Functional Pancreatic Isolated Islets from Cryopreserved Porcine and Human Pancreatic Tissues Based on GLUT2 Receptor Sensitivity

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Background: *Pancreatic islet transplantation is the milestone therapy to restore glucose homeostasis in type 1 diabetes. Despite advances in surgical techniques, patients in low and middle income countries do not have access to islet transplantation due to the steep cost of islet extraction. High quality islet isolation is the bottleneck because the standard enzymatic digestion method is too costly. Here we hypothesize that flash frozen pancreata still contain intact GLUT2 in endocrine islets.*
Material and Method: *We demonstrate that glucose sensitivity method of islet isolation from cryopreserved tissues of porcine and human pancreata results in functional islets. Islets were isolated based on the property of GLUT2 transporter in islets that enable glucose transport across the cell membrane. Minced pancreatic fresh or frozen tissues were incubated for 20 minutes in media supplemented with 600 mM glucose. After that, the medium was replaced with zero-glucose medium. We tested islet quality by immunostaining hormone contents, Glut2 receptor, and measuring insulin secretion using glucose induced insulin secretion assay. Importantly, islet isolated from frozen tissues showed ability to secrete insulin in response to glucose stimulation similar to freshly isolated islets. These isolation and purification method applied to cryopreserved tissues is much simpler and selective for functional islets.*
Results: *By omitting the enzymatic digestion step, this results more than 90% cost reduction in islet isolation and purification method.*
Conclusion: *These data bring about islet transplantation feasibility and accessibility to many more patients.*

Keywords: *Diabetes, Islet, Islet isolation, Transplantation*

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Islet transplantation is a gold standard therapeutic treatment for a quality of life in type 1 diabetic patient according to the ability for glycemic control. This is important because in natural islets; insulin-producing β-cells and glucagon-producing α-cells are two main cell types in islet which are responsible for downregulation and upregulation of blood glucose levels (Schwartz et al, 2013)[1]. People with type 1 diabetes tend to be brittle type 1 diabetes which is defined as type 1 diabetes with the characteristic of instable glycemic levels. This means that patient can undergo frequent unpredictable of hypoglycemic after hyperglycemic in a short period of time (Rheinheimer et al, 2015)[2], (Bertuzzi et al, 2007)[3], (Christina Voulgari 2011)[4]. These hypoglycemic episodes due to lack of properly functional glucagon-producing cells can cause life threatening experiences to type 1 patients as it can render the patients unconscious. Restoring the entire islets is a better cure for type 1 patients than exogenous insulin treatment. Allogenic islet transplantation can be done via infusion of islets directly into the liver through the portal vein account (Bertuzzi et al, 2007)[3], (Bruni et al, 2014)[5], (Rheinheimer et al, 2015)[2]. Before islets are transplanted, a quality in islet preparation step is required as the important step that can affect the islet function and the insulin-independent state in patients in post-transplantation period (Illani Atvvater 2010)[7], (Harvey ZAR 2014)[6], (Bruni et al, 2014), et al, 2015)[5]. Islet isolation and islet culture are the significant and critical steps for the successful of islet transplantation.

Enzymatic digestion has been the key

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mechanism used to discharge exocrine tissues from endocrine islets in donor pancreas with a process called semi-automated method by Ricordi. In this method, Ricordi® Chamber and enzymatic digestion has been a gold standard for clinical treatment in terms of islet isolation. To obtain intact islets with their viability and integrity of function, optimal conditions including time and temperature of incubation with collagenase enzyme are necessary (Illani Atvvater 2010)[7], (Harvey ZAR 2014)[6]. Obtained islets from the digestion step is further purified from enzyme and collected by histopaque gradients. In addition, University of Wisconsin solution is needed to re-suspend the islet-containing tissue pellet for increasing the difference in densities between the endocrine and exocrine fractions of the digest.

However, current islet isolation method is very expensive by accounting approximately 30% of overall islet transplant cost due to the cost of enzyme by costing over $4,000 US for each isolation. Moreover, the isolation also required expert skills and high quality equipment for execution (Guignard et al, 2004)[8], (Atwater et al., 2010)[9], (Moassesfar et al, 2016)[10].

In this research, we hypothesize that GLUT2 transporter of pancreatic beta cells of porcine and human are still intact in structures and functions despite having been frozen. This hypothesis has not been tested and is important for practicality of islet isolation and transplantation. In 2010, selective osmotic shock (SOS) method was demonstrated as the alternative islet isolation method by using the property of GLUT2 transporter, a specific transporter in islets that enables glucose transport across the cell membrane. GLUT2 transporter is located in only a membrane of islet cells in pancreas. GLUT2 is one of the glucose transporter isoforms that is responsible for sensing the glucose for stimulation of insulin secretion from pancreatic β-cells. However, GLUT2 is not found in exocrine cells of pancreas. Pancreata from organ donors are regularly discarded without any use. The economic burden to improve patients' long-term quality of life is heavily weighted on the Thai society because of the frequent daily usages of insulin injection and diabetes complications. Islet transplantation could save a number of people with type 1 diabetes and save economic cost to help the patients for the Thai society. However, islet isolation and purification are still the limiting step in Thailand because of its skyrocketed cost. Therefore, identifying an islet isolation method that can extract porcine and human islets efficiently and inexpensively is a major unmet need (Federation 2012)[11], (Deerochanawong and Ferrario 2013)[13], (World Health Organization, 2013)[12].

This work is the first to isolate frozen porcine and human islets by using GLUT2 receptor selectivity without the requirement of University of Wisconsin (UW) solution to preserve pancreas before isolation and purification steps. With this simplified and cost saving method, the islets show functional quality by GLUT2 and hormone markers, and most importantly, the ability to secrete insulin in response to various glucose concentrations. Therefore, frozen porcine and human pancreas can be used for efficient quantities and qualities of endocrine islet with this method of islet isolation for future islet transplantation.

Material and Method

Materials

Porcine pancreata were collected from porcine butchery in Nakhon Pathom, Thailand. The pancreata were chopped into small pieces and stored at -20°C. Three human pancreatic tissues from donors were provided from Ramathibodi hospital, Thailand. Tissue handling and Dithizone staining were performed according to Atwater et al, 2010. Islet isolation via high-to-low glucose media switching method and islet culture. Pancreatic tissues were cleaned by standard practice, then mixed and incubated with approximately 35 to 40 ml of RPMI 1,640 medium supplemented with 600 mM glucose in 20 minutes at room temperature. The minced pancreatic tissue was washed and switched to zero-glucose RPMI medium. The supernatant phase was filtered using a nylon mesh with a pore size of 440-μm to remove non-islet tissues and 70-μm strainer to retain islets. To recover isolated islets were cultured on the 40-μm strainer with RPMI 1,640 medium supplemented with 5.6 mM glucose and 3% penicillin/streptomycin.

Insulin secretion assay

At day 1 of culture, 100 islets were counted for measurement of insulin secretion by using glucose stimulation of insulin secretion assay (GSIS) performed according to protocol ELISA kit (Abnova) to measure insulin secretion respectively.

Immunohistochemistry

Human frozen islets were fixed and processed tissues for immunohistochemistry as described (Kitamura et al, 2009). The islets were applied perfused fixation (Padilla et al, 2010). A list of antibodies used in these studies is Anti-Glut-2 antibody, Anti-insulin, Anti-Chromagranin A, and three-hormone cocktails:

Glucagon, PP, and Somatostatin were used. Image acquisition, analysis have been described (Xuan et al, 2010). For electron microscopy, a cell mass morphometry and immunofluorescent colocalization analysis were performed as described (Gao et al, 2007). Coexpression of Gfp and insulin or glucagon, somatostatin, and Pp cells, was verified and quantified as ratio of overlapping area/total immunoreactivity area (single-stained regions -overlapping area) as automatically assigned by confocal microscopy and Laser Scanning Microscope Software (Zeiss LSM 510 and 710).

Results

Isolation of porcine islets

Here, we present high-to-low glucose media switching method to isolate islets from frozen tissues of porcine and human pancreata with additional simplified step of islet isolation, and isolated islet purification. By using the property of GLUT2 transporter when the pancreatic tissues are exposed to high glucose media the islets establish the balance with the hypertonic state by consuming glucose though their GLUT2 transporter while the exocrine cells consume water and ions such as potassium ion from the media. This situation causes exocrine cells to explode and become dissociated from islets when the media is rapidly replaced with low glucose media. This is because the exocrine cells cannot adjust themselves to release the consumed ions and water response the hypotonic state in time while the islets that do not consume any ions and water, but just glucose release their consume glucose out to response the hypotonic state (Urrego and others 2014)[15]. By this method, the washing step with several times to eliminate enzyme is omitted. And, the purification step with cell strainer cause the omission of using histopaque which increases toxicity to islet cells. Therefore, islet isolation and purification with this method impact the reduction of cost in the islet preparation step before undergoing islet transplantation.

Pancreatic tissues were divided. Some were separated for staining with dithizone solution to examine the ability of insulin-producing cells to produce insulin prior to islet isolation. Dithizone is a Zn chelating molecule that binds to insulin docking granules, and has been used as a marker for vital endocrine islets.When stained with dithizone, healthy insulin producing cells appeared in red color in porcine pancreatic tissue as shown in Fig. 1. In Fig. 1, we show that endocrine islets from both fresh and frozen tissues were positively stained with dithizone, thus indicating healthy tissue quality check.

After exposure to zero-glucose medium to release islets from other exocrine and ductal tissues, large pieces of pancreatic tissues were removed using a 440-μm cell strainer. As the size of porcine islets ranges between 50 μm to bigger than 200 μm (Taylor and others 2010)[14], a 70-μm strainer is therefore applicable for islet purification to separate islets from other tissues and microbial contaminants. Since large pieces of pancreatic tissues had been previously removed using a 440-μm nylon mesh, only tissues smaller than 440 μm were collected. When this cell mixture was filtered through a 70-μm strainer, only tissues that are larger than the strainer's pore collected on the strainer while cell debris and microbial contaminants passed through the strainer, thus increasing purity of islets from contamination with both microorganisms and cell debris. By using this method of islet purification, pure islets were successfully obtained. However, the islets showed diverse shapes; some of which have sphere or circular shape as shown in Fig. 2. The size of porcine islets is uncertain, but they generally range between 50 μm to more than 200 μm (Taylor and others 2010)[14]. Islets were cultured with RPMI 1,640 culture media supplemented with 5.6 mmol/L glucose on a 40 μm strainer. The strainer used in islet culture will provide an easy way of medium changing as well as help remove microbial contamination any times as needed. Note that the culture medium was replaced daily with fresh

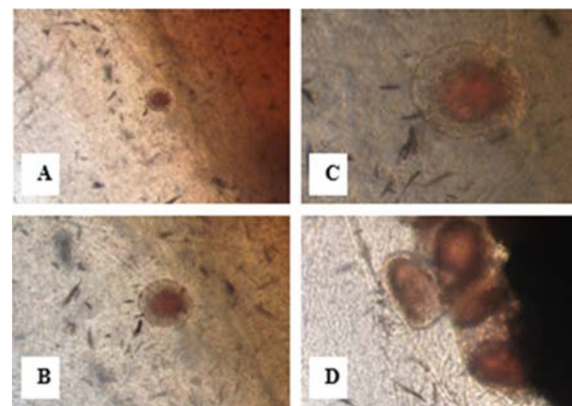


Fig. 1 Staining of porcine pancreatic tissues with dithizone solution. Porcine pancreas was chopped into small pieces and subsequently stained with dithizone, followed by imaging under inverted light microscope. Pancreatic tissues were observed and photographed with 4x (A) 10x (B) and 40x (C and D) magnification.

complete medium. Under this culture condition, islets could be cultured for at least 4 days without contamination. When observed under microscope, cultured islets displayed 2 different attributes. The first attribute is islets that loosely attach to the bottom of the 6-well plate and the second attribute is islets that form group and floated in the medium as shown in Fig. 3. Fig. 3 clearly shows that cell strainers help islets to group themselves and to form dimension as in their natural appearance. The islets appeared to build the extracellular matrix to contract between islet groups and also attached to strainer as shown in Fig. 3.

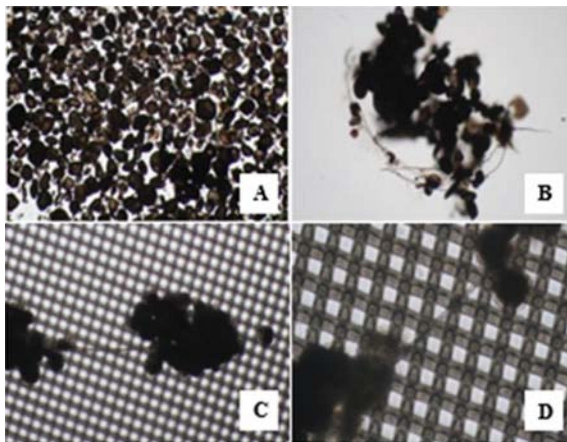


Fig. 2 Porcine islets cultured on the cell strainer. Islets were cultured in RPMI 1,640 supplemented with 5.6 mmol/L glucose. Two attributes of islets in the culture plates were observed under inverted light microscope with 4x magnification; first attribute is islets that attached to the bottom of the 6-well plate (A). Second attribute is islets that formed group and floated over the first attribute (B). The islets appeared to group themselves, formed dimension, build the extracellular matrix to contact between islet groups, and islets themselves also attached to strainer were observed under inverted light microscope with 4x (C) and 10x magnification (D).

Isolation of human islets

According to a satisfied result of islet isolation from porcine pancreatic tissues, human pancreatic tissues were further subjected to islet isolation using the same method. First we analyzed GLUT2 expression from fresh and frozen human islets obtained by this GLUT2 selective method, and we found that GLUT2 is properly expressed in human islets isolated from frozen tissues as shown in Fig. 4. This leads us to further hypothesize that the islets' ability to secrete insulin in response to different glucose levels are still intact.

Three frozen human pancreatic tissues with the weight of 1.26 g, 1.28 g and 2.58 g were used in islet isolation, and after isolated, islets were pooled. With the size ranging between 70 to 440 μm, 1007 islets were obtained. Human islets isolated using this method demonstrated to be clear from contamination with both cell debris and microorganism. The purity and morphology of human islets are shown in Fig. 5.

Islet viability and functional analysis

Isolated islets were tested for their insulin secretion at day 1 of culture to confirm their viability after isolation and also measure their ability to produce insulin. Glucose-stimulated insulin secretion was carried on in this test. Porcine islets were first tested. Following incubation in Krebs buffer supplemented with 2.8 mM glucose as a basal glucose concentration for 1 hour, 100 isolated islets were stimulated to secrete insulin in Krebs buffer supplemented with 16.7 mM

Fig. 3 Human isolated islets from frozen tissues by Glut-2 selective method secrete insulin in response to high glucose concentration.At day1 of culture 100 isolated islets were picked for testing ability to produce insulin when were stimulated with 1 ml. of Krebs buffer supplemented with 5.6 and 16.7 mM glucose for 1 hour, respectively. Both of Krebs buffer were compared with secreted insulin when the islets were pre-incubated for 1 hour in basal glucose concentration; Krebs buffer supplemented with 2.8.

glucose to stimulate insulin secretion. Insulin concentration was then measured in both Krebs buffers using ELISA technique. from two different porcine pancreata, termed A and B, displayed the ability to produce insulin after stimulated with high glucose concentration solution. Islet samples A and B secreted 5.052 µIU/ml and 3.639 µIU/ml of insulin in Kreb's buffer, respectively. The basal insulin secretions of islet samples A and B were 3.639 µIU/ml and 3.22 µIU/ml, respectively (Fig. 6).

In addition to measurement of insulin secretion by porcine islets, insulin secretion by human islets was also studied. In the present study, Krebs buffer containing 5.6 mM glucose was included in glucose stimulation of insulin secretion (GSIS) assay to observe the appearance of bi-phasic insulin secretion which is a natural appearance of insulin secretion. After islets from 3 different human pancreatic tissues were pooled, 100 islets were used in the assay. Human islets incubated with 2.8, 5.6 and 16.7 mM glucose secreted insulin with the concentration of 3,482 mIU/L, 3,534 mIU/L and 4,948 mIU/L, respectively (Fig. 7). This result demonstrated that insulin was augmented only when the concentration of glucose is high. The bi-phasic insulin secretion was shown, and the result also demonstrated the viability of islets after isolation.

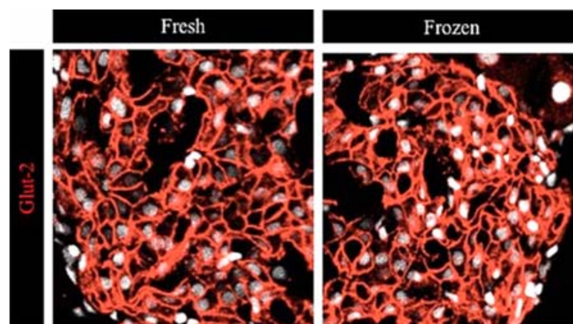
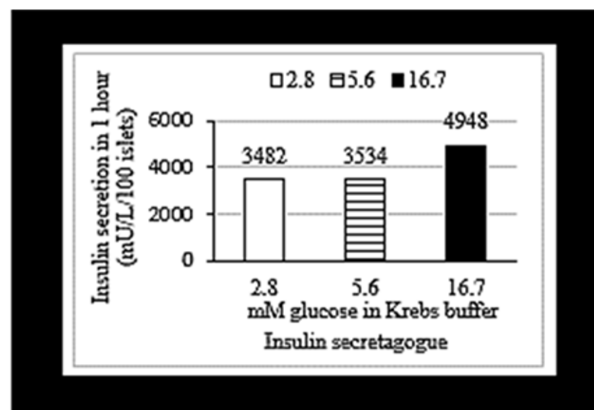


Fig. 4 Immunostaining of Glut-2 antibody to human islets obtained from high-to-low glucose media switching isolation method from fresh (A) and frozen (B) tissues. After isolation, intact expression of Glut-2 transporters were observed and captured under confocal microscope and photographed at 40x magnification.

Discussion

Approximately, 5,000 to 9,000 islets/ kg of body weight are needed for sustainable islet transplantation. Therefore, 0.5 to 1 million islets are required per patient. From the semi-automated islet isolation method based on enzymatic digestion, only 100,000 to 400,000 islets (islet equivalents; 150 µm diameter of islet) can achieve from whole donor pancreas (Harvey ZAR 2014)(6), (Rheinheimer et al, 2015)(2). For isolation, 2 gram of pancreatic tissue is required for more than 1,000 islets. Fortunately, the Edmonton group reported that patients who underwent transplantation with islets from multi-donors could achieve insulin independent, and 50% of patients who received islet transplantation were insulin independent after 3 years follow-up was reported by Collaborative Islet Transplant Registry (CITR) (Rheinheimer et al, 2015)(2). Human islets isolated from frozen pancreatic tissues by high-to-low glucose media switching



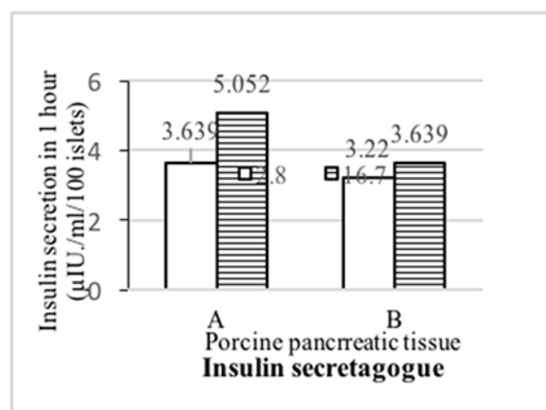


Fig. 5 Insulin secretion from porcine islets. At day 1 of culture, 100 islets were incubated with 1 ml. of Krebs buffer supplemented with 2.8 mM, prior to incubating with 1 ml of Krebs buffer supplemented with of 16.7 mM glucose. After 1-h incubation, insulin secretion was then measured using ELISA. A and B represent islet sample A and B, respectively.

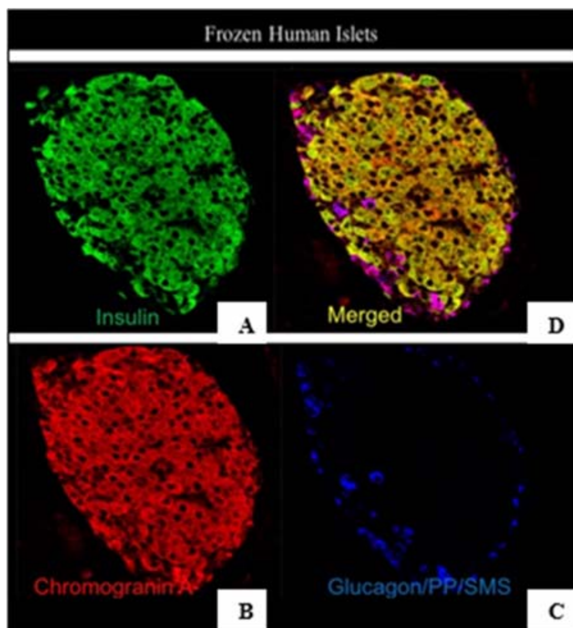


Fig. 6 Immunohistochemistry of insulin, chromagranin A, glucagon, PP, and Somatostatin in human frozen islets obtained from high-to-low glucose media switching isolation method. (A) Anti-insulin, (B) Anti-Chromagranin A (C) 3 hormone cocktails: Glucagon, PP, and Somatostatin, and (D) merged image. After isolation, proper expressions of the 4 hormones: insulin, glucagon, PP, and somatostatin as well as hormone-marker: chromogranin A were observed and captured under confocal microscope and photographed at 40x magnification.

method showed the number obtained at 1,007 islets (70 to 440 μm diameter) from 5.12 g total of tissue weight or equal to 196 islets per gram. Even there are some isolation that can obtain more than this extraction efficiency rate such as 500 islets per gram of pancreas (Botticher et al, 2011)[16]. But, there are key advantages from from high-to-low glucose media switching islet isolation method such that the pancreatic tissues are tissues. In contrast to our Glut2-based method, there are the pitfalls in enzymatic digestion method; the isolated islets are not selected for their viability or ability to sense glucose and produce insulin. Another pitfall is that the enzymes used can induce cellular damages in islets, expensive cost, and complicated (Atwater et al, 2010)[9], (Botticher et al, 2011)[16]. In our experiments, we observed substantial variation in the ability of islets to response insulin from two different porcine pancreata and also the pool of three human pancreatic tissues. This result could be due to the variation in pancreatic area that we partially cut the tissues to perform islet isolation. In addition, this variation could be from the health of pigs or human donors themselves.

More importantly, the selectively responsive to glucose point, isolated islets from high-to-low glucose media switching method showed ability to produce insulin higher than the values of insulin secretion that obtained from the enzymatic digestion method at 330 mIU/L comparing with 270 mIU/L from 20 islets incubated in 2 ml of buffer for stimulation of insulin secretion (Paget et al, 2007)[17]. Before transplantation, islet function is determined by stimulation of insulin secretion index (SI), and the SI should be over 1 (Rheinheimer et al., 2015)[2]. When we calculated the SI values of isolated islets from porcine pancreatic tissues A and B, and isolated islets from human pancreatic tissues, the values of SI from all are above 1 as 1.39, 1.13 and 1.42, respectively. Therefore, islets that isolate from frozen tissues with high-to-low glucose media switching method have the quality for further undergo transplantation.

Conclusion

By using high-to-low glucose media switching method, islet isolation of Porcine and human pancreatic tissues are achieved with the quality of isolated islets in term of satisfied number and function with much more feasible cost. Isolated islets showed ability to produce insulin response to glucose stimulation, and also showed the ability to reform from single islet into groups by building extracellular matrix which is an important characteristics for the revascularization of islets before being utilized in transplantation. Due to its feasibility and high quality islet function, this method potentially brings about islet transplantation in Thailand and low to middle income countries because the cost of islet preparation step is reduced about 90% cost as compared to enzymatic based method.

What is already known on this topic ?

High quality islet isolation is crucial in pancreatic islet transplantation. The standard enzymatic digestion method is costly in low and middle income countries.

What this study adds ?

The glucose sensitivity method for islet isolation and purification is simple and effective. It reduces the cost more than 90% which can make the islet isolation feasible for more patients.

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Potential conflicts of interest

None.

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ความสามารถในการทำหน้าที่ของเซลล์ไอซ์เลทที่สกัดแยกจากเนื้อเยื่อตับอ่อนหมูและมนุษย์ที่แช่แข็งบนพื้นฐานของความไวตัวรับ จี แอล ยู ที สอง

พิมพ์พลอย รัตนอำนวยชัย, เยาวลักษณ์ มะปราง รสหอม, จุมพล วิลาศรัศมี, ไชยรัตน์ ทรัพย์สมุทรชัย, จักรพันธ์ จิรสิริธรรม, ณปภัช โพธิ์พรหม, ณีวาตรา ชุติมา ตาลชัย

ภูมิหลัง: *การปลูกถ่ายเซลล์ไอซ์เลทจากตับอ่อนเป็นการรักษาที่สำคัญ ซึ่งสามารถช่วยสร้างภาวะสมดุลของระดับน้ำตาลในผู้ป่วยเบาหวานประเภทที่ 1 แม้ว่าวิทยาการของการปลูกถ่ายเซลล์ไอซ์เลทจากตับอ่อนจะก้าวหน้าไปมาก แต่ยังมีปัญหาเกี่ยวกับค่าใช้จ่ายที่สูงในกระบวนการ แยกเซลล์ไอซ์เลทจากตับอ่อน ซึ่งทำให้เกิดข้อจำกัดในประเทศที่ประชากรมีรายได้ต่ำถึงปานกลาง*

วัสดุและวิธีการ: *การศึกษานี้เป็นการศึกษาที่พิสูจน์สมมติฐานที่บ่งชี้ว่าการแยกเซลล์ไอซ์เลทจากตับอ่อนที่แช่แข็งได้เซลล์ที่มีความสามารถ ในการหลั่งอินซูลินที่ตอบสนองต่อการกระตุ้นด้วยกลูโคสไม่แตกต่างจากการแยกเซลล์ไอซ์เลทจากตับอ่อน*

ผลการศึกษา: *พบว่าการแยกเซลล์ไอซ์เลทจากตับอ่อนของมนุษย์และหมูแช่แข็ง โดยอาศัยคุณสมบัติพื้นฐานของการขนส่ง GLUT2 ผ่านเยื่อหุ้มเซลล์ซึ่งมีคุณภาพที่ยอบรับได้และทดสอบโดยวิธีอิมมูโนสเตนนิ่ง (immunostaining) บอกปริมาณฮอร์โมนตัวรับ GLUT2 และการวัดการหลั่งอินซูลินหลังการกระตุ้นด้วยกลูโคสโดยสามารถลดค่าใช้จ่ายลงได้มากกว่าร้อยละ 90 เมื่อเทียบกับการใช้วิธีการแยกเซลล์โดยเอนไซม์*

สรุป: *ข้อมูลจากการศึกษานี้ช่วยยืนยันถึงความเป็นไปได้และเพิ่มการเข้าถึงการปลูกถ่ายเซลล์ไอซ์เลทจากตับอ่อนในผู้ป่วยเบาหวานชนิดที่ 1*