are related to the FDA-stained (live) cells (B). Merged images of PI and FDA staining (C), (scale bars = 50 μm).

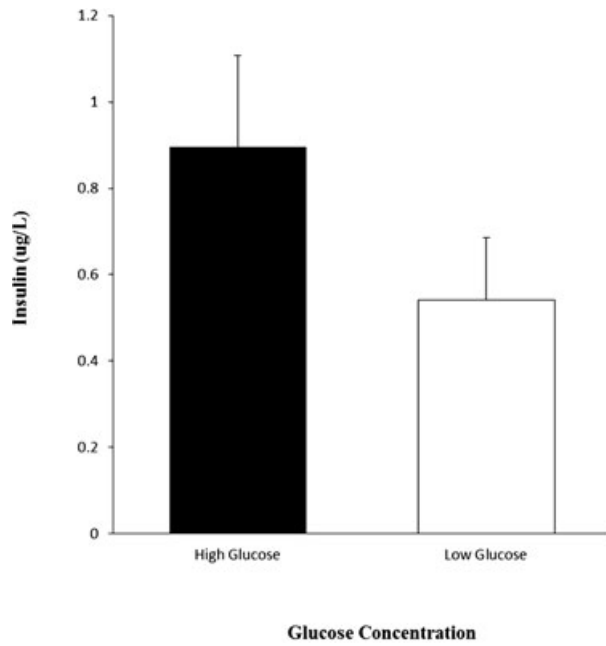


Fig. 2. In vitro insulin secretion of caprine islets in response to glucose challenge. The three replicates of 100 purified islets from each batch of islet isolation were exposed to 25 mM (high glucose) for 1 h and, respectively, to 1.67 mM glucose (low glucose) for another hour. Values are means \pm SEM. Cultured islets showed a good response for insulin secretion to different glucose concentrations.

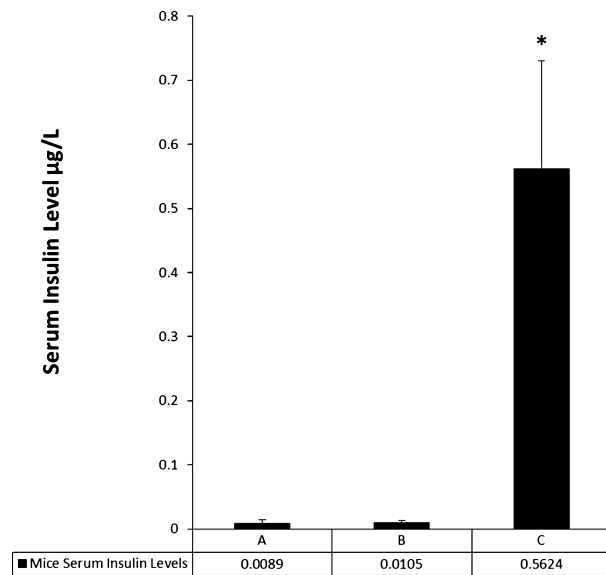


Fig. 3. Insulin secretion of diabetic mice. Insulin levels in diabetic control mice without islet graft, diabetic mice before islet transplantation (“A” and “B” columns) and also after islet transplantation (“C” column). All data represent the means \pm SEM, and significance was tested using ANOVA with LSD post hoc test, n = 10 mice. (P < 0.05).

until day 4 after transplant (to 11.18 mM), but subsequently increased (to 31.2 mM), which was diagnosed as islet graft rejection. The mean blood

glucose level in the GDT group was about 67% lower than in the SDT and DC groups (P < 0.05).

The mean body weights of the NC and GDT groups increased continuously for 4 weeks after transplantation. However, the mean body weight of the DC group started to decline at week 1 and was significantly (P < 0.001) lower than that of the NC group from 1 to 4 weeks after STZ administration (Fig. 5). Correlation analysis showed that body weight was negatively correlated with blood glucose levels in the GDT group (r = -0.582). Body weight was also negatively correlated with blood glucose levels in the DC and SDT groups (Fig. 5). Water intake in the DC and GD groups increased profoundly (not shown here).

Caprine islet recipients showed improved glucose tolerance

Figure 6 shows the IPGTT profile of the GDT and control groups. IPGTTs were performed on four groups of mice at the second week after transplantation (n = 5 per group). Mean fasted blood glucose levels for the GDT, NC, SDT, and DC groups were 8, 6.92, 29.42, and 32.28 mM, respectively. The GDT and NC groups responded to the glucose challenge in a similar fashion with mean blood glucose levels peaking at 15 min and quickly dropping to near baseline within 60 min. This demonstrates that the islet grafts preserved their capacity to regulate blood glucose levels (Fig. 6).

The mean fasted blood glucose levels for the GDT, NC, SDT, and DC groups were 12.4, 12.67, 42.8, and 40.9 mM, respectively. The mean values for the GDT and NC groups were significantly lower than those for the SDT and DC groups (P < 0.001) at 120 min.

Islet xenograft histology

Histological examination of islet grafts removed from the GDT group at day 30 showed that some caprine islets were remained viable. Immunostaining of islet graft sections from the GDT group was positive for insulin (Fig. 7). The insulin-positive cells represent a small fraction of islets graft.

Discussion

The major obstacle to successful xenotransplantation is the immunological incompatibility between donor and recipient. In the present study, we found that the transplantation of isolated and purified caprine islets, in combination with thalidomide treatment, reversed diabetes in STZ-induced diabetic BALB/c mice as evidenced by increased serum insulin and restoration of euglycemia. These

Fig. 4. Caprine islet graft effect on glycemic control in diabetic mice. Non-fasting blood glucose levels of GDT (n = 10), NC (n = 5), SDT (n = 5), DC (n = 5) and GD (n = 5) groups over experiment period. All data are represented the means \pm SEM. GDT, grafted diabetic thalidomide-treated mice; NC, normal control mice; SDT, sham-grafted diabetic thalidomide-treated mice; DC, diabetic control mice; and GD, grafted diabetic group. ("Died" represents all mice).

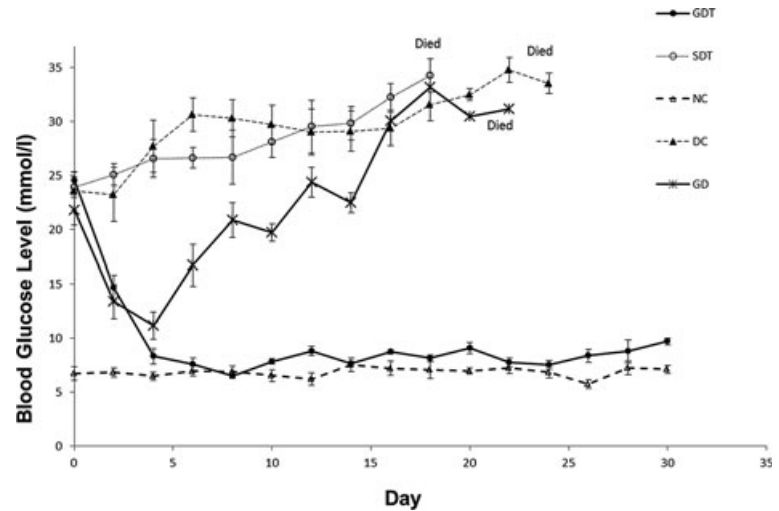
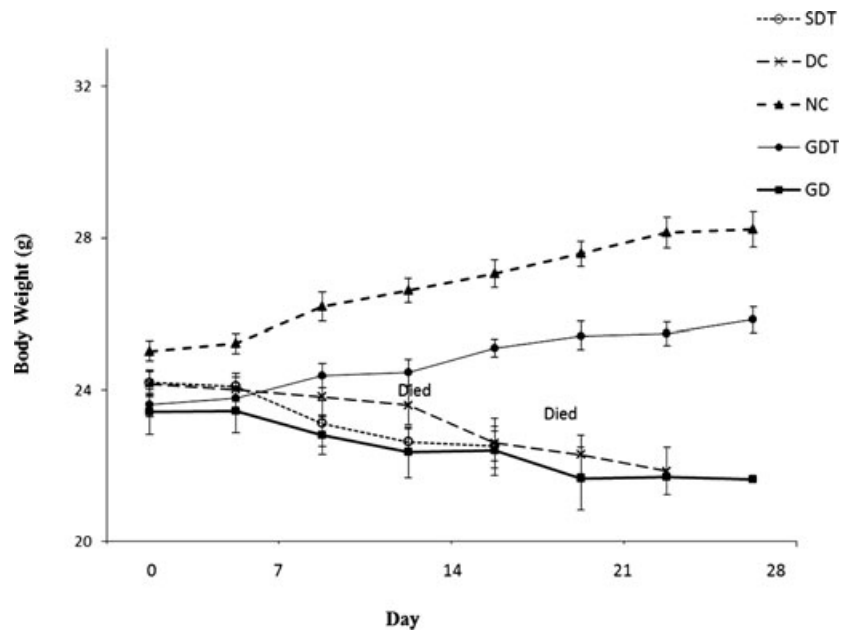


Fig. 5. Mice body weight monitoring during the experiment period. Changes in body weight of the mice after streptozotocin, thalidomide, and islets transplantation treatments. All data represent the mean values \pm SEM. GDT, grafted diabetic thalidomide-treated mice; NC, normal control mice; SDT, sham-grafted diabetic thalidomide-treated mice; DC, diabetic control mice; and GD, grafted diabetic group. ("Died" represents all mice).



results are consistent with other studies indicating the efficacy of thalidomide treatment in preventing islet graft rejection, improving graft function and survival in the recipient [12].

Caprine islet grafts in the immunosuppressed group resulted in approximately similar non-fasting blood glucose levels as in the normal control mice which were near to glucose level in caprine (3.2–8.2 mM). As previously reported, islet xenotransplantation in diabetic animal models establishes normoglycemia in the recipients to the point that is normal for the donor instead of the recipient [31,32]. The results of this study showed that caprine islet grafts are capable of reversing diabetes and normalizing blood glucose levels in the recipient's body.

The IPGTT profiles revealed the glucose responsiveness of the caprine islet grafts, findings that are comparable to results obtained with islet cells recovered from other mammalian species including pigs, primates, and rodents [33–38]. In addition, caprine islets elicited an insulin response under both basal- and glucose-stimulated conditions, with insulin levels equivalent to those obtained with islets from other mammals such as pigs, cows, and dogs, but less than those obtained with rodent islets [39]. Rodent islets secrete much more insulin than islets from human or porcine sources, either basally or under glucose stimulation [40]. Caprine islet cells are efficient insulin-producing cells, similar to human and porcine islet cells, because they were able to maintain euglycemia and glucose

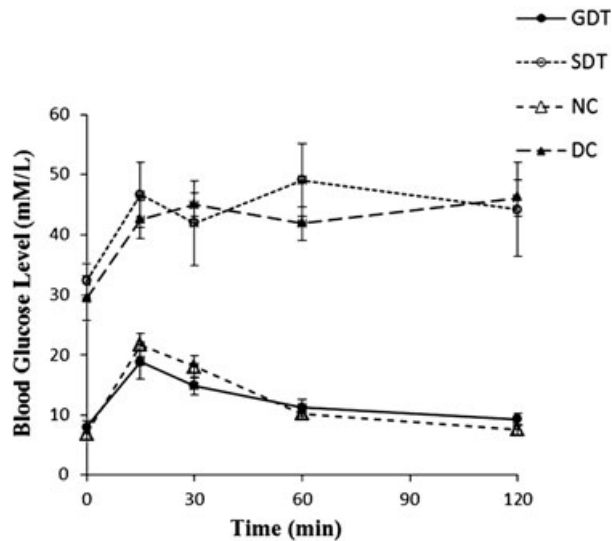


Fig. 6. Intraperitoneal glucose tolerance tests in fasted mice at week two post-transplantation of caprine islets. Fasting blood glucose levels (0 min) for GDT, NC, SDT, and DC were 8, 6.92, 29.42, and 32.28 mM. At 30 min, GDT and NC were non-significant while significantly lower than SDT and DC ($P < 0.001$). At 60 min, GDT and NC were significantly lower than SDT and DC ($P < 0.001$). At 120 min, GDT and NC were significantly lower than SDT and DC ($P < 0.001$). GDT, grafted diabetic thalidomide-treated mice; NC, normal control mice; SDT, sham-grafted diabetic thalidomide-treated mice; and DC, diabetic control mice.

responsiveness in recipients. It has been found that several thousand IEQ of the donor pancreas from human, bovine, porcine, or caprine sources are required to normalize blood glucose levels after transplantation in STZ-induced diabetic mice (assuming 1000–3000 islet cells/IEQ \times >2000 IEQ/transplant = $>2.0\text{--}6.0 \times 10^6$ islet cells/transplant). Normalization of blood glucose can be accomplished with fewer rodent islet cells (500 islets = 1.5×10^6) or fish islet cells (1.75×10^6), because of better compatibility of donor islets and recipients or higher insulin content and secretion [30].

In this study, the body weights of the mice were about 25 g before injection of STZ, and a weekly survival rate of $>80\%$ was observed in diabetic mice until caprine islet transplantation. Body weights are an essential consideration in STZ-induced diabetic models. In this study, male mice

with a close range of body weights were used because they have higher sensitivity to STZ induction than female mice [41,42].

One of the main reasons for performing this preliminary study was that religious and cultural factors in countries including Malaysia may favor goats over pigs as xenograft donors. However, potential limitations associated with the use of caprine donors must be recognized. First, the mean yield of 120 000 islets per adult goat is somewhat lower than the reported yield of up to 360 000 islets obtained from adult pigs [13,43,44]. Second, the efficacy of goat insulin in humans is not known. The amino acid sequence of goat insulin differs from that of human insulin at four residues, compared with pig insulin which differs at only one. However, bovine insulin, which differs at three of the same four residues as goat insulin, has been shown to control diabetes in humans [45]. Furthermore, should there be a problem with goat insulin, it would be feasible to genetically modify goats [46] to produce human insulin.

In conclusion, a single dose of STZ (200 mg/kg) induced diabetes in BALB/c mice, and hyperglycemia persisted until caprine islet transplantation. An increase in body weight, in parallel with a reduction in blood glucose levels (despite an increase in food consumption), of recipient mice signaled a reversal of diabetes.

In xenotransplantation, foreign tissues will face rejection by the immune system of an immunocompetent recipient individual. However, with the tremendous advancement and progress in immunosuppressive drugs, immunomodulation, and tissue engineering, the maintenance of xenotransplanted islet survival is possible. Caprine species may provide a new source of pancreatic islets for transplantation in the future, if xenogeneic compatibility is addressed. Genetic modification to minimize xenogeneic rejection is also possible.

Acknowledgments

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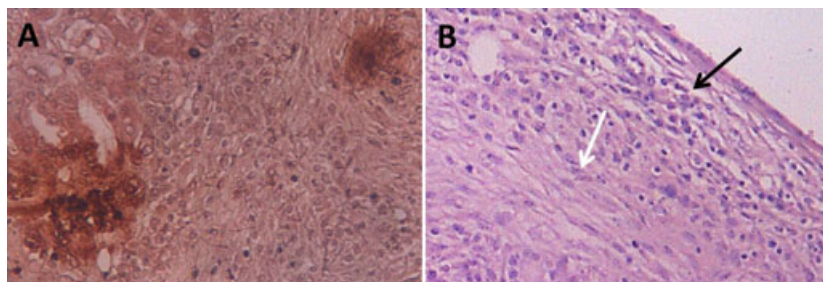


Fig. 7. Immunohistological staining of grafted caprine islets in BALB/c mice after 30 days. (A) Immunostaining of insulin in recovering islets graft section displayed that caprine islets in immunosuppressed mouse were capable to produce insulin (dark brown color area). (B) in rejected graft leukocyte infiltration and necrotic tissues were clearly observed (black and white arrows).

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Authors' contributions

Homayoun Hani, the first author, is the person who did the experimental design, data analysis and wrote the manuscript. Zeenathul Nazariah, the corresponding author, who reviewed and corrected the manuscript. Mohd-Azmi Mohd-Lila assisted in reading the manuscript. Tengku Azmi Tengku Ibrahim and Abas Mazni Othman reviewed the manuscript.

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