Experimental studies on islets isolation, purification and function in rats

Xinlu Pang¹, Wujun Xue², Xinshun Feng², Xiaohui Tian², Yan Teng², Xiaoming Ding², Xiaoming Pan², Qi Guo², Xiaoli He²

¹Department of Kidney Transplantation, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China; ²Hospital of Nephrology, The First Affiliated Hospital of Medical College, Xi’an Jiaotong University, Xi’an 710061, China

Abstract: To develop a simple and effective method of islet isolation and purification in rats. Collagenase P was injected into pancreatic duct followed by incubation in water bath to digest the pancreas and isolate islet, then discontinuous gravity gradient purification was used to purify the islet. The purified islets were identified by dithizone staining. The viability of islets was assessed by fluorescence staining of acridine orange (AO) and propidium iodide (PI). The function of purified islets was determined by glucose-stimulated insulin release test and transplantation of rat with streptozocin-induced diabetes. 738±193 islets were recovered after purification. The average purity was 77±13%, the viability of islets was more than 95%. When inspected by glucose stimulation, the secreted insulin concentration was 24.31±5.47 mIU/L when stimulated by low concentration glucose and 37.62±4.29 mIU/L by high concentration glucose. There was significant difference between the two phases (P<0.05). The blood sugar concentration recovered to normal level after two days in the animals with islet transplantation. In conclusion, islets can be procured with good function and shape by using the method of injecting collagenase into pancreatic duct followed by incubation in water bath and purification using discontinuous gravity gradient.

Keywords: Islet, isolation, purification

Introduction

There are nearly 200,000,000 diabetes mellitus patients worldwide in 2003 which is thought still increasing [1]. Diabetes mellitus not only cause vascular disease, but also associates with substantial premature death from several cancers, infectious diseases, external causes, intentional self harm, and degenerative disorders, independent of several major risk factors [2]. Type 1 and part of type 2 diabetes must use insulin therapy. Synthetic insulin injections can cause hypoglycemia, weight gain, edema, refractive changes, fat atrophy and other side effects. Severe hypoglycemia can even be life-threatening. Pancreas transplants or islet transplantation that can fully recover the physiological function of pancreas is the ideal choice for therapy. Pancreas transplantation is relatively mature, however, there is need to address the issue of exocrine complications. Islet transplantation only uses endocrine cells, thus has fewer complications [3]. The success of islet transplantation is dependent upon the quantity and quality of islets used in the transplantation procedure. But the success of islet isolation and purification is critical for transplantation. Many studies had focused on this topic [4-7]. In this study, we experimentally investigated the reliability of islet isolation and purification techniques and conducted islet transplantation experiments on rat with diabetes.

Materials and methods

Animals

Thirty SD rats, male or female, body mass 250–300 g were bought from Xi’an Jiaotong University School of Medicine Laboratory Animal Center. All protocols were in conformity with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health 86-23, revised in 1985.
Digestion and separation solutions

Digestion solution was prepared from HBSS with 0.5 mg/ml collagenase (Sigma-Aldrich, MO, USA), 20 mg/ml BSA (Sigma-Aldrich, MO, USA), and 2.0 mg/ml Soybean trypsin inhibitor (STI) (Sigma-Aldrich, MO, USA). One hundred mg of collagenase was completely dissolved in 180 ml HBSS and adjust PH to 7.8. Then set the volume to 190 ml and added 10 ml glycerol, mixed thoroughly and filtered through 0.22 μm pore size. The aliquot was stored at -20°C.

Separation solution preparation method: Weigh 25 g, 23 g, 20 g, 11 g of Ficoll 400, respectively and dissolve in 75 ml HC-A liquid completely, then set the volume to 100 ml and store at 4°C after high pressure steam sterilization.

Islet isolation

Pancreas anatomy and pancreas duct intubation: Rats were anesthetized by 2% thiopental intraperitoneal injection. Pancreas and common bile duct were fully exposed. Then, common bile duct was intubated with self made catheter and ligation fixed. Fifteen ml of Collagenase was sucked into a 20 ml syringe and the syringe was attached to the end of cannula. Inject a small amount of collagenase to the common bile duct to move the bile into the intestine and ligate the distal common bile duct. Then cut rat chest and broke heart to death. After that, retrograde inject 10 ml of collagenase solution to common bile duct slowly until the pancreas fully expanded. Then remove the pancreas rapidly and completely. After remove the fat and connective tissue from, use tweezers carefully torn the pancreas up on ice, transfer into 50 ml conical flask and add 5 ml pre-warmed collagenase solution.

Islet isolation: The flask was placed in 38°C water bath and shake gently about 20 min to until the pancreas looks like fine sands. Then a large volume of 4°C Hanks solution was added immediately to terminate the digestion. The solution was transferred to a centrifuge tube and homogenized by pipetting, before centrifuged 1,000 g×3 min at 4°C.

Islets purification: The supernatant was discarded and the pellet was purified by the discontinuous Ficoll density gradient centrifugation. First, the pellet was suspended in 10 ml 250 g/L Ficoll solution (density 1.084 g/ml). Thereafter, 5 ml of 230 g/L (density 1.077 g/ml), 200 g/L (density 1.068 g/ml) and 110 g/L (density 1.038 g/ml) solution was added subsequently. The solution then divided into four layers. Islet cells were retrieved from interface between 1.038~1.068 and 1.068~1.077. Purified islet cells were washed with cold RPMI 1640 media and centrifuged at 4°C, 1,000 g×4 min 2 times. Purified islet is obtained after centrifugation.

Determination of islet viability, specificity and purity

Islet viability was identified by AO/PI staining with living cells stained in green and dead cells stained in red. Pancreatic islet cell specificity were indentified with DTZ solution in which islet will be dyed scarlet at 37°C for 10 min. The purity of pancreatic islet cells was evaluated based on the ratio of the amount of endocrine versus exocrine tissue.

Assessment of islet function

Insulin release test: A concentration of 3.3 mM of glucose and 16.7 mM of glucose culture media was prepared with Hanks solution. Fifty islets of each rat were placed in a single well of 24-well plate with either 1 mL low or high glucose medium. The plates were incubated at 37°C for 4 h. Supernatant was collected and insulin concentration was examined by radioimmunoassay according to the kit's instruction (CAS Atomic Energy Research Institute, Beijing, China).

Islet transplantation in diabetic rats

Streptozotocin (60 mg/kg) (Sigma-Aldrich, MO, USA) was injected intraperitoneal to recipient SD rats to induce diabetes. Rats with blood glucose levels at or above 16.8 mM (measured via tail vein blood in the non-fasting state) were identified as diabetic rats and prepared for islets transplantation. Rats were randomly divided into two groups, the test group were injected with 1 ml purified islet cell suspension (about 2,000 islets) intraperitoneally, while the control group were injected with 1 ml saline.

The non-fasting blood glucose levels of the recipient rats were measured daily to monitor the function of the islet grafts for 9 days after
transplantation. Blood glucose level less than 11.1 mM in the non-fasting state was considered islet graft survival and function well.

Statistical analysis

All the experimental data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 18.0 software (SPSS, CHI, USA). Student’s t test was used for comparison between two independent groups. P<0.05 was considered statistically significant.

Results

Isolation and purification of islets

Retrograde injection of collagenase for the full expansion of the pancreas makes the pancreas easy to distinguish from surrounding tissues (Figure 1A). After intraductal collagenase digestion, 831 ± 127 islets were obtained from each pancreas. Dithizone staining showed the morphology of intact islet cell (Figure 1B). After purification 738 ± 193 islets from each of pancreas were obtained, the average purity is 77 ± 13%, and the purified cell morphology is intact (Figure 2A). After staining, more than 95% of cell mass can be seen green under the fluorescence microscope, indicating the survival of the islet preparation was greater than 95% (Figure 2B).

In vitro function assessment of purified islets

Fifty islets were selected to examine the function in vitro using insulin secretion assay. The
amount of insulin secretion was $24.31 \pm 5.47$ mIU/L under low glucose condition and $37.62 \pm 4.29$ mIU/L under high glucose condition, respectively and difference was significant ($P<0.05$). The in vitro result demonstrated that purified islets have good function in term of their response to high glucose stimulation.

**Islet graft function**

The blood glucose levels of control group and islets transplanted group are shown in Figure 3. In control group, the blood glucose level maintained at high basal level throughout the experiment. Contrastly, in rats receiving islet transplantation, the non-fasting blood glucose level decrease dramatically starting at day-3 after transplantation and it reached to the lowest level at day-5. The blood glucose maintained at low level until it re-elevated to the basal level at day-8. Experimental group maintain normal blood sugar for about $3.9 \pm 1.7$ days. The in vivo data demonstrates that islet graft is functioning well.

**Discussion**

Islet cell transplantation has opened a new way for the treatment of insulin-dependent diabetes mellitus especially after the success of Edmonton in 2000 [8]. Currently, animal studies of islet transplantation are mainly focusing on islet isolation. There are many factors affecting the function of transplanted islets, including islet mass, islet purity, pellet size, and in vitro insulin secretory capability. Body weight of recipient mouse should also be considered when evaluating islet function [9]. The ideal method for islet cell preparation should be able to provide adequate, clean and functional islet cells [10].

Collagenase perfusion method can fully expand the pancreatic duct. After injection of collagenase, it contacts with the exocrine pancreatic tissue first, and then the tissue between islet and exocrine pancreatic tissue. Therefore, the full expansion of pancreatic duct becomes very important. It is difficult to isolate islets from pancreatic tissue with poor expansion. Some other research also indicated the same point [11]. Rat bile duct is relatively thin, so it is very important to fast the rats for 12 hours before the experiment. Suitable conduit also increases the success rate of intubation. In this study, the new epidural catheter was stretched slowly after heating in boiling water to make the diameter more consistent with the rat bile duct.

Tissue dissociation enzymes are critical reagents that affect the yield and quality of pancreatic islets prepared for islet transplantation. The type of digestion enzyme and the time for digestion are two key points. If digestion time is too short, islet cannot release from exocrine pancreatic bubble, and if digestion time is too long, it can easily damaged islet activity [12, 13]. Therefore, the choice of the optimum digestion condition is critical for islet isolation. In this study, $0.5 \text{mg/ml}$ collagenase was used for a digestion in the period of 20 min to get high amount and good quality of islets. The digestion time may be changed in different species and different physical procedure [14, 15].

During islet separation process, the exocrine pancreas will be inevitably destructed, which leads to the release of a large number of trypsinogen and other digestive zymogen. Trypsinogen can activate itself and has the ability to change the molecular structure of trypsin, and through a positive feedback to cascades the effect. Trypsin and other active zymogens have a strong destructive effect on islet cells.
Soybean trypsin inhibitor (STI) has a special structure that can inactivate trypsin, thereby inhibited the activity of activated trypsin and thus avoid activation of a large number of trypsinogen and other digestivezymogens. Addition of STI can protect islet tissue from Trypsin digestion during isolation process. STI is also used to reduce inflammation in other diseases [16-18]. In addition, serine protease inhibitors also protect islet during pancreatic digestion [19].

In the process of islet separation, the content of oxygen free radicals significantly increased. While the oxygen radical itself cause DNA damage and protein sulphydryl oxidation, it can damage islets directly. Oxygen free radicals can cause decrease of cell membrane stability, so that pancreatic cells release lysosomal enzymes and active digestive enzymes, thus destroying the islets. Therefore, reducing the content of oxygen free radicals can protect islet cell from damage [20].

Free sulphydryl groups (-SH) on the surface of BSA-Fraction V react with oxygen radicals to clear it. This will reduce the damage caused by trypsin and oxygen free radical, therefore obtaining islets with good yields and high viability. Short digestion time, minimal mechanical stress and low temperature are also important in reducing oxidative stress. Perfluorohexyloctane and other ingredients are also beneficial to islet isolation and preservation [21, 22].

A viscous jelly often forms in the process of pancreatic digestion. It captures a large number of islets, thereby reducing the yield of islets. Based on Burghen’s method [23], the experimental conditions were strictly controlled, including digestion temperature (38.0 ± 1.0)°C and pH value (7.8 ± 0.1). Glycerol was added to the collagenase solution with an appropriate amount (volume ratio of about 2:1). At the end of digestion, cold Hanks solution (volume ratio of about 4:1) was immediately added to stop digestion. With all the processes above, the formation of viscous jelly was avoided. Many other factors can also impact the yield of islet isolation, such as donor strain, the source of inhibition of proteolytic activity, and the culture condition [24, 25]. Islet should be kept and treated under low-temperature before transplantation [26].

As described previously, the activity and purity of islets are also important to evaluate the effect of islet isolation. Islet transplantation features the best testing standards. In this experiment we did the AO/PI staining, glucose stimulation test and islet transplant. AO/PI staining showed that islet viability was above 95%. Glucose stimulation test showed a significant difference between high glucose-stimulation and low glucose-stimulation insulin release which suggested that islet β-cell function was well. Blood glucose decreased to normal after experimental allograft was transplanted 48 hours and continued 3-4 d which described purification islet with better functional in rats. The function loss of islet may because of rejection. Rejection and fibrosis are main reasons for allograft lost. Immunosuppression regimes may help maintain the function of allograft [27].

In summary, the present study suggests that combining intraductal collagenase digestion and Ficoll400 retrograde perfusion discontinuous density gradient centrifugation is a feasible method to isolate rat islets with a high yield, high purity and good activity. Transplantation with purified islets can reverse the hyperglycemic state in experimental diabetic mice for a certain period of time.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wujun Xue, Hospital of Nephrology, The First Affiliated Hospital of Medical College, Xi’an Jiaotong University, No. 277 Yanta West Road, Xi’an 710061, China. E-mail: wujunxuecn@163.com

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