UNIVERSITI PUTRA MALAYSIA

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CAPRINE PANCREATIC ISLETS

HOMAYOUN HANI

IB 2009 2

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CAPRINE PANCREATIC ISLETS

By

HOMAYOUN HANI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2009

Dedicated with love and gratitude to:

Father
Javad Hani

Mother
Maryam Gerogan

Brothers
Reza Hani and Hadi Hani

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Sciences

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CAPRINE PANCREATIC ISLETS

By

HOMAYOUN HANI

May 2009

Chairman: **Zeenathul Nazariah Bt Allaudin, PhD**

Faculty: **Institute of Bioscience (IBS)**

Diabetes mellitus is a major late consequence of chronic pancreatitis. Diabetes mellitus type 1 or insulin-dependent diabetes mellitus (IDDM) is characterized by the failure of the pancreatic islets of Langerhans to synthesis or secrete insulin. The long-term complications of IDDM are major health problems, and in these diabetics exogenous insulin may not adequately regulate blood glucose homeostasis and thus fail to avert the late complications. In this case, transplantation of pancreatic islets is arguably the most logical approach to restoring metabolic homeostasis. The limited availability of human donors makes the search for alternative source of islet cells mandatory for future developments in pancreatic transplantation. The present study investigates the potential of goats as an alternative source of pancreatic islets. The objectives of the study were to optimize techniques for goat islet isolation and purification for culture establishment, and to perform functional, morphological and

viability assessment of goat islets. Goat pancreatic tissues were collected within 15 min of slaughter, placed in Hanks balance salt solution (HBSS) and maintained at 4^{o}C. Goat islets were obtained successfully using a collagenase-based digestion and isolation technique at an optimized pH of 7.2 to 7.4 and temperature of 37^{o}C. Digested pancreatic islets were purified by Euro-Ficoll density gradients. Islet cell purity and viability were determined by dithizone and trypan blue staining, respectively. Islet clusters of different sizes were positively identified by both staining methods and it was observed that 90% of clusters were viable in the culture system. Following the static incubation, an *in vitro* insulin secretion assay was carried out by ELISA to determine the islets viability. The islets remained viable for 5 days in the culture system following regular media changes.

Pancreatic tissues were fixed in Bouin's solution stained with hematoxylin-eosin (H&E) and immunohistochemistry (IHC) stains and examined microscopically to estimate the islet mass and determine insulin secretion ability. Under the light microscope, there were minimal connective tissue cells separating the islets from the surrounding exocrine component. The nuclei of islets cells were uniform in size and surrounded by eosinophilic cytoplasm. Purified islets of Langerhans were fixed in a mixture of paraformaldehyde and glutaraldehyde solution for transmission (TEM) and scanning electron microscopic (SEM) examination. Under TEM, the caprine islet cell exhibited their characteristic secretory granules, which were of various sizes and electron opacity. The cells also showed characteristic abundance of rough endoplasmic reticulum (RER) and mitochondria. In β-cells, the rough endoplasmic reticulum was inconspicuous. The α-cells are characterized by their peripheral location in the islet,

being larger and having electron-dense secretory granules. Based on morphological criterion, intermediate cells are shown to be present in both the endocrine and exocrine tissue of the normal pancreas of goats. Under SEM the size of the islet clusters was shown to range from 50 to 250 µm. Cells with secretory sacs on the surface could possibly be the isolated islet cells. The study had provided an optimized isolation and purification techniques for goat pancreatic islets to be further developed and used for xenotransplantation in diabetic animal models. The findings can lead to further research in identification and sequencing of insulin indicator genes, pancreatic hormones biomarkers and long-term cryopreservation of goat pancreatic islets.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENGASINGAN, PENULENAN DAN PENCIRIAN GUGUSAN PANKREAS KAMBING

Oleh

HOMAYOUN HANI

Mei 2009

Pengerusi: **Zeenathul Nazariah Bt Allaudin, PhD**

Fakulti: **Bioscience Institute (IBS)**

Diabetes melitus merupakan akibat lambat utama kepada pancreatitis kronik. Diabetes melitus tip 1 atau diabetes melitus bersandar insulin (IDDM) dicirikan oleh kegagalan islet Langerhans pankreas untuk sintesis atau rembes insulin. Kesulitan jangka panjang IDDM merupakan masalah kesihatan utama, dan pada pengidap diabetes tersebut insulin eksogenus mungkin tidak mencukupi untuk mengawalatur homeostasis glukosa darah, dan seterusnya gagal untuk mengelak kesulitan lambat ini. Untuk kes ini, pentransplanan islet pancreas boleh dipertikaikan sebagai pendekatan munasabah untuk mengembalikan semula homeostasis metabolisme. Kerana terhadnya penderma manusia membuatkan usaha mencari sumber alternative sel islet supaya kaedah pentransplanan pancreas boleh terus mengembang, menjadi mandatori. Kajian ini menyelidik potensi kambing sebagai sumber alternative islet pankreas. Objektif kajian ini bertujuan mengoptimum teknik pemencilan dan penulenan islet kaprin untuk menubuhkan kultur, dan menjalankan penilaian fungsi, morfologi dan kebolehhidupan islet kaprin. Tisu islet

kaprin diperolehi dalam tempoh 15 min selepas penyembelihan, diletakkan dalam larutan garam seimbang Hanks (HBSS) dan disengara pada suhu 4°C. Islet kaprin berjaya diperolehi melalui pencernaan dan pemencilan berasas kolagenase pada pH teroptimum 7.2 hingga 7.4 dan suhu 37°C. Islet pankreas tercerna ditulenkan mengguna cerun ketumpatan Euro-Ficoll. Ketulenan dan kebolehhidupan sel islet ditentukan masing-masing melalui pewarnaan ditizon dan tripan biru. Gugusan islet pelbagai saiz telah dikenalpasti secara positif melalui kedua-dua kaedah pewarnaan ini, dan apa yang dilihat ialah 90% daripada gugusan ini boleh hidup dalam sistem kultur. Berikutan penginkubatan statik ini, satu assai perembesan insulin *in vitro* telah dijalankan melalui ELISA untuk menentukan kebolehhidupan islet. Islet ini terus hidup selama 5 hari dalam sistem kultur selepas dilakukan penukaran media secara regular.

Tisu pankreas ditetapkan dalam larutan Bouin, diwarna dengan pewarna hematoksilin-eosin (H&E) dan imunohistokimia (IHC) dan diperiksa secara mikroskopi untuk menyukat jisim islet dan menentukan keupayaan rembesan insulin. Di bawah mikroskop cahaya, terdapat sedikit sahaja sel tisu penyambung yang mengasingkan islet daripada unsur eksokrin disekelilingnya. Nukleus sel islet sekata saiznya dan dikelilingi oleh sitoplasma eosinofilik. Islet Langerhans tertulen ditetapkan dalam larutan campuran paraformaldehid dan glutaraldehid untuk pemeriksaan mikroskopi elektron pancaran (TEM) dan imbasan (SEM). Di bawah TEM, sel islet kaprin menunjukkan granul rembesan cirian, iaitu pelbagai saiz dan kelegapan elektron. Sel ini juga menunjukkan retikulum endoplasma kasar (RER) dan mitokondrion banyak cirian. α-sel juga cirian dengan terletaknya secara periferi pada islet, dengan besarnya dan granul rembesan yang

elektron-tumpat. Berasaskan criteria morfologi ini, sel pengantara dilihat wujud dalam kedua-dua tisu endokrin dan eksokrin pada pankreas normal kambing. Di bawah SEM saiz gugusan islet didapati berjarak 50 hingga 250 µm. Sel mempunyai kantung rembesan pada permukaannya mungkin merupakan sel islet terpencil. Kajian ini menyediakan suatu teknik pemencilan dan penulenan teroptimum islet kambing untuk dikembangkan seterusnya dan diguna untuk xenotranplanan dalam model haiwan diabetes. Penemuan ini boleh menggalak penyelidikan lanjutan dalam pengenalpastian dan penjujukkan gen petunjuk insulin, biopetanda hormon pankreas dan krioawetan islet pankreas kambing.

ACKNOWLEDGMENTS

My utmost appreciation and gratitude are conveyed to my supervisor Dr. Zeenathul Nazariah Bt Allaudin for her invaluable guidance, constructive advice, comments and suggestions, patience and encouragement throughout the study. I would like to express my heartfelt thanks and appreciation to my co-supervisors, Prof. Dr. Tengku Azmi Tengku Ibrahim and Prof. Dr. Mohd Azmi Mohd Lila for their invaluable advice, suggestions and discussion, constructive criticisms, patience and support which were really helpful towards the completion of my study. Additionally, their efforts spent to improve the quality of the thesis are very much appreciated.

My sincere thanks and gratitude are extended to Dr. Abas Mazni Othman, Prof. Dr. Mohd Hair Bejo and Dr. Noorjahan Bano Alitheen, for granting permission to use the equipments and facilities in their laboratories and their precious support. I am grateful to the staff members of Virology Laboratory of Faculty of Veterinary Medicine, Animal Laboratory of Agro-biotechnology Institute (ABI) and Institute of Biosciences' Electron Microscopy Department (IBS, EMD), especially Mr. Mohd Kamarudin Awang Isa, Ms. Amlizawaty Amzah, Mr. Asraf Jamalludin, Dr. Fazly Ann and Mr. Ho Oi Kuan for their valuable critical and also technical assistances. And here I would like to express my special thanks to Pedram Kashiani for his great assistance in statistical analysis of this investigation data and also showing the right way to reach to scientific results. Also special thanks to Prof. Rasedee for sharing expertise in editing.

My greatest gratitude and thanks are dedicated to Nik Mohd Afizan, Lo Sewn Cen, Tam Yun Joon, Caryn Lim Shen Ni, Noraini Abd Said, Siti Nur Farahiyah, Siti Nazrina Camalxaman, Tan Seok Shin, Hadi Hajarian, Morvarid Akhvan Rezaie, Kazhal Sarsaifi, Parisa Honari, Nor Hidayah Mustafa, Ruzila Ismail and other members in Virology Laboratory, Faculty of Veterinary Medicine, Laboratory of Immunotherapeutic and Vaccine (LIVES) for their friendship, assistance and encouragement throughout the course of study.

Last but not least, I am indebted to my beloved parents, brothers for their endless encouragement, moral support, patience, understanding and unconditional love all the time.

I certify that a Thesis Examination Committee has met on 6 May 2009 to conduct the final examination of Homayoun Hani on his thesis entitled "Isolation, Purification and Characterization of Caprine Pancreatic Islets" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science of Medical Biotechnology.

Members of the Thesis Examination committee were as follow:

Abdul Rani Bahaman, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohd Hair Bejo, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Mohamed Ali Rajion, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Musalma Mazian, PhD
Professor
Faculty of Medicine
Universiti Kebangsaan Malaysia
(External Examiner)

_____

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 18 June 2009

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follow:

Zeenathul Nazariah Bt Allaudin, PhD
Senior Lecturer
Faculty of Veterinary Medicine/ Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Tengku Azmi Tengku Ibrahim, PhD
Professor
Faculty of Veterinary Medicine/ Institute of Bioscience
Universiti Putra Malaysia
(Member)

Mohd Azmi Mohd Lila, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 7 July 2009

DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously, and it not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other Institution.

HOMAYOUN HANI

Date:

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

µm	Micrometer
ABI	Agro-Biotechnology Institute
BBs	Brockmann Bodies
BCG	Bacillus Calmette-Guerin
BMI	Body Mass Index
DAB	3-3′-diaminobenzidine tetrahydrochloride
dl	Deciliter
DMSO	Dimethyl sulfoxide
DTZ	Dithizone
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscope
ENDIT	European Nicotinamide Diabetes Intervention Trial
EUR	European
g	Gravity
GDP	Gross Domestic Product
GH	Growth Hormone
GI	Gastrointestinal
H&E	Hematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
HRP	Horseradish Peroxidase
hr	Hour
IBS	Institute of Biosciences
IDDM	Insulin Dependent Diabetes Mellitus
IEQ	Islet Equivalent
IHC	Immunohistochemistry
L	Liter
M	Molar
mg	Milligeram
min	Minute

ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
OD	Optical Density
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
PI	Principal Islets
PP	Pancreatic Polypeptide
PRL	Prolactine
PYY	Peptide Tyrosine-Ttyrosine
RER	Rough Endoplasmic Reticulum
rpm	Revolution per Minute
RT	Room Temperature
SEA	South-East Asian
SEM	Scanning Electron Microscope
SST	Somatostatin
SST-14	Salmon Somatostatin-14
SST-25	Salmon Somatostatin-25
TBS	Tris Buffered Saline
TEM	Transmission Electron Microscope
UPM	Universiti Putra Malaysia
v/v	Volume/volume
WP	Western Pacific

CHAPTER 1

INTRODUCTION

Diabetes mellitus is the major late subsequences of chronic pancreatitis (Margener *et al*., 1997; Amman *et al*., 1999; Bernades *et al*., 1983; Malka *et al*., 2000). Some 246 million people worldwide have diabetes in 2007 (International Diabetes Federation, 2008). It is now one of the most common non-communicable diseases globally. Diabetes is the fourth or fifth leading cause of death in most developed countries and there is substantial evidence that it is epidemic in many developing and newly industrialized nations (International Diabetes Federation, 2005). It may cause life-threatening complications such as severe hypoglycemia or to chronic microvascular and macromascular complications (Levitt *et al*., 1995; Ziegler *at al*., 1994). A study of quality of life after pancreatic resections found that diabetes and its complication had the greatest negative influence on everyday well-being (Petrin *et al*., 1995). Diabetes may fasten and affect mortality rates in patients with chronic pancreatitis (Miyake *et al*., 1989; Traverso *et al*., 1997; Levy *et al*., 1989).

Complications from diabetes, such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure, and blindness result in increasing disability, reduced life expectancy and enormous health costs for virtually every society. Diabetes is certainly one of the most challenging health problems of the 21st century.

Diabetes mellitus is a syndrome which is characterized by abnormally high glucose levels in the blood. The most common types of diabetes are Type 1 (immune-mediated diabetes mellitus), and Type 2 (insulin-resistant diabetes mellitus). A third type, (gestational diabetes mellitus), occurs during some pregnancies. All these types of diabetes have similar symptoms, because all forms of the disease are due to the high level of sugar, or glucose, in the blood, resulting in the incapability of the body to remove glucose from blood and deliver it to the cells. The cells use glucose as a source of energy to continue their metabolism. The reasons why the body cannot use glucose from the blood are different for Type 1 and Type 2 diabetes (American Diabetes Association, 2006).

Type 1 diabetics do not produce sufficient insulin, a small protein produced by the pancreas that helps the body utilize or store glucose from food. These diabetics can be treated with insulin. In contrast, Type 2 diabetics, viz women with gestational diabetes, produce insulin, but for some reason, either the cells in their bodies are resistant to insulin's action or they do not produce enough insulin. In all types of diabetes, glucose that do not reach the cells and tissues accumulates in the blood (American Diabetes Association, 2006).

Incidence of Type 1 diabetes in children and adolescents is increasing with an estimated overall annual increase of around 3% (Onkamo *et al*., 1999; Eurodiab ACE Study Group, 2000; Green *et al*., 2001). The increase in incidence in Type 1 diabetes has been shown in countries with both high and low prevalence with an indication of a steeper increase in some of the low prevalence countries (Tuomilehto *et al*., 1995; Gardner *et*

al., 1997; Dahlquist *et al*., 2000). In Malaysia, nearly 1.2 million people have diabetes, of which an estimated 24,000 people or 2% of the whole diabetic population with Type 1. This means approximately 0.1% of the whole population of Malaysia are suffering from Type 1 diabetes (Persatuan Diabetes Malaysia, 2006).

Considerable advances have been made in the technology of transplanting either pancreas or preparations of islet tissues, but major problems remain in obtaining donor tissues and in preventing immune rejections of the graft. Nevertheless, transplantation is the only available treatment that can lead to insulin independence (Shapiro *et al*., 2000), but human islet allograft transplantation could not be used on a large scale in clinical practice. Results following whole pancreas transplantation are very encouraging with about one-year graft survival rate of 85-90% (Landgraf, 1996). Islet transplants appeared to be much more vulnerable (Steven, 1999; Cretin *et al*., 1998; Sutherland *et al*., 1989; Sibley *et al*., 1985; Morris *et al*., 1989; Davalli *et al*., 1996) as many failed within few weeks or months after engraftment and most islet transplants (> 90%) failed within one year (Nakagawa *et al*., 1999; Steven, 1999; Cretin *et al*., 1998). The reasons for these functional failures are unknown, although insufficient numbers of islets, engraftment difficulties, chronic rejection and recurrence of autoimmune disease were suggested to be contributing factors (Cretin *et al*., 1998; Sutherland *et al*., 1989; Sibley *et al*., 1985; Morris *et al*., 1989). Hyperglycemia in the recipient after transplantation has been shown to deteriorate islet graft survival and function (Davalli *et al*., 1996).

One of the major obstacles for clinical islet transplantation is the lack of donors and optimization of the number of β-cells harvested from each donor. Another possibility is

to stimulate the growth and/or differentiation of β-cells, or to genetically manipulate insulin producing cell lines for transplantation. Several studies have shown that differentiated β-cells still have the ability to proliferate at a low pace (Hellerström *et al*., 1976; Hellerström, 1984; Scharfmann and Czernichow, 1996; Rane and Reddy, 2000). The proliferation rate can be affected in many ways, for example by growth stimulating hormones and prolactin (Sorenson *et al*., 1993; Stout *et al*., 1997; Carlsson *et al*., 1997; Tyrberg *et al*., 1996). The size and composition of the graft and the blood glucose level in the recipient are of crucial importance for β-cell replication (Tyrberg, 1999).

Xenotransplantation using islets derived from animals is one critical approach that might solve these problems. Over the last 40 years, chimpanzee kidneys have been transplanted into patients with renal failure (Reemtsma *et al*., 1964); a baboon liver has been transplanted to a patient with hepatic failure (Starzl *et al*., 1993); porcine islet cells of langerhans have been injected into patients with Type 1 diabetes (Rood *et al*., 2006); porcine skin has been grafted onto patients with burn (Chatterjee, 1978); and pig neuronal cells have been transplanted into patients with Parkinson's disease and Huntington's disease (Fink *et al*., 2000). Other strategies include immune modulation to reduce or prevent immune attack by the recipient's immune system, immunoisolation to prevent recognition of the islet graft, induction of tolerance and gene therapy (Thomas *et al*., 2000).

Ethical considerations have led to the selection of the pig as the most likely source of organs for humans, but there are significant biological barriers that hamper long term organ survival and function in recipients. It is clear that some genetic manipulation of

the donor animals will be required to overcome these barriers (Xenotransplantation Program, 2005).

The limited availability of human donors makes the research for alternative islet sources mandatory for future development in pancreatic islet transplantation. Xenotransplantation of islets has become a very crucial research area with commercial potential.

The objectives of present study were:

1. To establish feasible methods of isolation, separation and *in vitro* culture of goat pancreatic islet cells.

2. To assess and ascertain the functionality, viability and sustainability of the goat pancreatic islet cells in cell culture.

CHAPTER 2

LITERATURE REVIEW

2.1. Development of Pancreas

One of the most vital organs in the digestive and endocrine system of vertebrates is the pancreas which is an encapsulated, lobulated, compound tubuloacinar gland organ. It is divided into an exocrine (secreting pancreatic juice containing digestive enzymes) and an endocrine (producing several important hormones, including insulin, glucagon, and somatostatin) portion (Mac, 2000). During embryonic development of the pancreas, islet, acinar, and ductal cells are differentiated from a common multipotential precursor cell. In early embryonic development, the endocrine cells are integrated within the exocrine matrix of the pancreatic bud. They subsequently accumulate in nonvascularized clusters and later become isolated from the exocrine tissue and independently vascularized (William, 1995).

In the fetus, nutrients are carried across the placenta and not through the fetal gastrointestinal (GI) tract, and nutrient flow is moderately steady. A series of experimental studies on fetuses suggest that the endocrine pancreas is required for fetal nutrition and growth. However, both the endocrine and exocrine entities of the pancreas are relatively immature in structure and function, even in the late pregnancy period, and mature function is not present until several weeks after birth. Pancreatic development seems to be particularly rapid around the time of birth, and the first intake of enteral milk stimulates these maturational changes (Sangild, 1999).