การรักษาโรคเบาหวานด้วยเซลล์ต้นกำเนิดบำบัดในสัตว์ทดลองร่วมกับการใช้สารสกัดแป๊ะตำปึง

นางสาวปิยะภรณ์ รัตนนิลสรวง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DIABETIC TREATMENT BY STEM CELL BASED THERAPY *IN VIVO* IN COMBINATION WITH *GYNURA PROCUMBENS* EXTRACT

Piyaporn Rattananinsruang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

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Academic Year 2011

DIABETIC TREATMENT BY STEM CELL BASED THERAPY

IN VIVO IN COMBINATION WITH

GYNURA PROCUMBENS EXTRACT

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ปิยะภรณ์ รัตนนิลสรวง : การรักษาโรคเบาหวานด้วยเซลล์ต้นกำเนิดบำบัดในสัตว์ทดลองร่วมกับการใช้สารสกัดแป๊ะตำปึง (DIABETIC TREATMENT BY STEM CELL BASED THERAPY *IN VIVO* IN COMBINATION WITH *GYNURA PROCUMBENS* EXTRACT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ทนพญ.ดร.วิไลรัตน์ ลี้อนันต์ศักดิ์ศิริ, 182 หน้า.

เบาหวานเป็นโรคที่มีระดับน้ำตาลกลูโคสในกระแสเลือดสูงผิดปกติ ซึ่งการมีภาวะเบาหวานเป็นเวลานานจะส่งผลให้เกิดอาการแทรกซ้อนที่เป็นอันตรายต่อร่างกาย ดังนั้นการรักษาโรคเบาหวานจึงมีความสำคัญเป็นอย่างมาก ในการศึกษาครั้งนี้ได้ทำการทดสอบความสามารถของเซลล์ต้นกำเนิดบำบัดและสารสกัดจากแป๊ะตำปึงในการรักษาโรคเบาหวาน ซึ่งแบ่งออกเป็น 3 วิธีได้แก่ 1) การใช้เซลล์ที่ผลิตอินซูลินแบบไม่มีเยื่อหุ้มหรือมีเยื่อหุ้ม ซึ่งเซลล์นี้พัฒนามาจากเซลล์ต้นกำเนิดตัวอ่อนมนุษย์โดยทดสอบในหนูเมาส์ 2) สารสกัดแป๊ะตำปึงร่วมกับการใช้เซลล์ต้นกำเนิดชนิดมีเซ็นไคม์ที่แยกจากวาตันเจลลี่ของสายสะดือจากรกโดยทดสอบในหนูเมาส์ 3) การใช้เซลล์ต้นกำเนิดมีเซ็นไคม์เพียงอย่างเดียวโดยทดสอบในหนูแรท การศึกษาครั้งนี้ประสบผลสำเร็จในการสร้างเซลล์ที่ผลิตอินซูลินจากเซลล์ต้นกำเนิดตัวอ่อนมนุษย์โดยวิธีที่พัฒนาขึ้นมาใหม่ ผลการศึกษาพบว่าหนูที่เป็นเบาหวานเมื่อได้รับการฉีดรักษาด้วยเซลล์ที่ทำหน้าที่ผลิตอินซูลินซึ่งพัฒนามาจากเซลล์ต้นกำเนิดตัวอ่อนมนุษย์แบบไม่มีเยื่อหุ้มหรือมีเยื่อหุ้ม ให้ผลควบคุมระดับน้ำตาลในเลือดหลังจากทำการรักษา นอกจากนี้ยังสามารถลดระดับอินเตอร์ลิวคินวันเบต้าซึ่งเป็นไซโตไคน์ที่หลั่งออกมาเมื่อมีการอักเสบและเหนี่ยวนำให้เกิดการอักเสบเพิ่มขึ้นได้ รวมทั้งช่วยลดอัตราเสี่ยงต่อการเกิดภาวะหลอดเลือดแข็งตัว อย่างไรก็ตามการรักษาด้วยวิธีนี้ไม่มีผลต่อระดับยูเรียและครีเอตินินในเลือดของหนูเบาหวาน จากผลการทดลองยังพบว่าสารสกัดแป๊ะตำปึงเพียงอย่างเดียวหรือการรักษาแบบผสมผสานระหว่างสารสกัดแป๊ะตำปึงร่วมกับการฉีดรักษาด้วยเซลล์ต้นกำเนิดชนิดมีเซ็นไคม์สามารถควบคุมโรคเบาหวานในหนูที่เป็นเบาหวานได้ดี ยิ่งไปกว่านี้ยังพบว่าการฉีดรักษาด้วยเซลล์ต้นกำเนิดชนิดมีเซ็นไคม์เพียงอย่างเดียวส่งผลทำให้หนูที่เป็นเบาหวานมีระดับน้ำตาลในเลือดลดลง จากการทดลองนี้สรุปได้ว่าเซลล์ต้นกำเนิดบำบัดและสารสกัดแป๊ะตำปึงสามารถใช้เป็นการแพทย์ทางเลือกสำหรับป้องกันและรักษาโรคเบาหวานได้

สาขาวิชาจุลชีววิทยา ลายมือชื่อนักศึกษา_____

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PIYAPORN RATTANANINSRUANG : DIABETIC TREATMENT BY STEM CELL BASED THERAPY *IN VIVO* IN COMBINATION WITH *GYNURA PROCUMBENS* EXTRACT. THESIS ADVISOR : ASST. PROF. WILAIRAT LEEANANSAKSIRI, Ph.D. 182 PP.

HUMAN EMBRYONIC STEM CELLS-DERIVED INSULIN-PRODUCING CELLS/HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS/*GYNURA PROCUMBENS*/DIABETES

Diabetes mellitus is a group of diseases characterized by abnormally high levels of glucose in the blood stream. Diabetes can cause a wide range of chronic complications that affect almost every parts of the body. Thus, diabetes treatment is extremely important. The study was designed to investigate whether stem cell therapy and *Gynura procumbens* extract can be used as possible sources for diabetes treatment. Three approaches have been used in this project 1) human embryonic stem cell derived insulin-producing cells (hES-DIPCs) with either non-capsulation or encapsulation in mouse model 2) combination of human mesenchymal stem cell isolated from Wharton's jelly (hWJ-MSCs) in mouse model 3) hWJ-MSCs alone in rat model. This study revealed the achievement of hES-DIPCs *in vitro* by our new differentiation protocol. After transplantations, the ability of the cells in hyperglycaemic regulation and other blood chemistry tests has been evaluated. The results showed that subcutaneous transplantation of both non-encapsulated and encapsulated hES-DIPCs could control fasting blood glucose levels *in vivo*. In addition, transplantation of either non-encapsulated or encapsulated hES-DIPCs could

also reduce IL-1β inflammatory cytokine level and atherogenic index (AI). However, there is no significant effect on blood urea nitrogen (BUN) and creatinine. Our data also demonstrated that the administration of *G. procumbens* alone or combined with hWJ-MSCs transplantation revealed the ability to control the diabetes *in vivo*. Furthermore, the transplantation of hWJ-MSCs alone exhibited the ability to reverse hyperglycemia in diabetic recipients. Therefore, stem cell based therapy and *G. procumbens* extract can serve as alternative medicines for the prevention and treatment of diabetes mellitus.

School of Microbiology

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LIST OF ABBREVIATIONS

°C	=	degree Celsius
µl	=	microliter
µm	=	micromiter
3D	=	three dimensional
ADP	=	adenosine diphosphate
AI	=	atherogenic index
AS	=	adult skin
ATCC	=	American Type Culture Collection
BC	=	Before Christ
BETA2	=	β cell e-box transactivation 2
bFGF	=	basic fibroblast growth factor
bHLH	=	basic helix-loop-helix
BM	=	bone marrow
BSA	=	bovine serum albumin
BUN	=	blood urea nitrogen
CA	=	California
$CaCl_2$	=	calcium chloride
CAN	=	cardiac autonomic neuropathy
CD	=	cluster of differentiation
cDNA	=	complementary DNA

LIST OF ABBREVIATIONS (Continued)

cGMP	=	current Good Manufacturing Procedures
CIT	=	conventional insulin treatment
CO_2	=	carbon dioxide
COX2	=	cyclooxygenase 2
CSII	=	continuous subcutaneous insulin infusion
CVD	=	cardiovascular disease
CXCR4	=	chemokine receptor type 4
D	=	day
DAN	=	diabetic autonomic neuropathy
DAPI	=	4,6-diamidino-2-phenylindole
Dazl	=	deleted in Azoospermia
DCCT	=	Diabetes Control and Complications Trial
DE	=	definitive endoderm
DM	=	diabetes mellitus
DMSO	=	dimethylsulfoxide
DNA	=	deoxyribonucleic acid
DPN	=	distal symmetrical sensorimotor polyneuropathy
DPP-4	=	dipeptidyl peptidase-4
DR	=	diabetic retinopathy
DSME	=	diabetes self-management education
DTZ	=	dithizone
E	=	embryonic day

LIST OF ABBREVIATIONS (Continued)

e.g.	=	for example
EBs	=	embryoid bodies
EDTA	=	ethylenediaminetetraacetic acid
EGF	=	epidermal growth factor
ELISA	=	insulin enzyme-linked immunosorbent assay
ESCs	=	embryonic stem cells
FBS	=	fetal bovine serum
FGF2	=	fibroblast growth factor-2
FITC	=	fluorescein isothiocyanate
FM	=	fetal muscle
Foxa2	=	forkhead box protein A2
FPG	=	fasting plasma glucose
g	=	gram
g/kg	=	gram per kilogram
GAD	=	glutamic acid decarboxylase
GADA	=	glutamic acid decarboxylase
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
GCTM	=	germ cell tumor marker
Gdf3	=	growth differentiation factor-3
GDM	=	gestational diabetes mellitus
GI	=	gastrointestinal
GK	=	glucokinase

LIST OF ABBREVIATIONS (Continued)

GLP-1	=	glucagon-like peptide-1
GLUT1	=	glucose transporter-1
GLUT2	=	glucose transporter-2
GPE	=	*Gynura procumbens* extract
GSIS	=	glucose-stimulated insulin secretion
h	=	hour
HBO	=	hyperbaric oxygen
hBPCs	=	human adult breast parenchymal cells
HBSS	=	Hank’s balanced salt solution
HD	=	hanging drop
HDL	=	high-density lipoprotein
hEFs	=	human embryonic fibroblasts
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESC-dFs	=	hESC-derived fibroblasts
hESCs	=	human embryonic stem cells
hES-DIPCs	=	human embryonic stem cell-derived insulin-producing cells
HFF-1	=	human foreskin fibroblast-1
HGF	=	hepatocyte growth factor
HIV	=	human immunodeficiency virus
HLA	=	human leukocyte antigen
hMSCs	=	human mesenchymal stem cells

LIST OF ABBREVIATIONS (Continued)

Hnf	=	hepatocyte nuclear factor
hnRNA	=	heterogeneous nuclear RNA
HSV-1	=	herpes simplex virus type 1
hUECs	=	human adult uterine endometrial cells
hWJ-MSCs	=	human Wharton's jelly derived mesenchymal stem cells
IA-2	=	islet antigen 2
IA–2A	=	IA-2 molecule
IAA	=	insulin autoantibodies
IAPP	=	islet amyloid polypeptide
ICA	=	islet cell antibodies
ICAs	=	islet-like cell aggregates
ICM	=	inner cell mass
ID	=	idiopathic type 1 diabetes
IDDM	=	insulin-dependent diabetes mellitus
IDO	=	indoleamine 2,3-dioxygenase
IFG	=	impaired fasting glucose
IFNγ	=	interferon-gamma
IGF	=	insulin-like growth factor
IGT	=	impaired glucose tolerance
IL	=	interleukin
ILCs	=	insulin-producing islet-like clusters
INS	=	insulin

LIST OF ABBREVIATIONS (Continued)

InterASIA	=	International Collaborative Study of Cardiovascular Disease in Asia
IP	=	intraperitoneally
IPF-1	=	insulin promoter factor-1
IPGTT	=	intraperitoneal glucose tolerance test
iPSCs	=	induced pluripotent stem cells
Isl1	=	ISLET1
ITSF	=	insulin-tranferin-selenium-fibronectin
IV	=	intravenously
IVF	=	*in vitro* fertilization
JDS	=	Japan Diabetes Society
kD	=	kiloDalton
KIR6.2	=	KATP channel subunits
KO-DMEM	=	knockout Dulbecco's modified Eagle's medium
KO-SR	=	knockout serum replacement
KRBH	=	Krebs-Ringer bicarbonate HEPES
LDL	=	low-density lipoprotein
M	=	molar
MC	=	methylcellulose
MCM	=	methylcellulose semisolid medium
MDD	=	mean minimum detectable dose
MEF	=	mouse embryonic fibroblasts

LIST OF ABBREVIATIONS (Continued)

mESC	=	mouse embryonic stem cells
mg/dl	=	milligram per desiliter
mg/kg	=	milligram per kilogram
MHC	=	major histocompatibility complex
mHC	=	minor histocompatibility complex
MIT	=	multiple daily insulin injections therapy
ml	=	milliliter, volume
mM	=	millimolar
mmol/l	=	millimoles per liter
MODY	=	maturity-onset diabetes of youth
MOPS	=	3-(N-morpholino) propanesulfonic acid
mRNA	=	messenger RNA
MSCs	=	mesenchymal stem cells
NaCl	=	sodium chloride
NAFLD	=	non-alcoholic fatty liver disease
Nanos 1	=	Nanos Homologue 1
NeuroD1	=	neurogenic differentiation 1
ng/ml	=	nanogram per milliliter
Ngn3	=	neurogenin 3
NGSP	=	National Glycohemoglobin Standardization Program
NIDDM	=	non-insulin-dependent diabetes mellitus
NOD	=	nonobese diabetic

LIST OF ABBREVIATIONS (Continued)

NT	=	nuclear transfer
NY	=	New York
O_2	=	oxygen
Oct4	=	octamer-4
OGTT	=	oral glucose tolerance test
PANC-1	=	human pancreas carcinoma cell line
Pax	=	paired box
PBS	=	phosphate buffer saline
PC	=	prohormone convertase
PCR	=	polymerase chain reaction
PDM	=	pregestational diabetes mellitus
Pdx1	=	pancreatic-duodenal homeobox 1
PG	=	plasma glucose
pg/ml	=	pictogram/milliliter
PGE2	=	prostaglandin E2
pH	=	log of hydrogen concentration
PP-cell	=	pancreatic polypeptide-cell
Ptf1a	=	pancreas transcription factor 1
Pum	=	pumilio gene
rER	=	rough endoplasmic reticulum
Rex-1	=	RNA exonuclease 1
RNA	=	ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

RNAi	=	RNA interference
RPE	=	rat pancreatic extract
RT	=	reverse transcription
S.E.M.	=	standard error of mean
SC	=	subcutaneously
SCID	=	severe combined immunodeficiency
Shh	=	Sonic Hedgehog
Sox	=	SRY-box containing gene
SSEA	=	stage-specific antigens
STZ	=	streptozotocin
SUR1	=	sulfonylurea receptor 1
T1D	=	type 1 diabetes
T2D	=	type 2 diabetes
Tdgf1	=	teratocarcinoma-derived growth factor 1
TEM	=	transmission electron micrograph
TG	=	trophoblast giant
TGF-β	=	transforming growth factor-β
Thy-1	=	thymus cell antigen 1
TNFα	=	tumor necrosis factor-alpha
TRA	=	tumor recognition antigen
U	=	unit
U/ml	=	unit per milliliter

LIST OF ABBREVIATIONS (Continued)

UCB	=	umbilical cord blood
US$	=	The United States dollar
w/w	=	weight per weight
WBC	=	white blood cell count
WHO	=	World Health Organization
WJ	=	Wharton’s jelly
α-cell	=	alpha-cell
β-cell	=	beta-cell
δ-cell	=	delta-cell

CHAPTER I

INTRODUCTION

1.1 Background

Diabetes mellitus is one of the most common chronic diseases that threaten the health and health economics in every country of the world. The prevalence of diabetes in the world's population will rise from 151 millions in the year 2000, to 221 millions by the year 2010 and to 300 millions by 2025 (Zimmet, Shaw, and Alberti, 2003). In Thailand, the estimated prevalence of diabetes in Thai adults was 9.6% (2.4 millions people) (Aekplakorn et al., 2003). Type 1 diabetes (T1D) is an autoimmune destruction of the pancreatic β-cells and affects children and young adults. T1D known as insulin-dependent diabetes mellitus (IDDM) that can be treated by exogenous insulin treatment. Whereas the most common form of diabetes is type 2 diabetes (T2D). In T2D, the body does not produce enough insulin or the cells do not use insulin properly. This type of diabetes is called non-insulin-dependent diabetes mellitus (NIDDM), obesity related diabetes or adult-onset diabetes. This type can be prevented or delayed by management of diet, exercise and weight reduction (Kahn et al., 2005). When the disease progresses, T2D patients finally require insulin to maintain blood glucose level (Bethel and Feinglos, 2005). The clinician will include the insulin therapy in treatment strategy for improving symptoms, enhancing quality of life and provide a sense of well-being (White et al., 2003). Although

diabetic patients have benefited from insulin therapy, the intensive treatment can cause hypoglycemia (Bernroider et al., 2007).

At present, islet cell transplantation is the most potential source for diabetes treatment. Transplantation of islet cells has demonstrated normoglycemia in the absence of exogenous insulin therapy. Nevertheless, the limitation of islet cell replacement are the following factors: non-functioning of isolated islets, the small number of transplanted islets, the immunogenicity of isolated islets, transplantation to inappropriate sites, recurrence of auto-immunity in the transplanted islets and rejection and immunosuppression (Gunasekaran, 2003). Therefore, the scientists are interested in human embryonic stem cells (hESCs). The hESCs are more interesting because they have the differentiation potential to become several tissues in the body, while adult stem cells have limited differentiation capacity. The strategies for differentiation of hESCs into insulin-producing cells have been demonstrated by many research groups. The insulin-producing cells are expressed the markers that are associated with pancreatic β-cell differentiation pathway. These cells can also produce and secrete insulin in response to glucose concentration (Assady et al., 2001; Baharvand, Jafary, Massumi and Ashtiani, 2006b; Segev, Fishman, Ziskind, Shulman and Itskovitz-Eldor, 2004). However, recent evidence indicates that these cells may not be capable of de novo insulin synthesis and in fact are principally secreting insulin absorbed from the culture medium (Rajagopal, Anderson, Kume, Martinez and Melton, 2003). Moreover, these methods maintain undifferentiated hESCs on mitotically inactivated mouse embryonic fibroblasts (MEF) feeder layers which can provide the animal pathogen contamination to hESCs (Mallon, Park, Chen, Hamilton

and McKay, 2006). Therefore, the insulin-producing cells differentiation protocols are still subjected to improvement.

Recently, human mesenchymal stem cells (hMSCs) have provided an attractive source for tissue engineering, regenerative medicine and autoimmune disease treatment. The ability of self-renewal and multipotency make hMSCs an effective tool for cell-replacement therapy. In addition, several characteristics of hMSCs such as migration and homing potential, immunomodulatory properties and trophic effect, could raise their clinical applications in various disorders. Moreover, its ability to secrete bioactive molecules such as growth factors, cytokines and chemokines has been shown to mediate paracrine mechanisms of hMSCs (Xu et al., 2008). Also, it is considered that they lead to reformation of tissues at sites of injury (da Silva Meirelles, Fontes, Covas and Caplan, 2009). These cells from multiple sources were found to improve a variety of disease conditions in animal models. Interestingly, hMSCs can be isolated from the Wharton's jelly of the umbilical cord (hWJ-MSCs). They have self-renewal and have a higher rate of proliferation when compared to adult stem cells (Secco et al., 2008). Human umbilical cord is taken at the time of a newborn's delivery that would normally be discarded as a medical waste. Therefore, Wharton's jelly is an easily accessible source and has no ethical controversy for the isolation of MSCs (Nekanti et al., 2010; Secco et al., 2008).

There have several reports of morbidity and mortality associated with the use of drug treatment in diabetic patients (Eurich et al., 2007). These effects have contributed to the increase in the use of medicinal plants as an alternative treatment of diabetic patients. Many medicinal plants have been reported the properties such as antihyperglycemic and antihyperlipidemic activities in streptozotocin (STZ)-induced

diabetic rats. In diabetic rats, the improvement in body weight, blood glucose, urine glucose and lipid profile were observed when treated with medicinal plants extract (Eidi, Eidi and Esmaeili, 2006; Hsu et al., 2003; Noor, Gunasekaran, Soosai Manickam and Vijayalakshmi, 2008; Salahuddin and Jalalpure 2010; Singh, Kesari, Gupta, Jaiswal and Watal, 2007). In folk medicine, the fresh form of leaves of *Gynura procumbens* is routinely used to treat diabetes. The ethanolic extract of *G. procumbens* significantly reduced serum cholesterol and triglyceride levels as well as suppressed the elevated serum glucose levels in diabetic rats (Zhang and Tan, 2000). The aerial part of *G. procumbens* exhibited anti-inflammatory activities (Iskander, Song, Coupar and Jiratchariyakul, 2002). It has been shown that the hypotensive effect of *G. procumbens* extract mediated by nitric oxide (NO) production in blood vessels (Kim, Lee, Wiryowidagdo and Kim, 2006). Moreover, this plant has been reported to protect the gastric mucosa against ethanol-induced injury in rats (Mahmood, Mariod, Al-Bayaty and Abdel-Wahab, 2010).

1.2 Research objectives

This study aims to investigate new approaches for the treatment of diabetes. There were three major objectives in this study; 1) to produce insulin-producing cells from undifferentiated hESCs (hES-DIPCs) and evaluate the capability of hES-DIPCs for diabetic treatment *in vivo*; 2) to examine the antidiabetic effect of the combination of *G. procumbens* extract (GPE) and Wharton's jelly derived mesenchymal stem cells (hWJ-MSCs) in diabetic animal model; and 3) to investigate whether hWJ-MSCs can be used as a possible source for diabetes treatment.

CHAPTER II

LITERATURE REVIEW

2.1 Diabetes mellitus

The first description of diabetes started in approximately 1550BC. An ancient Egyptian papyrus is thought to be the first reference to the disease. It mentions a rare disease that causes the patient to lose weight rapidly and urinate frequently. Diabetes mellitus comes from the Greek words for ‘siphon’ and ‘sugar’ and describes the most obvious symptom of uncontrolled diabetes: the passing of large amounts of urine, which is sweet because it contains sugar. Research in the last hundred years has led to a greatly improved understanding of diabetes. Two Canadian Scientist, Frederick Banting and Charles Best, discovered in 1921 that insulin was produced in the pancreas by the islets of Langerhans. They were awarded the Nobel Prize in Medicine for their discovery two years later (Sattley, 2008).

2.1.1 Epidemiology

The prevalence of diabetes in the world’s population will rise from 151 millions in the year 2000, to 221 millions by the year 2010 and to 300 millions by 2025 (Zimmet, Shaw and Alberti, 2003) (Figure 2.1). The expected population growth between 2000 and 2003 will be focused in developing countries (Wild, Roglic, Green, Sicree and King, 2004). There will be a 170% increase, from 84 to 228 millions in the developing countries and the majority of people with diabetes are in the age range of 45-64 years (King, Aubert and Herman, 1998). In contrast, the majority of people

with diabetes in developed countries are > 64 years of age (Wild, Roglic, Green, Sicree and King, 2004).The global diabetes prevalence is similar in men and women but it is slightly higher in men < 60 years of age and in women at older ages (Wild, Roglic, Green, Sicree and King, 2004). There are more women than men with diabetes in many countries particularly in the developed countries, a probable explanation is the greater long life of women.

The International Collaborative Study of Cardiovascular Disease in Asia (InterASIA) has demonstrated that diabetes and impaired fasting glucose (IFG) are common in Thai adults. These conditions are associated with adverse cardiovascular risk factors. The estimated national prevalence of diabetes in Thai adults was 9.6% (2.4 millions people), which included 4.8% of previously diagnosed and 4.8% of newly diagnosed subjects. The prevalence of IFG was 5.4% (1.3 millions people). The prevalence of diabetes and IFG in Thailand have risen to Western levels which appears to be ascribing to change in demographic factors including the greater age of the population, the increased proportion living in an urban area and increasing levels of obesity (Aekplakorn et al., 2003).

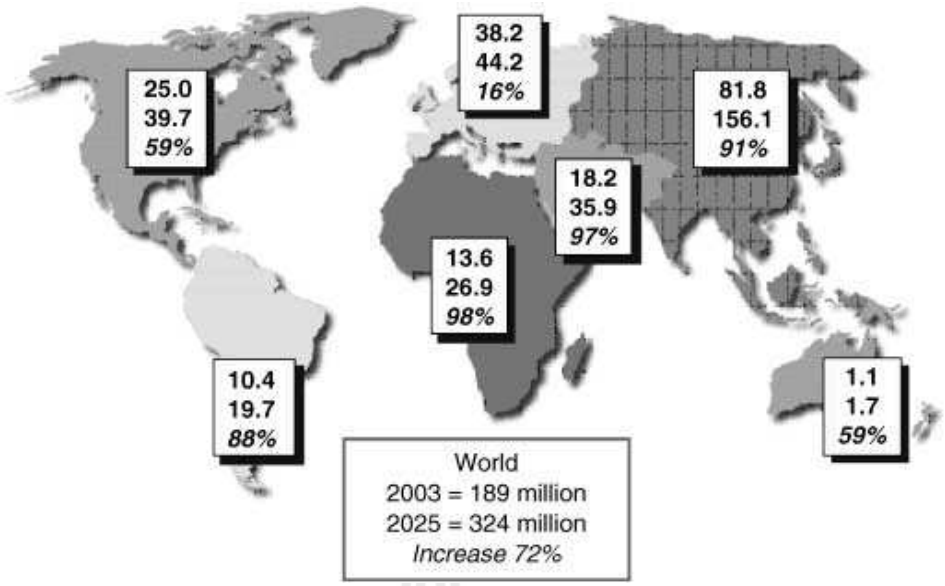

Figure 2.1 Prevalence of diabetes in the world's population (Zimmet, Shaw and Alberti, 2003).

2.1.2 The classification of diabetes mellitus

Diabetes mellitus is a group of diseases presented with chronic hyperglycemia and other metabolic disorders, which are due to the inability of body to produce or properly use insulin. The diabetic state is associated with complications (retinopathy, nephropathy, neuropathy and arteriosclerosis) after a long duration of the disease (Gavin, 1998; Kuzuya et al., 2002). In the Committee of the Japan Diabetes Society (JDS) report, the etiological classification of diabetes and related disorders of glycemia includes, 1) type 1; 2) type 2; 3) other types of diabetes due to specific causes; and 4) gestational diabetes mellitus (Kuzuya et al., 2002).

Type 1 diabetes (T1D) was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. T1D is an autoimmune disease of which

the immune system attacks and destroys the insulin-producing β cells in the pancreas. This type of diabetes usually progresses to the absolute insulin deficiency stage. It develops most often in children and young adults but may appear at any age, sometimes with slow progression (Knip and Siljander, 2008; Kuzuya et al., 2002).

T1D is characterized by selective loss of insulin-producing β-cells in the pancreatic islets in genetically susceptible subjects. It has been demonstrated that only 10-20% of the β-cells are still functioning at the time of diagnosis. In addition, in family studies and also in general population cohorts, the number of detectable autoantibodies is unequivocally related to the risk of progression to overt T1D. The active players contributing to β-cell destruction are autoreactive T cells, both CD4 and CD8 cells. The autoantigens including insulin, glutamic acid decarboxylase (GAD), the protein tyrosine phosphatase-related islet antigen 2 (IA-2), and most recently the zinc transporter Slc30A8 residing in the insulin secretory granule of the β-cell have been identified in T1D. The important genes located in the HLA class II locus on chromosome 6 have been implicated in disease susceptibility. In addition, there are ten other genes or genetic regions that have been observed to be associated with T1D. Furthermore, it has been documented that islet cell antibodies (ICA), insulin autoantibodies (IAA), auto-anti-bodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-related IA-2 molecule (IA–2A) antibodies can be used to predict clinical T1D (Knip and Siljander, 2008). Autoantibodies to islet cell antigens can be detected in 70-90% of the patients, particularly at the early period after onset. Autoantibodies or the loss of acute insulin response to intravenous glucose may be detected before the occurrence of clinical symptoms or hyperglycemia. However, autoantibodies to islet antigens are never

detected in some T1D patients (idiopathic type 1 diabetes). In the future, T1D patients may be classified into subtypes according to mode of onset (i.e. acute or slowly-progressive), HLA antigens or epitopes of autoantigens (Kuzuya et al., 2002).

Type 2 diabetes (T2D) was previously called non-insulin-dependent diabetes mellitus (NIDDM), obesity related diabetes or adult-onset diabetes. It usually preserves some extent of pancreatic β-cells mass and their function. It seldom needs insulin injection to sustain life. However, ketoaciosis could occur in the presence of severe infection or other stress. This state is ranging from predominantly insulin secretory defect, to a predominantly insulin resistance with varying degree of insulin secretory defect. Its pathogenesis involves the combinations of decreased insulin secretion and decreased insulin sensitivity (insulin resistance) (Kuzuya et al., 2002).

It is the most common form of diabetes and is associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity, and race/ethnicity. African Americans, Hispanic/Latino Americans, American Indians, and some Asian Americans and Native Hawaiians or other Pacific Islanders are at particularly high risk for T2D. T2D is increasingly diagnosed in children and adolescents. Studies show that people at high risk for T2D can prevent or delay the onset of the disease by maintaining a healthy diet and regular exercise (Gavin, 1998; Kahn et al., 2005; National Diabetes Information Clearinghouse, 2008).

Other types of diabetes due to specific causes

This diabetic state is further classified into two major groups; A) those in which specific mutations have been identified as a course of genetic susceptibility and B) those associated with other specific diseases or conditions.

A) Diabetes in which specific mutations have been identified as a course of genetic susceptibility. The genetic susceptibility for diabetes includes genetic abnormalities of pancreatic β-cell function and insulin action. This group with known genetic defects of β-cell function comprise of insulin gene abnormalities and MODY (maturity-onset diabetes of youth) cases. Abnormal insulinemia and proinsulinemia are characteristic features of insulin gene abnormalities condition. MODY is related to the mutations of genes for Hnf4α (MODY 1), Glucokinase (MODY 2), Hnf1α (MODY 3), IPF1 or Pdx1 (MODY 4) and Hnf1β (MODY 5). Abnormalities of mitrochrondrial DNA and amylin gene are also identified as genetic defects of β-cell function. A number of insulin receptor gene mutations such as type A insulin resistance, leprechaunism and Rabson-Mendenhall syndrome are included in this category (Garvin, 1998; Kahn et al., 2005; Kuzuya et al., 2002).

B) Diabetes associated with other pathologic conditions or disease. This group includes persons with exocrine pancreatic diseases, endocrine diseases, liver diseases, drug- or chemical-induced pathology, viral infections, rare forms of immune-mediated diabetes and various genetic syndromes. With known underlying etiology, the disease can be assigned to a more specific, pathophysiology-based category (Garvin, 1998; Kuzuya et al., 2002).

Gestational diabetes mellitus (GDM) is a state referred to any woman who develops glucose intolerance during pregnancy. It is more common among obese women and women with a family history of diabetes. Although this form of diabetes usually disappears after the birth of the baby, women who have had GDM have a 20 to 50 percent chance of developing T2D within 5 to 10 years. About 7.05% of pregnant women in Thailand develop GDM. GDM is caused by the hormones of

pregnancy and a shortage of insulin. The maternal risk factors for GDM include increasing age, racial origin, family history of diabetes mellitus and pre-pregnancy body mass index. Older mothers and overweight women are more likely to get GDM. GDM has serious adverse consequences for both mothers and their children. Patients who have been diagnosed with diabetes before pregnancy are considered to have the 'pregestational diabetes mellitus (PDM)' condition. Among women who develop T1D during pregnancy or those with undiagnosed, asymptomatic T2D discovered during pregnancy are classified as having GDM. Mothers with PDM and their children face accelerated diabetes complications, such as hypertension and retinopathy, increased risk for many adverse outcomes including birth defects, perinatal mortality (Garvin, 1998; Kahn et al., 2005; Kuzuya et al., 2002; National Diabetes Information Clearinghouse, 2008; Buchanan, 1995).

The stage of diabetes can be assessed following the conditions that will help evaluate the degree of insulin deficiency including, clinical information (history of the disease, glycemic level and its stability, ketosis-proneness and response to diet and drug therapy), plasma insulin assays (fasting and after glucose load, and after intravenous glucagon), and C-peptide assays in plasma and urine (Kuzuya et al., 2002).

2.1.3 Diagnosis of diabetes mellitus

The diagnosis of diabetes should be performed in the subjects and considering the current degree of glycemia. An early assessment and treatment of patients are also essential for preventing diabetes complications. The diabetes complications are closely associated to the degree and duration of hyperglycemia. (Gavin, 1998; Hall and Davies, 2008; Kuzuya et al., 2002).

Individuals within an intermediate group were diagnosed as having glucose criteria did not specifically meet those for diabetes. These individuals therefore have an IFG [fasting plasma glucose (FPG levels 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)], or impaired glucose tolerance (IGT) [2 h values in the oral glucose tolerance test (OGTT) of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)]. Patients with IFG and/or IGT are referred to as having pre-diabetes, indicating the relatively high risk for the future development of diabetes. IFG and IGT are associated with obesity (especially abdominal or visceral obesity), dyslipidemia (high triglycerides and/or low HDL cholesterol) and hypertension. Indeed, individuals with A1C levels 5.7-6.4% should be considered to be at high risk for developing diabetes (Diagnosis and classification of diabetes mellitus, 2012).

Current diagnostic criteria for diabetes are summarized in Table 2.1. The established glucose criteria for the diagnosis of diabetes [FPG and 2 h plasma glucose (PG)] remain valid as well. The new diagnostic cut point for diabetes is FPG $\geq$ 126 mg/dl (7.0 mmol/l) and confirmed the long-standing diagnostic 2 h PG value of $\geq$ 200 mg/dl (11.1 mmol/l). Moreover, the A1C with a threshold of $\geq$ 6.5% is recommended as the cut point for diagnosing diabetes. Furthermore, patients with classic symptoms of hyperglycemia or hyperglycemia crisis with a random PG $\geq$ 200 mg/dl (11.1 mmol/l) are defined as diabetes (Diagnosis and classification of diabetes mellitus, 2012).

The diagnostic tests should be repeated to rule out laboratory error, unless the diagnosis is clear on clinical grounds, such as a patient with classic symptoms of hyperglycemia or hyperglycemic crisis. That is, the same test be repeated for confirmation since there will be a greater likelihood of concurrence in

this case. For example, if the A1C is 7.0% and a repeat result is 6.8%, the diagnosis of diabetes is confirmed. However, if the two different tests (e.g., FPG and A1C) are both above the diagnostic thresholds in the same patient, the diagnosis of diabetes is confirmed. On the other hand, in an individual with the results of two different tests are discordant, the test whose result is above the diagnostic cut point should be repeated and the diagnosis is made on the basis of the confirmed test. For example, if a patient meets the diabetes criterion of the A1C (two results ≥ 6.5%) but not the FPG (< 126 mg/dl or 7.0 mmol/l), or vice versa, that person should be considered to have diabetes. It should be noted that the test which use to assess a patient for diabetes should be at the discretion of the health care professional, taking into account the availability and practicality of testing an individual patient or groups of patients (Diagnosis and classification of diabetes mellitus, 2012).

GDM carries risk for the mother and neonate. The risk of adverse maternal, fetal and neonate outcomes in GDM also increases with maternal glycemia at 24-28 weeks, even within ranges previously considered normal for pregnancy. In pregnant women not known to have diabetes, the diagnostic test using a 75 g OGTT should be performed at 24-28 weeks of gestation. The diagnostic cut points were as follows: fasting ≥ 92 mg/dl (5.1 mmol/l), 1 h ≥ 180 mg/dl (10.0 mmol/l) and 2 h ≥ 153 mg/dl (8.5 mmol/l). Admittedly, the diagnosis of GDM should then be based on only one blood glucose value above the specified cut points. These diagnostic criteria for GDM will made the worrisome worldwide increases in obesity and diabetes rates, with the intent of optimizing gestational outcomes for women and their babies (Diagnosis and classification of diabetes mellitus, 2012).

Table 2.1 Criteria for the diagnosis of diabetes (Diagnosis and classification of diabetes mellitus, 2012).

A1C ≥ 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥ 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.*
OR
2 h plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dl (11.1 mg/dl).

*In the absence of unequivocal hyperglycemia, criteria 1-3 should be confirmed by repeat testing.

2.1.4 Complications of diabetes mellitus

Diabetes can cause problems in so many parts of the body that it may damage large blood vessels (called macrovascular disease) and damage to small blood vessels such as capillaries (called microvascular disease). These complications may considerably affect the quality of life, patient's survival and also health care direct and indirect cost. Preventing diabetes complications will improve the patient's quality of life and survival as well as reducing medical costs, thus it will benefit society and save resources for other purposes (Morsanutto et al., 2006).

In macrovascular disease, high blood glucose causes hardening of the arteries (arteriosclerosis), which can lead to a heart attack, stroke or poor circulation in the feet. Heart disease is the leading cause of diabetes-related death. Adults with diabetes have heart disease death rates about 2 to 4 times higher than adults without diabetes. The risk of stroke is also 2 to 4 times greater for people with diabetes. Moreover, the microvascular disease is closely linked to high blood glucose which results in thickens capillary walls, makes blood stickier and can cause small blood vessels to leak. Together, these effects reduce blood circulation to the skin, arms, legs, and feet. They can also change the circulation to the eyes and kidneys. Reduced capillary blood flow may cause some brown patches on the legs (Kahn et al., 2005; National Diabetes Information Clearinghouse, 2012).

The microvascular complications of diabetes such as retinopathy, nephropathy and neuropathy are an increased burden in the individual with diabetes (Girach and Vignati, 2006). Retinopathy (eye disease) is the most common cause of impaired eyesight and loss of vision in diabetic patients. Risk factors of diabetic retinopathy (DR), include the duration of diabetes, diabetes type, and poor glycemic

and blood pressure control (Delcourt, Massin and Rosilio, 2009). The presence of DR is associated with an increased risk of mortality. This association in patients with T2D is more consistently seen than that in T1D. It was associated with an older age and possibly with the higher prevalence of cardiovascular risk factors in T2D. In addition, there is emerging evidence indicated that DR reflects widespread microcirculatory disease in vital organs elsewhere in the body. The association of diabetic retinopathy with a range of systemic vascular complications should be provided as a biomarker of underlying widespread deleterious effects from abnormal glucose metabolism (Cheung and Wong, 2008). The major component of DR is retinal microvascular dysfunction which is characterized by hemorrhages, microaneurysms, cotton-wool spots, lipid exudates, macular edema, capillary occlusion and ultimately neovascularization. The development and progression of DR are also related to systemic metabolic and cardiovascular parameters. Thus, the treatment must include active management of systemic diabetes and cardiovascular risk factors (Gardner, Antonetti, Barber, LaNoue and Levison, 2002).

Nephropathy is the most common cause of kidney damage in both T1D and T2D. The clinical manifestations include proteinuria, decreased glomerular filtration rate and increasing blood pressure. In T1D patients, the renal morphologic lesions occur in the glomeruli, arterioles, interstitium and tubules. In contrast, T2D patients have normal glomerular structure with or without tubulo-interstitial and arteriolar abnormalities, despite the presence of microalbuminuria or proteinuria. It has been suggested that genetic factors may be important in conferring diabetic nephropathy risk and/or protection in both T1D and T2D (Dabla, 2010; Vestra and Fioretto, 2003). Several family studies have shown the cases of diabetic nephropathy

cluster in families and a parental history of hypertension is more common in patients with diabetic nephropathy. It demonstrates that hypertension has an important role in the genetic susceptibility to diabetic nephropathy. The susceptibility to diabetic nephropathy is caused by at least one or more major locus within the susceptibility gene(s), these are helpful in predicting those patients at risk of diabetic nephropathy (Krolewski, Fogarty and Warram, 1998). Diabetic nephropathy is a multi-stage condition that takes several years to become clinically overt. In the individual with diabetic nephropathy, the changes in renal function such as glomerular hyperfiltration, increased renal blood flow and hypertrophy of the kidney usually appear at the onset of diabetes. Good glycemic control can reverse these changes at an early stage. However, the persistent of those changes in renal function may be important in the later development of clinical nephropathy (Thomas and Viberti, 2006).

Neuropahty is one of the most common long term complications of both T1D and T2D. The spectrum of clinical and subclinical syndromes differs in anatomical distributions, clinical courses, and possibly underlying pathogenetic mechanisms. The syndromes may be grouped into diffuse and focal neuropathies. The diffuse neuropathies are comprised of distal symmetrical sensorimotor polyneuropathy (DPN) and diabetic autonomic neuropathy (DAN) (Edwards, Vincent, Cheng and Feldman, 2008). DPN is a ‘length-related’ pattern of sensory deficits, with the symptoms starting in the distal portions of the extremities. Indeed, it progresses in a “glove-stocking” distribution that appears extending to involve the feet and legs. Based on epidemiological studies, the risks for DPN are increased with increasing age, increasing duration of diabetes, poor glycemic control, hypertension, smoking, obesity and hyperlipidaemia. DAN is very common in subjects with long-standing

diabetes, but clinically significant autonomic dysfunction is uncommon. The abnormalities of autonomic function affect several systems such as cardiovascular, gastrointestinal and genito-urinary (Tesfaye, 2010). Cardiac autonomic neuropathy (CAN) is a significant risk of mortality due to its association with several negative outcomes. CAN can result in orthostatic hypotension, changes in peripheral blood flow, exercise intolerance, silent ischaemia and sudden death (Tesfaye, 2010). The production of oxidative stress is a common factor that leads to nerve damage. It is assumed that several metabolic changes occur as a consequence of high levels of glucose and thus lead to oxidative stress (Lincoln and Shotton, 2008). Combinations of oxidative stress and hyperglycemia have an effect on biochemical pathways which induce inflammation and neuronal dysfunction. It should be emphasized that strict glycemic control is the only proven method currently available to prevent DPN and DAN or slow progression (Edwards, Vincent, Cheng and Feldman, 2008). There are two forms of focal neuropathies including mononeuritis and entrapment syndromes. Mononeuropathy tends to occur in older patients, is acute in onset, usually involve single or multiple nerves, is self-limiting and resolves spontaneously. Median and ulnar neuropathies are the most common peripheral nerve mononeuropathies. It also suggests that the diabetic nerve has increased susceptibility to compression. The entrapment neuropathies can occur in median, ulnar, peroneal, medial and lateral plantar nerves. Its treatment is wrist resting by the placement of a wrist splint in a neutral position, and the addition of anti-inflammatory drug medications. The treatment with surgery should be considered based on severity of symptoms, appearance of motor weakness and failure of nonsurgical treatment (Edwards, Vincent, Cheng and Feldman, 2008; Vinik and Mehrabyan, 2004).

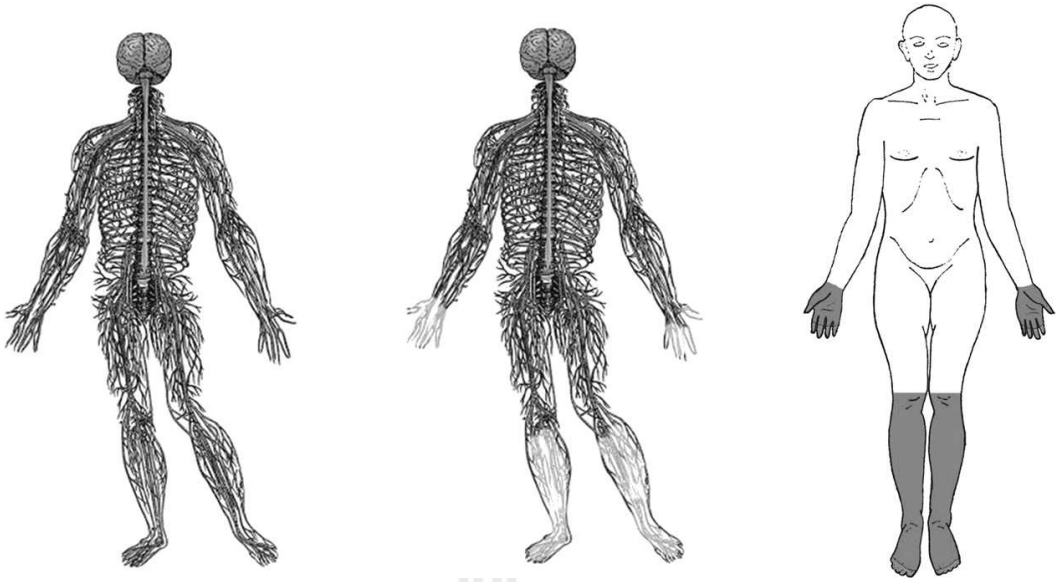

Figure 2.2 Glove-stocking configuration of diabetic neuropathy (DPN). DPN is dependent on axon length, initiating in the toes and progressing upward until reaching the calf. Neuropathy presents at the fingertips at this point (Edwards, Vincent, Cheng and Feldman, 2008).

The major cause of morbidity and mortality for diabetic individuals is cardiovascular disease (CVD). Both T1D and T2D individuals have a 2- to 3-fold incidence of CVD (Cameron, 2010; Marks and Raskin, 2000). People with diabetes have more cerebrovascular accidents, coronary artery disease, and peripheral arterial disease (Kengne, Turnbull and MacMahon, 2010). The risk of cardiovascular death is associated with increasing fasting plasma glucose and 2 h post-challenge glucose levels. In addition, the clustering of impaired fasting glucose with hypertension, visceral obesity and atherogenic dyslipidemia (low HDL cholesterol or high triglycerides), known as metabolic syndrome, increases the risk of CVD (Deedwania

and Fonseca, 2005). Moreover, it is important to note that women with diabetes have higher risk of CVD than men. Since obesity, hypertension and dyslipidemia are more common and more severe in diabetic women. There is evidence that diabetic women are less responsive than diabetic men to treatment of cardiovascular risk factors (Rivellese, Riccardi and Vaccaro, 2010). Based on these observations, an early identification and intensive glycemic control have the potential to reverse the progression and can reduce the morbidity and mortality of CVD (Deedwania and Fonseca, 2005). It appears that aggressive control of blood pressure in diabetic individuals may prevent the development of CVD (Marks and Raskin, 2000).

2.1.5 Diabetes treatment

The major goal of diabetes treatments is to control elevated blood glucose without causing abnormally low levels of blood glucose. T1D treatments include insulin, exercise, a diabetes diet and oral medications are useless in this type. T2D treatments include weight reduction, a diabetes diet and exercise. When these treatments fail to control the elevated blood glucose, oral medications are used. If oral medications are still insufficient, insulin medications are considered (Kahn et al., 2005; National Diabetes Information Clearinghouse, 2008).

Antidiabetic drugs have the potential to prevent diabetes-related sequelae, by normalizing glycemic levels while minimizing adverse events. There are distinct classes of drugs for glycemic regulation, which include biguanides, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, GLP-1 agonist, DPP-4 inhibitors and insulin. Their unique pharmacologic properties have provided a number of choices for physicians to individualize treatment of diabetic people (Lorenzati, Zucco, Miglietta, Lamberti and Bruno, 2010). These drugs may be

used in combination (Melikian, White, Vanderplas, Dezii and Chang, 2002). However, it is important to note that many antidiabetic agents could result in serious morbidity if administered to unsuitable patients (Fowler, 2007).

Insulin therapy has been considered the appropriate treatment for glycemic control in patients with diabetes. Insulin is required for everyone with T1D and some people with T2D. In order to maintain strict control of blood glucose in patients with T1D, continuous subcutaneous insulin infusion (CSII) is used as the treatment strategy. A meta-analysis was performed to evaluate the selected insulin regimens that included 1) multiple daily insulin injections therapy (MIT) or CSII versus conventional insulin treatment (CIT), 2) two versus four daily insulin injections and 3) rapid-acting insulin analogues versus human soluble insulin. The current evidence showed that a significant reduction in A1C was seen during CSII treatment. Interestingly, when comparing the effect of rapid-acting insulin analogues versus human soluble insulin, no difference of A1C reduction was observed between the two insulin types. However, a reduction in the hypoglycemic events was found when using rapid-acting insulin analogues (Jacobsen, Henriksen, Hother-Nielsen, Vach and Beck-Nielsen, 2009).

Due to inadequate oral treatment regimens, insulin has been recommended as a treatment option in T2D. The earlier use of insulin can help patients achieve long-term glycemic control, and reduce the risk of diabetes complications (Eldor, Stern, Milicevic and Raz, 2005; Funnell, 2008; Vinik, 2006). The effects of insulin on quality of life are inconsistent (Funnell, 2008).

Despite an improvement in glycemic control with insulin therapy, there has been reported on the allergic reactions with respect to insulin products. Insulin

reactions were reported in many patients treated with insulin preparations ranging from local injection site reactions to severe generalized anaphylactic reactions (Ghazavi and Johnston, 2011).

Regarding the need to eliminate exogenous insulin, β-cell replacement therapy for diabetic patients can be achieved by either pancreas or islet transplantation (Kandaswamy and Sutherland, 2006). The first successful pancreas transplantation was performed in 1966 (Shyr, 2009). The pancreas transplant recipients are able to stabilize or improve the long-term complications of diabetes (e.g. retinopathy, nephropathy, and neuropathy) (Vogel and Friend, 2011). However, it should be noted that this treatment is associated with the risk of pancreatic graft rejection and the consequent burden of pharmacological immunosuppression. Despite having the immunosuppressive regimens, the recurrence of T1D has been identified in some recipients, partly due to persistent autoimmunity (Dennedy and Dinneen, 2010). Because pancreas transplant is associated with surgical morbidity, islet cell transplantation has become an option for the patient. Islet cell transplantation appears to be less invasive with fewer complications (Kandaswamy and Sutherland, 2006). There are long-term endogenous insulin production and glycemic stability associated with islet transplantation using the Edmonton protocol. However, insulin independence was depended on the numbers of isolating islets from available pancreas donors (Shapiro et al., 2006). Furthermore, other limitations are related to the harmful effect of immunosuppressive agents and the limited number of pancreas donors (Naftanel and Harlan, 2004).

The requirements for an effective and unlimited supply for pancreatic β-cell replacement has led to explore the way of generating insulin-producing cells to

use in this treatment. Recently, scientists develop cell-based therapy as the alternative treatment to conventional donor dependent transplantation. Stem cells are self-renewing cells and are able to differentiate *in vivo* to produce the desired kind of cell. Laboratories all over the world have been exploring the possibility of using stem cells to restore damaged or lost tissue (Beattie and Hayek 2004; Stojkovic, Lako, Strachan and Murdoch, 2004). The understanding of the developmental biology of pancreas would be helpful for *in vitro* differentiation of stem cells towards the functional insulin-producing cells.

2.1.6 Diabetes management

The disease and case management interventions in diabetes are improving health and quality of life. The goal of disease management is to improve short- and long-term health or economic outcomes or both in the entire population with the disease. This intervention is composed of 1) the identification of the population with diabetes or a subset with specific characteristics (e.g., cardiovascular disease risk factors), 2) guidelines or performance standards for care, 3) management of identified people and 4) information systems for tracking and monitoring. Additional approaches are focused on the patient or population (e.g., diabetes self-management education [DSME]), the provider (e.g., reminders or continuing education), or the healthcare system or practice (e.g., practice redesign in which “planned improvements” are made in “the organization of practice to better meet the needs of the chronically ill”). An important intervention for people at high risk for adverse outcomes and excessive healthcare utilization is case management intervention. The essential features of case management are 1) identification of eligible patients, 2) assessment, 3) development of an individual care plan,

4) implementation of the care plan and 5) monitoring of outcomes. The effectiveness of case management results in improving both glycemic control and provider monitoring of glycemic control. Case management can be combined with other interventions include self-management education, home visits, telephone call outreach, telemedicine, and patient reminders (Norris et al., 2002).

According to the economic impact, interventions for diabetes to decrease complications can be classified as 1) clearly cost-saving, 2) clearly cost-effective, 3) possibly cost-effective, 4) non-cost-effective or 5) unclear. Retinopathy screening to prevent blindness and pre-conception care to prevent birth defects are classified as clearly cost-saving. For nephropathy prevention in T1D and improved glycemic control, the economic impact of these interventions has been clearly cost-effective. Interventions that provide nephropathy prevention in T2D and self-management training are reported as possibly cost-effective. Interventions with unclear economic impact included case management, medical nutrition therapy, self-monitoring of blood glucose, foot care, blood pressure control, blood lipid control, smoking cessation, exercise, weight loss, A1C measurement, influenza vaccination, and pneumococcus vaccination (Klonoff and Schwartz, 2000).

Nevertheless, the various barriers to diabetes management among persons with T2D have been identified from the perspectives of both patients and clinicians. The failure of diabetes self-management by patients as well as inadequate intervention strategies by clinicians may be affecting glycemic control. The patient factors included adherence, beliefs, attitudes, knowledge, ethnicity/culture, language ability, financial resources, co-morbidities and social support may relevant to T2D management. In addition, adherence to self-management is influenced by an

individual's financial resources, beliefs and attitudes about the disease, and effectiveness of the treatment regimen. The clinician's barriers can influence the diabetes care outcome. Clinician factors which included beliefs, attitude, knowledge, communication skills and health care system may affect diabetes self-management education and quality of diabetes care. Therefore, the quality of diabetes care including metabolic control and diabetes self-management can be improved by identifying barriers to diabetes management (Nam, Chesla, Stotts, Kroon and Janson, 2011).

2.2 The pancreas

2.2.1 Pancreas structure

The pancreas is an organ located in the abdomen behind the stomach. It contains the exocrine and endocrine functions (Ellis, 2006). The secretory units of the exocrine part are groups of cells, known as acini, surrounding the ends of small ducts. The acinar cells secrete digestive enzymes, which can digest proteins, carbohydrates, nucleic acids and fat. The endocrine part is a small proportion of pancreatic cells that aggregates into small clusters and scatters throughout the exocrine cells. These small clusters of cells called the islets of Langerhans (Tan, 2005).

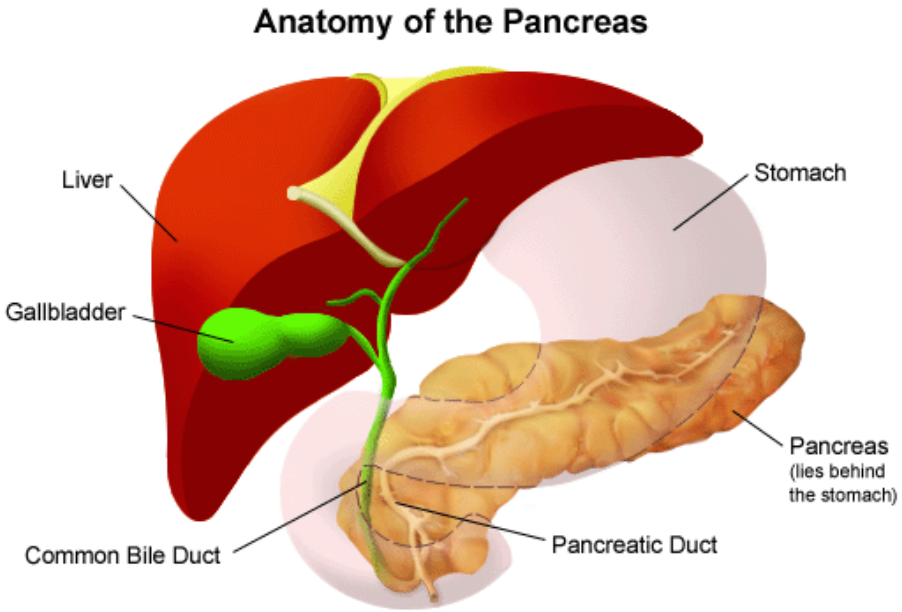

Figure 2.3 Anatomy of the pancreas.

Source : http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW023328

2.2.2 Pancreas development

The mature pancreas is composed of two different structures: endocrine and exocrine, which consists of the various cell types (Table 2.2). These pancreatic cell types are derived from endodermal origin (Figure 2.4). During embryogenesis, the pancreas arises from dorsal and ventral buds of the forgut endoderm. Dorsal endoderm is in contact with the notochord and ventral pancreas is close to the cardiac mesoderm. The dorsal prepancreatic endoderm and the notochord remain in contact until about embryonic day (E) 8.5 in mice (13 somites stage) (Soria, 2001). Subsequently, the dorsal pancreatic bud can be detected on E9.5 (22-25 somites

stage). Then, the ventral pancreatic bud appears on E10.5 (approximately 30 somites stage) (Gangaram-Panday, Faas and de Vos, 2007). The pancreatic bud undergoes branching morphogenesis by E12.5 and the dorsal and ventral buds later fuse to produce an epithelial tubular complex that contains the precursor cells for islets, acini and ducts (Kim and MacDonald, 2002; Wells, 2003). The well-defined islet architecture is observed in E18, which forms insulin-secreting cells in the center and non β-cells in the periphery (Soria, 2001).

Table 2.2 Pancreatic cells: their phenotypes and function (Gangaram-Panday, Faas and de Vos, 2007).

Pancreas	**Cells**	**Function**
Endocrine	β-cells	Production of insulin for sustaining euglycemia
	α- cells	Secrete glucagon, which counteracts insulin hypoglycemia effects
	δ-cells	Secrete somatostatin, which inhibits insulin secretion
	PP cells	Pancreatic polypeptides function remains unclear
	Ghrelin cells	Production of ghrelin can inhibit glucose-induced insulin release and stimulate glucagon secretion
Exocrine	Acinar cells	Produce at least 22 digestive enzymes such as proteases, amylases, lipases and nucleases
	Duct cells	Produce non-enzymatic components of the pancreatic juice, including bicarbonate

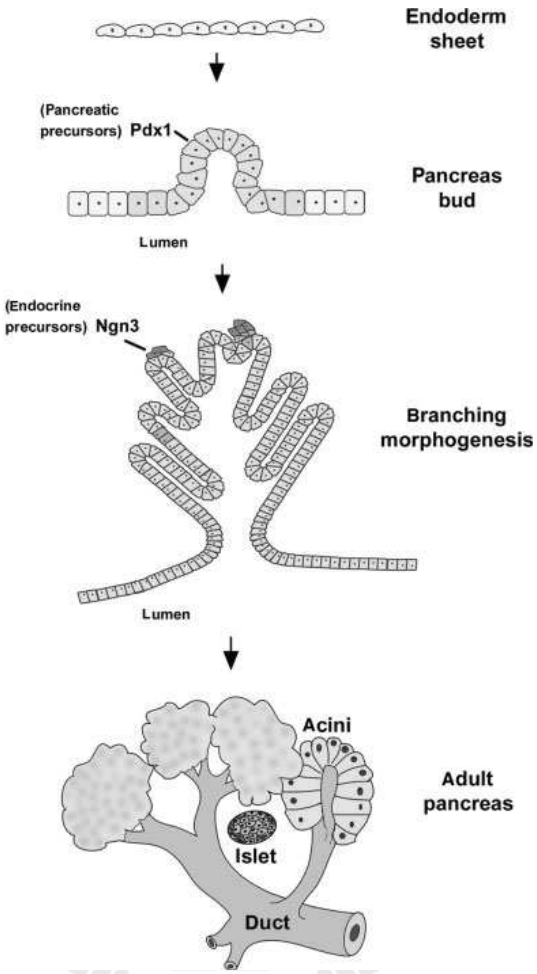

Figure 2.4 Cells that give rise to the endocrine pancreas. The pancreas derives from an unspecified sheet of endoderm cells. The endoderm sheet is transformed into an epithelium, leading to the expression of Pdx1. Pdx1 expression marks both the dorsal and ventral domains of the developing pancreas and defines where the pancreatic buds will appear. Pdx1-expressing cells can give rise to all the cells of the adult pancreas and therefore represent a pancreatic precursor. The pancreatic bud undergoes branching morphogenesis and endocrine progenitor cells begin to express Ngn3. Ngn3-expressing cells can give rise to all endocrine cells of the adult islet (Wells, 2003).

2.2.3 Transcription factors involved in pancreatic development

The steps that control endocrine pancreas in specified cell types or developmental stages are restricted to the pancreas-specific transcription factors (Figure 2.5).

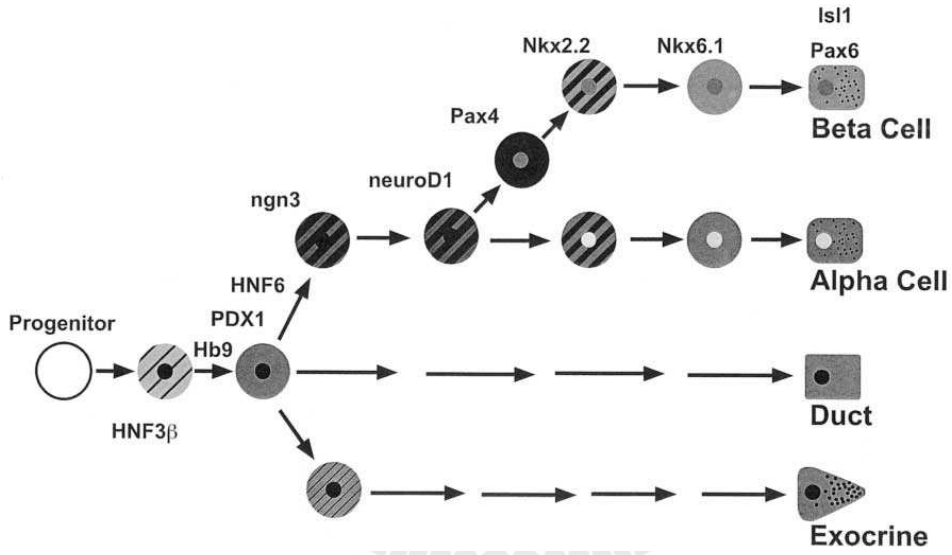

Figure 2.5 A proposed model of the hierarchy of transcription factors involved in pancreatic development (Schwitzgebel, 2001).

The winged-helix transcription factor Foxa2 [formerly hepatocyte nuclear factor 3β (Hnf3β)] is a transcriptional regulator of *Pdx1* and regulates fetal endocrine pancreas development (Gao et al., 2007; Soria, 2001). *Foxa2/Hnf3β* is first expressed at E5.5-6.5 in the anterior part of the early primitive streak and later in the definitive endoderm (Schwitzgebel, 2001). In adult pancreatic β-cells, Foxa2/Hnf3β

also plays a major role in the assembly and maintenance of the glucose metabolism and insulin secretion machinery (Gao et al., 2007). It has been demonstrated that *Foxa2/Hnf3β* deficiency may contribute to the relative hyperinsulinemia (Lantz et al., 2004). Another homeodomain transcription factor, Hnf6, is expressed in the epithelial cells that are precursors of the exocrine and endocrine pancreatic cells. It is the first positive regulator of the proendocrine gene *Ngn3* in the pancreas (Jacquemin et al., 2000).

The protein Hb9 expression is observed at E8.0 in the epithelium at the forgut-midgut junction. It is expressed in the dorsal and ventral portion by E13.5. Hb9 is expressed at E17.5 restricted to β-cells only in the adult pancreas (Schwitzgebel, 2001).

As pancreatic buds form, the cells are morphologically uniform and are expressed for the pancreatic-duodenal homeobox 1 (Pdx1). Pdx1-expressing cells represent a pancreatic precursor that will give rise to all types of adult pancreatic cells. The islet transduction with dominant-negative Pdx1 (RIPDN79PDX1) results in impaired mitochondrial metabolism and glucose-stimulated insulin secretion (GSIS) (Gauthier et al., 2009).

Ngn3, a member of the basic helix-loop-helix (bHLH) family of transcription factors, is expressed in the pancreatic anlage at E9.0- 9.5 (Schwitzgebel, 2001). At E12.5, these pancreatic buds become to form the branching morphogenesis and endocrine progenitor cells are found to express Ngn3. Ngn3-expressing cells are considered as the endocrine progenitor cells that specifically give rise to the endocrine pancreas (Wells, 2003). Ngn3-deficient embryos have a dramatic alteration in the

morphology of the developing ductal tree in the pancreas of E13.5 and E15.5 (Magenheim et al., 2011).

The β cell e-box transactivation 2 (*BETA2*) [also known as neurogenic differentiation 1 (*NeuroD1*)] is associated with the activation of the *insulin* (*INS*) gene and islet cells differentiation (Gangaram-Panday, Faas and de Vos, 2007; Soria, 2001). At E9.5, the expression of *BETA2/NeuroD1* is presented in a subset of pancreatic epithelial cells and becomes restricted to the adult islets. It was shown that the cooperation of *NeuroD1* and *Pdx1* maintained the property of insulin-producing cells derived from embryonic stem cells (Saitoh, Yamato, Miyazaki and Miyazaki, 2007). In NeuroD1-null mice, the reduction of α- and β-cells is presented after E17.5 (Chao, Loomis, Lee and Sussel, 2007).

The paired box gene named *Pax4* and *Pax6* are required for the differentiation of certain endocrine cell lineages during pancreas development. During the early stages of pancreas development, *Pax4* and *Pax6* are expressed in the cellular subsets of the endocrine lineage (Dohrmann, Gruss and Lemaire, 2000). *Pax6* expression is required for the generation of glucagon secreting α-cells, which can be detected around E9.0 in a small subset of cells in the prepancreatic endoderm. *Pax6* can be detected throughout pancreas development, whereas *Pax4* can only be detected during embryogenesis. *Pax4* expression is first detected in E10 embryos, which is restricted to insulin producing β-cells and somatostatin producing δ-cells (Dohrmann, Gruss and Lemaire, 2000; Soria, 2001). In *Pax6* knock-out mice, glucagon producing α-cells are absent throughout all developmental stages. In contrast, *Pax4* knock-out mice are unable to develop mature β-cells (Dohrmann, Gruss and Lemaire, 2000).

It has been shown that *Nkx2.2* and *Nkx6.1* genes are required for pancreatic development (Chiang and Melton, 2003). Nkx2.2 is expressed in the whole pancreas bud at E9.5, but later it becomes restricted to α-, β- and PP-cells. Moreover, Nkx2.2 is required for maintenance of Nkx6.1 expression. Nkx6.1 expression starts at E10.5 in the pancreatic bud and then becomes restricted to insulin-producing cells by E15.5 (Gangaram-Panday, Faas and de Vos, 2007; Schwitzgebel, 2001). Nkx2.2 mutant mice show immature or partially differentiated β-cells, coupled with the absence of insulin production (Sussel et al., 1998). RNAi-mediated suppression of Nkx6.1 mRNA in rat insulinoma INS-1-derived cell lines demonstrates a decrease in glucose-stimulated insulin secretion (GSIS) (Schisler et al., 2005).

Isl1 is involved in the early formation of the pancreas. During development, Isl1 is expressed in dorsal pancreatic epithelium at E9.0. Isl1 is presented at E9.5 in glucagon positive cells and later in all the islet cells (Gangaram-Panday, Faas and de Vos, 2007; Schwitzgebel, 2001).

2.2.4 Pancreatic endocrine cells development

During pancreatic organogenesis, the population of pancreatic progenitor cells receives multiple signals in order to differentiate into the endocrine cells (Dhawan, Georgia and Bhushan, 2007). The early marker of pancreatic organogenesis is the homeobox transcription factor Pdx1. The secreted factors including the transforming growth factor-β (TGF-β) members (e.g. activin) and fibroblast growth factor-2 (FGF2) are known to influence pancreatic development. These factors can also induce transcription of pancreatic marker genes in isolated endoderm (Burant and Simeone, 2002). The repression of Sonic Hedgehog (Shh) by both activin and FGF2 leads to Pdx1 expression in adjacent pancreatic endoderm (Burant and Simeone,

2002; Soria, 2001). In addition, the expansion and differentiation of progenitor cells has been shown to involve Notch signaling. The Notch signaling involves in maintaining the progenitor cell population and also prevented endocrine differentiation. It should be noted that Notch signaling maintains the progenitor cells status by inhibiting Ngn3 expression (Dhawan, Georgia and Bhushan, 2007). Accordingly, Ngn3 expression in pancreatic progenitors is required for the differentiation of the entire pancreas into endocrine cells. However, these Ngn3-expressing cells are mostly developed to glucagon-producing cells (Collombat, Hecksher-Sorensen, Serup and Mansouri, 2006). The transient expression of Ngn3 initiates endocrine differentiation and further activates NeuroD1 that can drive islet differentiation in progenitor cells (Gasa et al., 2008).

A number of transcription factors that are expressed selectively in the endocrine lineage in the developing pancreas, and that could play a role in endocrine cell subtype fate decisions, have been identified. These factors contain homeodomains and can be divided into early factors (Pax4, Nkx2.2 and Nkx6.1) that are coexpressed with Ngn3 in endocrine progenitor cells and late factors (Pax6, Isl1, Hb9 and Pdx1) that are found in more mature cells (Wilson, Scheel and German, 2003).

The paired-homeodomain factors, Pax4 and Pax6, have important roles in the differentiation of specific endocrine cell lineages during pancreas development. Pax4 is expressed selectively in cells restricted to the β- and δ-cell lineage (Wilson, Scheel and German, 2003). In addition, *Pax6* is expressed in all endocrine cells of the developing pancreas. There is further evidence that *Pax4* expression can only be detected during embryogenesis, while *Pax6* can be detected throughout pancreas

development (Dohrmann, Gruss and Lemaire, 2000). However, the precursor cells expressing only *Pax6* are restricted to the α-cell fate (Chakrabarti and Mirmira, 2003).

In the final differentiation of β-cells, the homeobox proteins Nkx2.2 and Nkx6.1 appear to be crucial for directing developing cells to the β-cell lineage. These factors cause differentiation of β-cells by activating directly *Ins* gene in precursor cells, as the *Ins* promoter contains binding sites for both Nkx2.2 and Nkx6.1 (Chakrabarti and Mirmira, 2003). Sander et al. (2000) propose that downstream of *Nkx2.2*, *Nkx6.1* ensures the expansion and progression of Nkx2.2-expressing β-cell precursors to mature β-cells. In addition, Pdx1 also plays a role in the final differentiation of mature islet cells in which it regulates the expression of mature β- and δ-cell gene products. Furthermore, the studies support Pax6 and Isl1 in islet hormone gene expression since Pax6 implicates in glucagon, insulin and somatostatin expression and Isl1 implicates in glucagon and somatostatin expression (Wilson, Scheel and German, 2003).

2.4 Stem cells

Stem cells are often classified with regards to their origin, into embryonic stem cells and adult stem cells. Depending on where the stem cells originate, they have different properties (Kiatpongsan, Tannirandorn and Virutamasen, 2006; Stojkovic, Lako, Strachan and Murdoch, 2004).

Embryonic stem cells (ESCs) are derived from embryos that develop from *in vitro* fertilized eggs e.g. *in vitro* fertilization [IVF] clinic and then donated for research purposes with informed consent of the donors. Once the inner cell mass (ICM), which is part of the early (4-5 days old) embryo called the blastocyst is

removed, the cells can be cultured into embryonic stem cells. ESCs have the intrinsic ability to become mesoderm, ectoderm, or endoderm, thus giving rise to every differentiated cells in the body. In addition, it is also immortal by self renewal capability. Consequently, ESCs are promising as a therapeutic tool because it has less limitation on differentiation potential.

Human adult stem cells are stem cells isolated from various parts of the body from fetus to adult stages of human development. The adult stem cell can renew itself, and can differentiate to yield the major specialized cell types of the tissue or organ. However, they contain less differentiation capacity than ESCs. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. It is generally believed that adult stem cell therapies will complement but not replace embryonic stem cell therapies. Certain kinds of adult stem cells seem to have the ability to differentiate into a number of different cell types under the right conditions. If the differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of therapies for many serious common diseases.

Moreover, stem cells can be classified by a hierarchal system (Kiatpongsan, Tannirandorn and Virutamasen, 2006).

1. Totipotent stem cell is the first stage stem cell that can be found in zygote. It can develop into both embryonic and extraembryonic tissues.

2. Pluripotent stem cell is the later stage of totipotent stem cell. This cell type can develop into all kinds of cells of an embryo (except the extraembryonic tissues). Pluripotent stem cell can be found in an embryo, fetus and developing organism.

3. Multipotent stem cell is a stem cell in any specific tissues, organs or systems. This stem cell has more limited differentiation potential.

2.3.1 Human embryonic stem cells (hESCs)

2.3.1.1 Derivation and culture of hESCs

The human embryonic stem cells (hESCs) are derived from the ICM of blastocyst-stage embryos that are isolated by immunosurgery or mechanically methods. The hESCs lines have been efficiently derived from ICM isolated by immunosurgery that involves animal-derived substances, mouse antibodies and guinea pig complement (Skottman and Hovatta, 2006). However, the immunosurgery procedure raises the probability of smaller or poorly defined ICMs. Importantly, there is a risk of the contamination of the hESCs with animal pathogens by the antibody and complement treatment (Kim et al., 2005). Alternatively, the use of Tyrode's acid for the removal of zona pellucida and mechanical isolation of ICM can serve as a potentially useful method for the establishment of hESCs line. Indeed, this technique also implies that the blastocyst could not contact with animal-derived pronase, antibodies, and complement factors (Skottman and Hovatta, 2006).

The hESCs lines can be maintained in an undifferentiated or pluripotency state *in vitro* for prolonged periods of time. The potential of hESCs to differentiate into representing ectoderm, mesoderm and endoderm derivatives has generated the possible use of hESCs in therapeutic applications (Trounson, 2006). The derivation process involves culturing of the ICM of blastocyst stage, induce to proliferate and differentiate into desired cell types (Stojkovic, Lako, Strachan and Murdoch, 2004) (Figure 2.6). The first successful derivation of hESCs was performed in the Thomson laboratory at the University of Wisconsin (Madison, WI, USA). They were isolated from the ICM of human blastocyst and placed on mitotically inactivated murine feeder cells (Thomson et al., 1998).

Continuous culture of isolated ICM cells and hESCs in an undifferentiated state requires the presence of feeder layers. Some previous reports have demonstrated that the growth of undifferentiated hESCs can be maintained on mouse embryonic fibroblast (MEF) feeders and on laminin- or Matrigel-coated-plastic surface with MEF conditioned medium. The xenosupport systems possibly transfer harmful animal pathogens to any human transplant recipients in clinical use of existing human cells (Mallon, Park, Chen, Hamilton and McKay, 2006). Furthermore, human feeder layers are used for hESCs culture which include human adult marrow cells, human fetal muscle (FM), human adult skin (AS), commercial human fetal skin (FS; D551/CCL-10, American Type Culture Collection [ATCC]), human adult uterine endometrial cells (hUECs), human adult breast parenchymal cells (hBPCs) and embryonic fibroblasts (hEFs). They are capable to support undifferentiated stage and proliferation state of hESCs (Cheng, Hammond, Ye, Zhan and Dravid, 2003; Kibschull, Mileikovsky, Michael, Lye and Nagy, 2011; Lee et al., 2004a; Richards et al., 2003). Conditioned medium from hESCs-derived fibroblasts (hESC-dFs) efficiently supports growth of hESCs in feeder-free culture systems (Stojkovic et al., 2005). A three-dimensional (3D) porous natural polymer scaffolds (chitosan and alginate) effectively support self-renewal of hESCs without the need of feeder cells or conditioned medium (Li, Leung, Hopper, Ellenbogen and Zhang, 2010). Recent study has demonstrated that a defined engineered 3D microfiber system allows adequate propagation and cryopreservation of hESCs under feeder-free chemically defined conditions (Lu et al., 2012). However, these culture conditions still have the ingredients from animal such as fetal bovine serum (FBS) and bovine albumin that contain in culture medium. For the clinical potential in cell replacement therapy,

differentiated cells from hESCs will be cultured in xeno-free systems (Kibschull, Mileikovsky, Michael, Lye and Nagy, 2011; Mallon, Park, Chen, Hamilton and McKay, 2006). Interestingly, Chen et al. (2012) reported the suspension culture system under defined and serum-free conditions provides a powerful approach for scale-up expansion of hESCs. It was demonstrated that cell banks of several hESCs lines are generated from this system under current Good Manufacturing Procedures (cGMP) or cGMP-equivalent conditions.

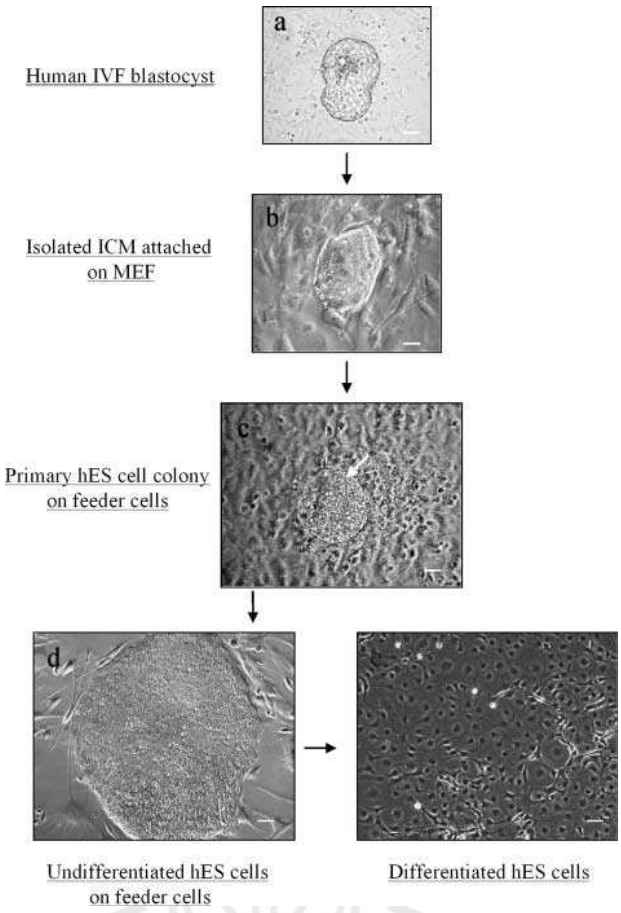

Figure 2.6 Derivation of hESCs. (a) Day 8 (IVF = day 0) hatching blastocyst derived after *in vitro* fertilization. (b) ICM isolated by immunosurgery or mechanical isolation and attached to the mouse feeder cells (MEF). (c) Thirteen-day-old primary hESCs colony grown on MEF. Note the presence of cells with typical hESCs like morphology (white arrow). (d) Undifferentiated hES-NCL1 colony grown on human feeder. (e) Spontaneous differentiation of hES-NCL1 cells with neuronal-precursors morphology. Induced differentiation of pluripotent hES cells could be achieved after addition of specific factor(s). Scale bars, 200 mm (a, c, d, e); 100 mm (b) (Adapted from Stojkovic, Lako, Strachan and Murdoch, 2004).

2.3.1.2 Characterization of hESCs

The hESCs lines form flat and compact colonies with distinct cell borders which have a high ratio of nucleus to cytoplasm and have prominent nucleoli. The hESCs exhibit high levels of telomerase activity and show normal karyotype (number and type of component chromosomes) after prolonged culture with multiple passages. They are survived and proliferated *in vitro* indefinitely under well-defined tissue culture conditions. Most of the cells can be subcultured after freezing, thawing, and replating. The cells can be differentiated into a variety of cell types both *in vitro* and *in vivo* conditions. The hESCs lines are expressed the stage-specific antigens (SSEA-3 and SSEA-4), the glycoproteins tumor recognition antigen (TRA-1-60, TRA-1-81 and TRA-2-54), germ cell tumor marker (GCTM-2), trophoblast giant (TG343 and 30), cluster of differentiation (CD9 and 133), Octamer-4 (Oct4), Nanog, SRY-box containing gene 2 (*Sox2*), teratocarcinoma-derived growth factor 1 (Tdgf1), left-right determination factor 2 (LeftyA), RNA exonuclease 1 (Rex-1), Stellar, Dazl, Nanos 1, pumilio gene (*Pum 1* and *2*), growth differentiation factor-3 (Gdf3), thymus cell antigen 1 (Thy-1) and alkaline phosphatase (Raikwar, Mueller and Zavazava, 2006; Stojkovic, Lako, Strachan and Murdoch, 2004). Markers that are common to characterize hESCs are following: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct4 and alkaline phosphatase. Nevertheless, there are differences between hESCs in their pluripotency or the genetic profile under the same conditions, their potential for large-scale culture and growth under feeder-free protocols, or their ability to form teratoma after injection into severe combined immunodeficiency (SCID) mice. Moreover, their capacity to differentiate into different cell types under *in vitro* conditions is variable (Raikwar, Mueller and

Zavazava, 2006; Stojkovic, Lako, Strachan and Murdoch, 2004). It is important to note that the difference in various hESCs lines is useful for the scientists to choose the appropriate hESCs line for their research.

2.3.1.3 Directed differentiation of hESCs

The hESCs represent an unlimited source of cells for cell replacement therapy because of their ability to proliferate and differentiate into cells of all three germ layers. Directing the differentiation of hESCs into desirable cell types requires the efficient differentiation protocols to derive tissue-specific progenitor cells. In addition, the culture conditions are also optimized to control and restrict the differentiation pathways of hESCs differentiation. As hESCs differentiates *in vitro*, the formation of embryoid bodies (EBs) represents an important step toward the generation of particular cell lineages. It should be noted that hESCs are able to differentiate through EBs parallels embryonic development due to EBs recapitulates events during embryogenesis (Hwang, Varghese and Elisseeff, 2008).

For the production of EBs, several methods have been designed specifically by researchers to provide a source of cells for their applications (Figure 2.7). Methylcellulose (MC) methods have been developed to form EBs from single ESCs. This method allows for the differentiation of hematopoietic cells and endothelial cells. Additionally, MC culture is also employed in hematopoietic colony-forming assays (Kurosawa, 2007). In contrast, hanging drop (HD) methods have been widely used to differentiate a variety of cell types from ES cells. HD is a two-step process consisting of the aggregation of ESCs in drops and the maturation of cell aggregates in suspension culture using bacterial-grade dishes. The HD culture can be efficiently generated a good environment for forming EBs, in which it allows the

aggregation of ESCs by the rounded bottom of a hanging drop. This method has the advantage of using small media volume and it can control the number of ESCs aggregated in a hanging drop. However, it is a troublesome multiple steps methods. Moreover, it is impossible to exchange the medium for a drop and the observations of forming EBs in drops by direct microscopic is difficult (Kurosawa, 2007). Beside the MC and HD culture, suspension culture in bacterial-grade dishes has been used for liquid suspension culture of ESCs to induce EB formation. By this method, the resulting EBs tends to be heterogeneous in size and shape because their self-organized aggregation in culture. The heterogeneity of EBs structures may influence cell fate differentiation (Kurosawa, 2007). Therefore, it is necessary to develop methods that can be used to form EBs homogenously and can control their differentiation (Karp et al., 2007).

The use of EBs to produce a variety of desired cell types represents an exciting approach for therapeutic applications. Several researchers have developed the protocols for deriving functional cell types from hESCs. Importantly, the culture conditions have been designed to obtain a number of cell types by an understanding of developmental biology. In order to mimic cell development, it is necessary to optimize various cytokines and growth factors that would control the differentiation towards specific cell lineages (Hwang, Varghese and Elisseeff, 2008).

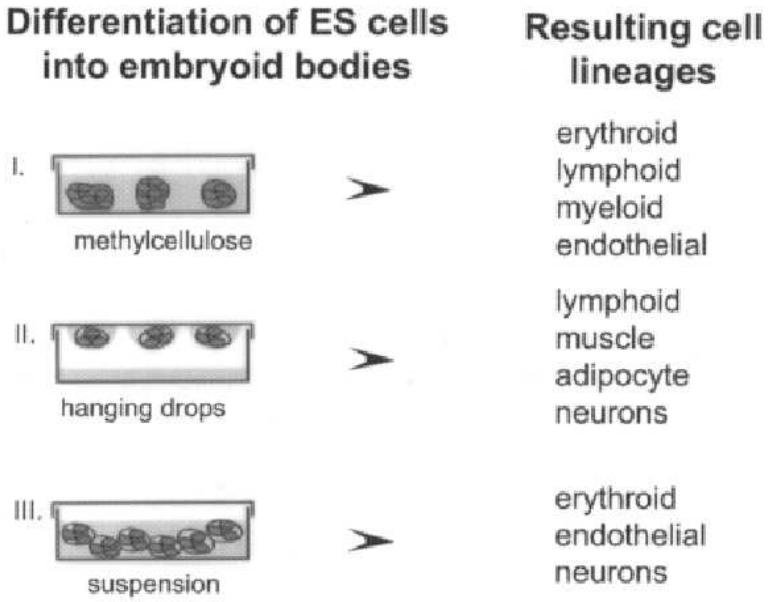

Figure 2.7 Methods for inducing EBs formation. (I) single cells are grown as EBs in a methylcellulose semisolid medium (MCM). Nucleated erythroid, erythroid and myeloid cells, neutrophils, mast cells and megakaryocytes and endothelial cells are developed from EBs cultured in MCM. (II) Drops containing 400-1000 ES cells are plated on the lids of Petri dishes. After 2 days of incubation, EBs are harvested and subsequently cultivated in suspension. Lymphoid progenitors, cardiomyocytes, skeletal and vascular smooth muscle cells, adipocytes formed in EBs. (III) ES cells are grown in nonadhesive tissue culture plates in standard cell culture medium. Erythroid and endothelial cells, neuronal and glial cells developed in suspension EBs. (Adapted from Desbaillets, Ziegler, Groscurth and Gassmann, 2000).

2.3.1.4 Differentiation of hESCs into insulin-producing cells

For diabetic therapies, T1D is mostly studied. Many scientists have focused on inducing islet neogenesis from stem cells *in vitro*. The cell replacement for islet destruction states would be available in unlimited supply. Consequently, embryonic stem cells are possibly induced to differentiate efficiently into insulin-producing cells and release insulin appropriately in response to glucose (Beattie and Hayek, 2004; Jones, Burns and Persaud, 2004).

Many scientists have established techniques to induce hESCs differentiation into insulin-producing cells. For instance, Assady et al. (2001) have used hESCs in both adherent and suspension cultures and observed spontaneous *in vitro* differentiation that included the generation of cells with characteristics of insulin-producing β-cells. The H9 line of hESCs was used. These cells grow as homogeneous and undifferentiated colonies when they are propagated on a feeder layer of MEFs. Accordingly, spontaneous *in vitro* differentiation of hESCs is investigated after removal of cells from the MEF feeder layer using two different model systems. Cells grew under adherent conditions in tissue culture plates, in the absence of MEFs, display a pleiotropic pattern with numerous morphologies. In contrast, *in vitro* differentiation in suspension culture results in a more consistent pattern, with the formation of discrete embryoid bodies (EBs). Organization of EBs starts as early as day 3 after removal from MEFs and transfer to suspension culture. More complex structures occur with progressive days in suspension culture such as epithelial- or epithelial-like cells lining hollow structure or cysts. Until day 19 after EBs development, occasional cells expressing insulin are evident as early as 14 days of differentiation, with a progressive increase in number through day 19.

Immunohistochemistry analysis using anti-insulin antibody, 60-70% of EBs is stained positively for insulin, and 1-3% of cells are positively stained at maximum density. Insulin release is measured by enzyme immunoassay in undifferentiated hESCs, differentiated hESCs, and MEF cells. The level of insulin release is significantly greater from 20- to 22-day of EBs (60-70 EBs per dish), as compared with undifferentiated hESCs. The expressions of other β-cell-related genes using RT-PCR analysis of undifferentiated and differentiated hESCs are examined. Insulin mRNA, islet glucokinase (GK) and glucose transporter-2 (GLUT2) genes are identified in differentiated but not undifferentiated hESCs. The glucose transporter-1 (GLUT1) isotype, a constitutive glucose transporter, is widely expressed in all forms of hESCs. In addition, expression of Oct4 mRNA, a marker of the pluripotent stage is detected in undifferentiated hESCs but decreases progressively during the subsequent differentiation. On the other hand, the differentiated hESCs express insulin promoter factor-1/pancreatic duodenal homeobox factor-1 (IPF1/PDX1) and neurogenin 3 (Ngn3) transcription factors. These 2 proteins have been shown to contribute to the regulation of pancreatic and endocrine cell differentiation.

It has been described that islet like clusters derive hESCs differentiation can be achieved in multi-step procedures; (i) formation of EBs (stage 1); (ii) selective differentiation of cell populations expressing nestin using fetal calf serum depletion and culture with ITSF (stage 2); (iii) proliferation and maintenance of precursor cells (stage 3); and (iv) the differentiation induction and maintenance of insulin-positive cells (stage 4). The resultant cells are positive for dithizone (DTZ), a zinc-chelating agent known to selectively stain pancreatic β-cells and are immunoreactive for antibodies against insulin, glucagons, and C-peptide. Insulin and

other pancreatic β-cell related genes such as glucagon, somatostatin, KIR6.2 and SUR1, IAPP, Isl1, PC1/3, PC2, GK, Nkx6.1, GLUT2 and Pax4 are expressed in the differentiating cells. The results indicate that differentiated cells can express genes involved in the β-cell differentiation pathway (Baharvand, Jafary, Massumi and Ashtiani, 2006b). Furthermore, insulin-producing islet-like clusters (ILCs) are generated from hESCs according to the method developed by Jiang et al (2007). The hESC lines are cultured under feeder-free conditions and direct differentiation toward ILCs by using a multi-step, serum-free protocol. The 36-day differentiation protocol consists of four stages which included definitive endoderm induction (stage 1), pancreatic endoderm formation (stage 2), endocrine induction (stage 3) and islet-like clusters maturation (stage 4). The hESCs generate definitive endoderm coexpressing CXCR4 and Sox17, and CXCR4 and Foxa2 when treated with sodium butyrate and activin A. The Pdx1-expressing pancreatic endoderm is then induced by the addition of bFGF, EGF, noggin and B27 supplement. Following withdrawal of bFGF, these cells are allowed to develop pancreatic endocrine cells. Gene expression analysis shows that pancreatic endoderm cells also start to express other pancreas-related genes, such as HlxB9, Ptf1a, Ngn3 and Nkx6.1. Upon further differentiation of Pdx1-positive cell clusters to day 36, immunocytochemical staining data demonstrated that the C-peptide-, glucagon- and somatostatin-positive cells were predominantly localized in the small bud-like clusters as well as in some of the smaller ILCs. When further characterized by TEM, the ILCs appear to have intact granulated cells containing secretory granules. In addition, the ILCs generated by this protocol are able to secrete C-peptide in response to 20 mM glucose.

Although several authors achieved insulin containing cells from hESCs, the other report suggests that these cells are uptake insulin from the culture medium (Rajagopal, Anderson, Kume, Martinez and Melton, 2003). In this report, the insulin positive cells did not stain with an antibody for C-peptide, a byproduct of de novo insulin synthesis. Moreover, electron microscopy of differentiated cells failed to demonstrate the granule characteristic of β-cells. Therefore, the convenient cultures systems for inducing insulin-producing cells from hESCs must be adjusted to obtain the endogenous insulin synthesis. In addition, the differentiation should be performed in xeno-free system in order to further develop potential medical application (Mallon, Park, Chen, Hamilton and McKay, 2006). Moreover, some factors of hESCs have to be overcome before it can be applied for therapeutic purposes. For examples, during differentiation stage, the major histocompatibility complex (MHC) is up-regulated, leading to the non-self proteins expression on the graft cells which may result in immune rejection of the graft in the absence of immunosuppressive therapy (Stojkovic, Lako, Strachan and Murdoch, 2004). In addition, the expression of high level of telomerase activity can lead to teratoma formation after injection which should be noted (Fujikawa et al., 2005). Furthermore, prolonged growth *in vitro* may also cause chromosomal aberrations. Therefore, complete characterization of hESC lines including their molecular status, a continuous genetic and chromosomal analysis is important. However, the results from many laboratories demonstrate the rapid progress for possible treatment of diabetes by using insulin-producing cells generated from hESCs.

2.3.1.5 The limitations of hESCs

The potential application of hESCs has been extensively studied due to their high differentiation capacity. However, the use of hESCs to generate functional cells raises the problems associated with technical limitations and ethical issues. One of the technical limitations is associated with the use of culture conditions using feeder layer in a serum-containing medium. The use of any feeder layer will always exhibit inherent variability, increases the work load and limits the large-scale culture of hESCs. Hence, developing a feeder-free culture system as well as eliminating xenogeneic products for culturing hESCs will be valuable tools for researchers (Mallon, Park, Chen, Hamilton and McKay, 2006). The issue of difficulty to maintain hESCs in culture is also highlighted by their spontaneous apoptosis and differentiation. It has been reported by Qin et al. (2007) that p53 knockdown hESCs reduces spontaneous differentiation and slows the differentiation rate. Indeed, teratoma formation has become a critical obstacle for the therapeutic applications of hESCs. It is generally believed that highly purified progenitors or terminally differentiated cell types derived from hESCs results in prevention of teratoma formation (Hentze et al., 2009). While subpopulations of hESCs have been characterized by the expression of distinct surface markers, their fates have provided a valuable tool for generating tissue-specific reagents for cell-based therapy (King et al., 2009). Moreover, the combined gene transfer/hESCs therapies can generate a pure population of genetically modified differentiated cells with the selection using lineage-specific markers (Strulovici, Leopold, O'Connor, Pergolizzi and Crystal, 2007). Furthermore, the encapsulation procedure has the potential to prevent the formation of tumors (Fong, Gauthaman and Bongso, 2010a).

The possible destruction of transplanted hESCs derivatives by the patient's immune system should also be considered before the transplantation of these cells. Recent reports have shown that the immunological properties of hESC-derived cells are related to the immune rejection processes. The most important classes of alloantigens that may cause rejection are the minor histocompatibility complex (mHC) antigens and the ABO blood group antigens. It has already been shown that undifferentiated hESCs express low levels of MHC-I molecules, while differentiated hESCs show elevation in this expression. Therefore, it is possible that MHC expression on differentiated hESCs leads to the rejection of the transplanted cells. However, there are several options for the prevention of graft rejection. In these regards, the immune-previleged sites including eye, brain and testes have been used to avoid immune response toward transplanted cells. Additionally, it might be possible to create a bank of immunotyped hESCs, to generate isogenic cell lines by somatic nuclear transfer (NT) or by parthenogenesis. However, these technical and ethical issues must be overcome before applying these cells in clinical applications. Other solutions to prevent the rejection of hESC-derived cells are the use of hematopoietic chimerism for tolerance induction and the creation of universal donor cell line (Boyd, Higashi and Wood, 2005; Drukker and Benvenisty, 2004).

The ethical controversies represent another issue associated the use of fresh human embryos. There is concern about guidelines on the use of fresh embryos as a source of new hESC lines. However, the Canadian pluripotent stem cell guidelines provide such donors and the public with the option of making a well-considered and voluntary decision about the donation of surplus fresh embryos for hESC research. Importantly, they offer a model for ethical oversight of the

donation of fresh embryos for this research that is available to oversight bodies in other countries (Cohen et al., 2008). Furthermore, induced pluripotent stem cells (iPSCs) technology provides a solution to the ethical debate surrounding hESCs since it does not require both the destruction of an embryo and the use of human oocytes (Kastenberg and Odorico, 2008).

2.3.1.6 Encapsulation technology: A power tool for hESCs applications

In the context of therapeutic applications, the encapsulation technology represents a powerful tool towards the implementation of hESCs in clinical and industrial applications. The encapsulated hESC results in high expansion ratio and high cell recovery yields after cryopreservation. This method also improves the culture of hESC aggregates by protecting cells from hydrodynamic shear stress, controlling aggregate size and maintaining cell pluripotency (Serra et al., 2011). It has been demonstrated that hESCs encapsulated in alginate hydrogels maintain the undifferentiated state and retain their pluripotent capabilities without any enzymatic treatment, mechanical expansion or manipulation in a feeder-free environment. This approach is well-suited for providing automated culture scale-up process and the opportunity of long-term culture, feeder-free and non-labor-intensive culture of hESCs (Siti-Ismail, Bishop, Polak and Mantalaris, 2008).

Alginate encapsulation systems have been shown to support the ability of ES cells to differentiate into specific cell types. The researchers use an alginate encapsulation process for the proliferation and growth of mESC aggregates, which further supports the differentiation of insulin-positive cells from mESCs (Wang, Adams, Buttery, Falcone and Stolnik, 2009). In addition, the other group

demonstrates 3D model to culture and differentiate hESCs that are encapsulated in calcium alginate microcapsules. This system promotes cellular interactions that are essential for both maintaining pluripotency and differentiation. In addition, encapsulated hESCs are separated from feeder cells during the process of differentiation, which mimics *in vivo* microenvironment and bypass the EBs formation step in a controlled manner. Thus, this 3D culturing of hESCs using alginate microcapsules may be useful for direct differentiation of hESCs toward particular cell types and also has potential for immunoisolation and prevention of teratoma formation of hESCs during transplantation (Chayosumrit, Tuch and Sidhu, 2010).

Cell encapsulation has been proposed to be a solution for treatment of diabetes since it potentially allows the cell protection from host immune system by a concept of immunoisolation. In particular, the microcapsules of islets provide a delicate balance of characteristics including physical strength, immunocompatibility and selective permeability that will block large immune components. Additionally, its membrane allow the passage of smaller molecules such as oxygen, glucose, water and insulin (Lee and Bae, 2000). In addition, encapsulated islets in a biocompatible alginates have protected the islets against immune rejection, which is confirmed by prolonged survival of encapsulated islet allografts up to 200 days (de Vos, Faas, Strand and Calafiore, 2003). Furthermore, Schneider et al. (2005) have developed a microcapsule system that protects adult rat and human islets against xenogenic rejection in immunocompetent diabetic mice without immunosuppression.

2.3.2 Mesenchymal stem cells (MSCs)

2.3.2.1 The characteristics of MSCs

Mesenchymal stem cells (MSCs) are multipotential nonhematopoietic progenitor cells that are initially isolated from bone marrow. It is later found that these cells are present in almost every type of connective tissue (Hocking and Gibran, 2010; Pountos and Giannoudis, 2005). It has been reported that MSCs are present in several sources including umbilical cord blood (UCB), adipose tissue, bone marrow (BM) and Wharton's jelly of umbilical cord (Bunnell, Flaat, Gagliardi, Patel and Ripoll, 2008; Laco, Kun, Weber, Ramakrishna and Chan, 2009; Lee et al., 2004b; Taghizadeh, Cetrulo and Cetrulo, 2011).

The MSCs are characterized by plastic adherence, colony forming capacity and rapid proliferation. These cells exhibit differentiation capacity that can differentiate into osteogenic, adipogenic and chondrogenic lineages under appropriate conditions (Figure 2.8) (Chen, Shao, Xiang, Dong and Zhang, 2008; Deans and Moseley, 2000; Hoogduijn et al., 2010). It is also recognized that MSCs are positive for CD105, CD73, and CD90 expression and are negative for hematopoietic cell surface markers CD34, CD45, CD11a, CD19 or CD79α, CD14 or CD11b expression and HLA-DR (Dominici et al., 2006). Additionally, there is evidence demonstrating that undifferentiated MSCs and MSCs differentiated into adipose, bone and cartilage express HLA class I, but not HLA class II. This suggests that MSCs can be transplantable in HLA-incompatible individuals (Le Blanc, Tammik, Rosendahl, Zetterberg and Ringden, 2003).

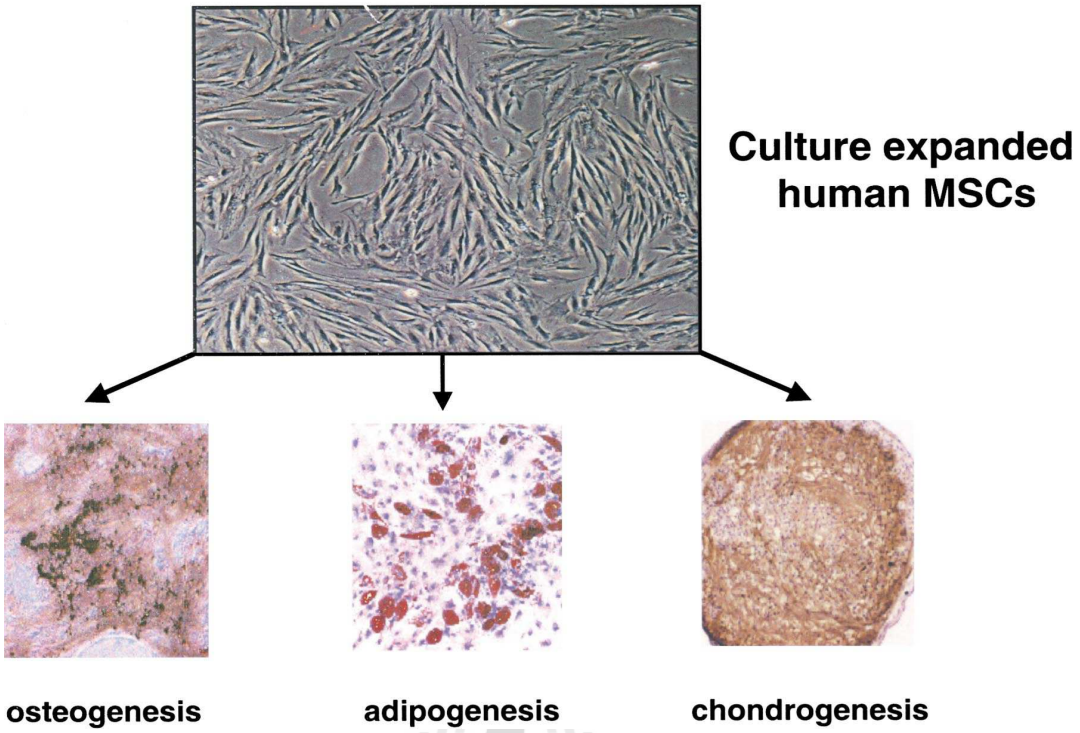

Figure 2.8 Human mesenchymal stem cells culture. The cells exhibit a spindle-shaped fibroblastic morphology following culture expansion *ex vivo* (top panel). Under appropriate inducing conditions, the cultures will demonstrate adipogenic differentiation evidenced by fat globules, chondrogenic differentiation as measured by staining for type II collagen, or osteogenesis as seen by calcium conditions (Deans and Moseley, 2000).

2.3.2.2 The therapeutic properties of MSCs

The MSCs have provided an attractive source for tissue engineering, regenerative medicine and autoimmune disease treatment. The ability of self-renewal and multipotency also make MSCs an effective tool for cell-replacement therapy. Several recent studies indicate that MSCs possess the capacity to differentiate into nonhematopoietic cells of multiple tissues including epithelial cells of the liver, kidney, lung, skin, gastrointestinal (GI) tract, and myocytes of heart and skeletal muscle (Herzog, Chai and Krause, 2003). In addition, several characteristics of MSCs such as migration and homing potential, immunomodulatory properties and trophic effect, could raise their clinical applications in various disorders. Moreover, the ability to secrete bioactive molecules such as growth factors, cytokines and chemokines has been shown to mediate paracrine mechanisms of MSCs (Meirelles Lda, Fontes, Covas and Caplan, 2009). The trophic effects of MSCs are distinct from the direct differentiation of MSCs into repair tissue. The MSCs accomplish this function by secreting a variety of cytokines and growth factors that have both paracrine and autocrine activities. It is proposed that MSCs supply bioactive factors that suppress the local immune system, inhibit fibrosis (scar formation) and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-intrinsic reparative or stem cells (Caplan and Dennis, 2006). Moreover, the data indicate that the presence of immunomodulatory properties may play specific roles as immunomodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumor evasion, as well as fetal-maternal tolerance (Nauta and Fibbe, 2007). It is also important to recognize that MSCs possess remarkable immunosuppressive properties and can inhibit the proliferation and function of the major immune cell populations.

This effect may be attributed to the interaction with a wide range of immune cells, which is evidenced by the *in vivo* and *in vitro* immunomodulatory properties of MSCs (Shi, Liu and Wang, 2011). MSCs could also contribute in the repair of focal tissue injury due to their ability to secrete bioactive factors that serve to limit the extent of tissue damage at the injured sites and to re-establish blood supply. Indeed, the mechanisms involved in MSC homing to injured sites followed by exertion of local trophic and immunomodatory effects could be the most important functions of systemically delivered MSCs (da Silva Meirelles, Caplan and Nardi, 2008). Furthermore, MSCs represent an easily harvested and expanded cell type without complications, thus providing an efficient cell sources for transplantation (Bieback, Schallmoser, Kluter and Strunk, 2008). When compared with embryonic stem cells, MSCs are relatively free of ethical issues, immune rejection and tumor formation (Kim, Jeon, Yang, Oh and Chang, 2010). Therefore, these MSC characteristics exhibit the potential for use in cell-based therapy.

2.3.2.3 Treatment of diabetes mellitus by MSCs

It has been an increasing interest in therapeutic effects of MSCs for diabetes mellitus treatment. Lee et al. (2006) reported that intracardiac infusion of human bone marrow cells (hMSCs) into diabetic NOD/*scid* mice engrafted immediately after systemic infusion into the mice, probably in response to specific signals from the injured tissues. The results indicated that the infused hMSCs improved the hyperglycemia and increased mouse insulin levels in the diabetic mice. There was an increase in pancreatic islets and β-cells producing mouse insulin in the hMSC-treated mice. Therefore, the effects of the hMSCs treatment could represent the selective homing of hMSCs and the ability of the cells to repair the tissues.

Additional study has noted that *in vitro* transdifferentiation of human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) into insulin-producing cells could provide an abundant source of cells for islet transplantation. This finding demonstrates that human UCB-MSCs-derived insulin-secreting cells express pancreatic β-cell markers including insulin, glucagon, GLUT2, Pdx1, Pax4 and Ngn3. These induced cells could synthesize and secrete functional islet proteins such as insulin, glucagon and C-peptide. However, the induced cells represent immature islet-like cells, because those cells are not responsive to glucose challenge very well (Gao et al., 2008). Recently, Xie et al. (2009) have studied the capacity of human bone marrow mesenchymal stem cells (human BM-MSCs) to generate insulin-producing cells. In that effort, human BM-MSCs were induced to transdifferentiate into insulin-producing cells both phenotypically and functionally. These differentiated cells expressed multiple genes related to the development or function of pancreatic β-cells including *Nkx6.1*, *Isl1*, *BETA2/NeuroD*, *Glut2*, *Pax6*, *Nestin*, *Pdx1*, *Ngn3*, *insulin* and *glucagon*. Immunofluorescence analysis indicated that these cells co-expression insulin and C-peptide. These insulin-producing cells could secrete insulin in a glucose-dependent manner *in vitro* and could ameliorate the diabetic conditions of streptozotocin (STZ)-treated nude mice.

2.3.2.4 Wharton's jelly derived mesenchymal stem cells (WJ-MSCs)

This context focuses specifically on the properties of MSCs that have been harvested from Wharton's jelly. The Wharton's Jelly that is contained within the human umbilical cord is typically discarded as medical waste post-delivery.

Wharton's jelly stem cells are isolated in a non-invasive procedure that is safe for both mother and newborn. The stem cells in Wharton's jelly exhibit MSC properties which possess plastic culture ware adherence, specific surface antigen expression and multipotent differentiation potential (Taghizadech, Cetrulo and Cetrulo, 2011). On the basis of MSC properties, these stem cells are also known as Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) (Peng et al., 2011). Interestingly, these cells exhibit specific markers consistent with ESCs, including hESC markers (Tra-1-60, Tra-1-81, SSEA-1, SSEA-4 and alkaline phosphatase), pluripotency markers (Oct4, Sox2 and nanog) and form EBs *in vitro*. However, WJ-MSCs show low expression levels of pluripotency markers compared to hESCs (Taghizadech, Cetrulo and Cetrulo, 2011).

The MSCs derived from the Wharton's jelly are considered as a potential alternative to human umbilical cord blood (UCB)-derived MSCs. Fong et al. (2010b) reported that human Wharton's jelly was a rich source of MSC compared with human UCB. In this study, the results showed that human WJ-MSCs could offer an invaluable source for regenerative purposes, because of the high derivation efficiency, availability of large numbers of fresh live cells, high expansion capabilities, prolonged maintenance of stem cell properties and differentiation potential over UCB-derived MSCs. Moreover, the other group demonstrates that different tissue derived MSCs could utilize unique mechanisms of immune-modulation. They have thus evaluated the immune properties of MSCs from bone marrow and Wharton's jelly when primed with inflammatory stimuli, IFNγ and TNFα. The results indicated that expression levels of known MSCs immune-modulatory molecules such as IDO, Hgf and COX2 generated PGE2 differed

in culture supernatants of both the MSCs. These factors were differently modulated by inflammatory stimuli. It is temping to suggest that inflammation can fine tune the immune properties of various tissue derived MSCs distinctly. Therefore, the effect of predominant inflammatory cytokines at the site of transplantation is considered to be important in modulating the immunogenicity and immunomodulatory behavior of different MSCs (Prasanna, Gopalakrishnan, Shankar and Vasandan, 2010).

2.4 Herbal medicine

Scientists remain interested in natural products including plants, animals and minerals that can be used for the treatment of human diseases. In developing countries, the applications of natural products to replace or reduce the toxicity and side effects of synthetic drugs have been developed significantly. (Verma and Singh, 2008). The use of natural products can represent an economic and safe alternative approach to treat infectious diseases (Rojas, Ochoa, Ocampo and Munoz, 2006). Indeed, a number of modern pharmaceutical drugs have their origin from herbs that are used in traditional medicine (Muthu, Ayyanar, Raja and Ignacimuthu, 2006). The World Health Organization (WHO) estimates that approximately 80% of the global population uses herbal medicine for their primary healthcare. As a consequence, the herbal market is around US$ 60 billion with an annual growth rate of 7% (Wakdikar, 2004).

In fact, substances derived from plants or plant-derived synthetic analogs offer one quarter of all prescription medications. Traditional knowledge and experiences have had an influence on modern allopathic medicine. It is likely that many important new remedies will be discovered and commercialized in the future. Accordingly, there

are many herbal remedies that have the power to prevent and treat a variety of diseases. The mixtures of different chemical compounds that are found in herbal plants exhibit various effects on the human body. For examples, bitter substances can stimulate digestion but anti-inflammatory compounds reduce swellings and pain rather than reveal the effect on digestive system. Phenolic compounds may act as an antioxidant and venotonics while antibacterial and antifungal tannins act as natural antibiotics. Diuretic substances can enhance the elimination of waste products and toxins. In addition, alkaloids have been found to enhance mood and give a sense of well-being (Gurib-Fakim, 2006).

In Thailand, many herbs have been used in medical applications since ancient time to present time. There is a great amount of publishing data regarding Thai herbs' properties in medicine. Many herbs have been scientifically evaluated for their medicinal values. For instances, root extracts of *Ananas comosus* and *Carica papaya* exhibited diuretic activities (Sripanidkulchai, Wongpanich, Laupattarakasem, Suwansaksri and Jirakulsomchok, 2001). The extract of *Moringa oleifera* showed therapeutic efficacy for HSV-1 infection (Lipipun, 2003). *Aeginetia indica* Roxbert extracts enhanced T cell-mediated immune responses (Auttachoat, Chitsomboon, Peachee, Guo and White, 2004). The extract from *Canna indica* rhizomes displayed HIV type 1 reverse transcriptase inhibitory activity (Woradulayapinij, Soonthornchareonnon and Wiwat, 2005). The *Mucuna collettii* extracts manifested antimutagenic potential (Cherdshewasart, Sutjit, Pulcharoen, Panriansaen and Chulasiri, 2008). *Garcinia cowa* leaf extracts exhibited antibacterial activity against gastrointestinal pathogenic bacteria (Sakunpak and Panichayupakaranant, 2012).

2.4.1 Herbal medicine for diabetes

The diabetic treatments using oral hypoglycemic agents and insulin therapy demonstrate promising results. However, these treatments are considered to cause side effects in some individuals (Lorenzati, Zucco, Miglietta, Lamberti and Bruno, 2010; Kuroe et al., 2007). Thus, there is an increased requirement of low side effect agents that can improve safety of diabetes treatment. The herbal plants have been good candidates for commonly used against diabetes. Several herbs have been evaluated for their antidiabetic activities. The biological actions of herbal products are related to their chemical compositions that are used to treat diabetes. It has been reported that the key compounds for diabetic improvement are phenolics, flavonoids, terpenoids, coumarins and other constituents which show blood glucose reduction (Rao, Sreenivasulu, Chengaiah, Reddy and Chetty, 2010). More important antidiabetic potential of herbal plants and their active principles are given in the Table 2.3.

In Thailand, several herbal medicines have been documented in Thai/Lanna Medicinal Plants/Recipe Database “MANOSROI II” as antidiabetes including *Anogeissus acuminata*, *Rauwolfia serpentina*, *Catunaregam tormentosa*, *Dioecrescis erythroclada* and *Mimosa pudica* (Manosroi, Moses, Manosroi and Manosroi, 2011). Moreover, a recent systematic review suggested that supplementation with *Ipomoea batatas*, *Silybum marianum* and *Trigonella foenum-graecum* may improve glycemic control in T2D patients (Suksomboon, Poolsup, Boonkaew and Suthisisang, 2011). Thus, these findings indicate that herbs may offer an alternative approach for preventing and treating diabetes.

Table 2.3 Important antidiabetic potential of herbal plants and their active principles (Rao, Sreenivasulu, Chengaiah, Reddy and Chetty, 2010).

Botanical name	**Family**	**Parts used**	**Main active components**
Allium sativum	Alliaceae	Bulbs	Allyl propyl disulphide, allicin
Annona squamosa	Annonaceae	Fruits	Liriodenine, moupinamide
Areca catechu	Arecaceae	Seed	Arecaine and arecoline
Artemisia pallens	Asteraceae	Leaves and flowers	Germacranolide
Azadirachta indica	Meliaceae	Leaves, flowers and seed	Azadirachtin and nimbin
Bauhinia forcata	Leguminosae	Leaf	Astragalin, kaempferitrin
Beta vulgaris	Amaranthaceae	Root	Phenolics, betacyanins
Boerhavia diffusa	Nyctaginaceae	Whole plant	Punarnavine and ursolic acid
Camellia sinensis	Theaceae	Leaves	Caffeine and catechins

Table 2.3 (Continued).

Botanical name	Family	Parts used	Main active components
Capparis decidua	Capparidaceae	Fruit	Spermidine Isocodonocarpine
Combretum micranthum	Combretaceae	Leaves	Polyphenols
Elephantopus scaber	Asteraceae	Whole plant	Terpenoid and 2,6,23 -trienolide
Ficus bengalensis Linn	Moraceae	Bark	Leucodelphinidin and leucopelargonin
Gymnema sylvestre	Asclepiadaceae	Leaf	Dihydroxy gymnemic triacetate
Gynandropsis gynandra	Capparidaceae	Root	*N*,*N*-diethyltoluamide
Lantana camara	Verbenaceae	Leaves	Lantanoside, lantanone
Liriope spicata	Liliaceae	Root	Beta-sitosterol, stigmasterol
Momordica charantia	Cucurbitaceae	Leaves	Charantin, sterol
Ocimum sanctum	Labiatae	Whole plant	Eugenol
Panax quinquefolius	Araliaceae	Root	Ginsenosides, protopanaxadiol

Table 2.3 (Continued).

Botanical name	Family	Parts used	Main active components
Parinari excelsa	Chrysobalanaceae	Bark	Myricetin, quercertin
Phyllanthus amarus	Phyllanthaceae	Whole plant	Phyllanthin
Prunus amygdalus	Rosaceae	Seeds	Amygdalin
Pterocarpus marsupium	Leguminosae	Whole plant	Kenotannic acid, pyrocatechin
Punica granatum	Lythraceae	Fruit	Punicalagin, punicalin
Ricinus communis	Euphorbiaceae	Root	Ricinolic acid
Salacia oblonga wall	Celastraceae	Root bark	Salacinol
Sarcopoterium spinosum	Rosaceae	Root	Catechin and epicatechin
Smallanthus sonchifolius	Asteraceae	Leaves	Sonchifolin, uvedalin, enhydrin, uctuanin
Swertia punicea	Gentianaceae	Whole plant	Methyl swertianin and bellidifolin
Tinospora cordifolia	Menispermaceae	Root	Tinosporone, tinosporic acid

Table 2.3 (Continued).

Botanical name	Family	Parts used	Main active components
Trigonella foenum-graecum	Fabaceae	Leaves and seeds	4-hydroxy isoleucine
Vernonia anthelmintica	Asteraceae	Seed	Epoxy acid or vernolic acid
Withania somnifera	Solanaceae	Cuscohygrine and withasomnine	Somniferine,withananine and cuscohygrine

2.4.2 *Gynura procumbens*

Gynura procumbens (family Compositae, common name paetumpung) is widely distributed in South East Asian countries such as Indonesia, Malaysia and Thailand. Its classification is as the following:

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Asterales

Family Asteraceae

Genus Gynura

Species *Gynura procumbens (Lour.) Merr.*

Source: http://www.homolaicus.com/scienza/erbario/utility/botanica_sistematica/hypertext/0791.html.

Figure 2.9 *Gynura procumbens (Lour.) Merr.*, paetumpung.

Gynura procumbens (Lour.) Merr., has traditionally been used for the treatment of eruptive fevers, rash, kidney disease, migraine, constipation, hypertension, diabetes mellitus and cancer. Several reports documented that *G. procumbens* has hypoglycemic activity (Hassan, Yam, Ahmad and Yusof, 2010; Zhang and Tan, 2000), anti-inflammatory (Iskander, Song, Coupar and Jiratchariyakul, 2002), anti-hypertensive (Hoe, Lee, Mok, Kamaruddin and Lam, 2011; Kim, Lee, Wiryowidagdo and Kim, 2006), anti-ulcerogenic (Mahmood et al., 2010), wound healing activity (Zahra et al., 2011), antioxidant and antitumor (Maw, Mon and Oo, 2011). A previous research has reported that phenolics and flavonoids are the major antioxidant components identified in *G. procumbens* leaf (Mustafa, Abdul Hamed, Mohamed and Abu Baker, 2009). Phenolic compounds exert multiple biological effects including antioxidant activity, antitumor, antimutagenic and antibacterial properties (Shui and Leong, 2002). Additionally, the flavonoids exhibit potential for antiviral, anti-allergic, antiplatelet, anti-inflammatory and antitumor activities (Buhler and Miranda, 2000). Moreover, another study has suggested that steroids might be one class of anti-inflammatory compounds in this plant (Iskander, Song, Coupar and Jiratchariyakul, 2002).

In the hypoglycemic activity aspect, the study has shown that an ethanolic extract of *G. procumbens* leaves (150 mg/kg) significantly lower serum glucose, cholesterol and triglyceride levels in diabetic rats. It has been demonstrated that the hypoglycemic effect of *G. procumbens* may be due to a biguanide-like activity, similar to metformin (a biguanide derivative) that can ameliorate abnormalities in lipid levels (Zhan and Tan, 2000). Furthermore, the water extract of *G. procumbens* leaves (1000 mg/kg) showed a significant decrease in blood glucose

levels and improved outcome of the intraperitoneal glucose tolerance test (IPGTT) in diabetic rats. It was observed that the *G. procumbens* water extract actively enhanced insulin-stimulated glucose transport across the membrane of skeletal muscle, similar to metformin in the absence of insulin (Hassan, Yam, Ahmad and Yusof, 2010). It is therefore possible that *G. procumbens* may provide a useful source of new hypoglycemic agent to treat diabetic patients.

2.4.3 Efficacy and safety of herbal products

Despite the widespread interest, herbs that lack ethnomedical verification of efficacy or safety should be of concern. The adverse reactions of herbal products are caused by adulteration, inappropriate formulation or lack of understanding of herb and drug interaction (Elvin-Lewis, 2001). The information about the effects of herbal remedies must provide safety, efficacy and reliable alternatives to conventional medicine. There have been reports of the adverse medical consequences from herbal plant products. Numerous herbal products are associated with multisystem organs failure such as cardiovascular, pulmonary, renal, liver, hematological and endocrine system (Tovar and Petzel, 2009). By analyzing the Johannesberg forensic database, the cause of death may be due to a traditional remedy with an unknown substance (Stewart, Moar, Steenkamp and Kokot, 1999).

In order to minimize adverse side effects, it is highly important to develop methods that ensure the quality and safety of herbal products. Thus, standardization and quality control for these products are essential to be measured for consumer safety. In addition, assessments of markers such as taxonomic, chemical, genomic and proteomic referring the identification/authentication of herbal product components also need to be investigated. The defined markers are also helpful for

identification of the adulterants, differentiation of herbal medicines with different sources, stability testing of proprietary products. However, marker-based analysis has limitations as the markers are not single compounds and a combination of methods is necessary for herbal component detection. Besides herbal marker analysis, the safety and efficacy of herbal products are established through their long historical use. Furthermore, the quality control of herbal medicines has a direct impact on their safety and efficacy. There are many parameters such as identification, water content, chemical assay of active ingredients, inorganic impurities (toxic metals), microbial limits, mycotoxins, pesticides and others, should also be certified for herbal products (Sahoo, Manchikanti and Dey, 2010).

Although there is an increase in the market of herbal products, the regulation of the quality assurance of both practitioners and product-related treatment for medicinal uses need to be well established. This requires a new category of cGMP license, meet standards of safety and quality requirements and local government health department regulation (Chan, 2003). Furthermore, the international regulatory requirements on specifications, standardization and classification of herbal products need to be met in order to ensure uniformity of quality, safety and efficacy of the same herbal medicines across the countries.

CHAPTER III

MATERIALS AND METHODS

3.1 Human embryonic stem cells (hESCs)

3.1.1 Culture of hESCs

The hESCs line H9 was purchased from Wicell Research Institute, Madison, USA. The cells were maintained in the undifferentiated state by culturing on the layer of mitomycin-C treated human foreskin fibroblast (HFF-1) feeder. Undifferentiated hESCs were grown in hESC medium containing 79% knockout Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen), 20% knockout serum replacement (KO-SR; Invitrogen), 1% non-essential amino acid (Sigma), 1 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Invitrogen) and 5 ng/ml basic fibroblast growth factor (bFGF; Peprotech) at 37°C, 5% O_2, 4.5% CO_2 and 95% humidity. The cells were mechanically passaged every 5-7 days.

3.1.2 *In vitro* differentiation of insulin-producing cells

For the differentiation of insulin-producing cells, undifferentiated hESC colonies were produced to generate embryoid bodies (EBs) using the hanging drop method and were cultured in hESC medium without bFGF. EBs were then cultured in 6-well plate for 14 days in stage 1 medium mainly composed of KO-DMEM containing 2% B27 (Invitrogen), 2 ng/ml bFGF and 20 ng/ml EGF (Peprotech) **(Stage 1)**. Then, the differentiated cells were cultured in culturing medium as stage 1 in the absence of bFGF for 7 days **(Stage 2)**. At day 29, the cells were cultured in a

maturation medium mainly composed of KO-DMEM, 10 mM nicotinamide (Sigma) and 50 ng/ml IGF II (Peprotech) to generate insulin-producing cells for 18 days **(Stage 3)**. Differentiated cells were incubated at 37°C, 5% O_2, 4.5% CO_2 and 95% humidity.

To verify whether differentiated cells are insulin-producing cells, differentiated cells were subjected to characterization methods including protein expression by immunofluorescence assay, gene expression by real-time PCR, dithizone staining and insulin secretion assay.

3.1.3 Immunofluorescence assay

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS and blocked with 3% serum and 0.2% triton X-100 in PBS. The cells were then incubated overnight at 4°C with primary antibody. After washing with PBS, cells were incubated with secondary antibody for 1 h at room temperature. The cells nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 3 minutes at room temperature and were mounted with mounting media (VECTASHIELD, Vector Labs, Burlingame, CA). The immunofluorescence images were analyzed using a fluorescence microscope (Olympus American Inc. Melville, NY).

The following primary antibodies and dilutions were used; mouse anti-hNestin 1:100 (R&D Systems, MAB1259); guinea pig anti-insulin, 1:100 (Dako, A0564); rabbit anti-glucagon, 1:100 (Dako, A0565); mouse anti-human pro-insulin c-peptide 1:100 (Chemicon, CBL94); mouse anti-somatostatin (Santa Cruz Biotechnology, SC-25262). Secondary antibodies were FITC-conjugated goat

anti-mouse 1:100 (BD Pharmingen), FITC-conjugated swine anti-rabbit (Dako) and Cy3-conjugated goat anti-mouse 1:100 (Jackson Immuno Research Labs).

3.1.4 Quantitative real-time PCR

Undifferentiated hESCs, EBs and differentiated stage 1-3 cells were collected for RNA extraction using RT100 Total RNA Mini kit (Geneaid). RNA concentrations were measured by NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies Inc.) and 50-100 ng of this RNA was used in a reverse transcription (RT) reaction with a cDNA Synthesis kit (Fermentas). The PCR mixture were carried out by mixing cDNA with SYBR Green master mix (Applied Biosystems), forward and reverse primers (listed in Table 3.1). The real-time PCR reactions were performed using an ABI 7900HT real time PCR system (Applied Biosystems). The relative gene expression values were calculated by normalization with housekeeping gene GAPDH.

Table 3.1 Primer sequences and PCR conditions used in the real-time PCR.

Gene	Primer sequence (5' ----> 3')	Annealing temp. (°C)	Product size (bp)	Reference
GAPDH	F: AGC CAC ATC GCT CAG ACA CC R: GTA CTC AGC GGC CAG CAT CG	60	302	Yao et al., 2006
OCT4	F: GAGCAAAACCCGGAGGAGT R: TTCTCTTTCGGGCCTGCAC	60	310	Yao et al., 2006
Nestin	F: CAGCTGGCGCACCTCAAGATG R: AGGGAAGTTGGGCTCAGGACTGG	55	208	Shim et al., 2007
Pdx1	F: CCC ATG GAT GAA GTC TAC C R: GTC CTC CTC CTT TTT CCA C	58	262	Seeberger et al., 2006
Hnf3β	F: CCA CCA CCA ACC CCA CAA AAT G R: TGC AAC ACC GTC TCC CCA AAG T	60	294	Baharvand, Hashemi, Ashtiani and Farrokhi, 2006a

Table 3.1 (Continued).

Gene	Primer sequence (5' ----> 3')	Annealing temp. (°C)	Product size (bp)	Reference
Ngn3	F: GGT AGA AAG GAT GAC GCC TC R: CCG AGT TGA GGT CGT GCA T	58	313	Seeberger et al., 2006
NeuroD1	F: GCC CCA GGG TTA TGA GAC TAT CAC T R: CCG ACA GAG CCC AGA TGT AGT TCT T	61	523	Khoo et al., 2005
Pax6	F: CCG AGA GTA GCG ACT CCA G R: CTT CCG GTC TGC CCG TTC	64	239	Segev, Fishman, Ziskind, Shulman and Itskovitz-Eldor, 2004
Nkx6.1	F: GTT CCT CCT CCT CCT CTT CCT C R: AAG ATC TGC TGT CCG GAA AAA G	53	381	Segev, Fishman, Ziskind, Shulman and Itskovitz-Eldor, 2004
GLUT2	F: AGGACTTCTGTGGACCTTATGTG R: GTTCATGTCAAAAAGCAGGG	55	231	Segev, Fishman, Ziskind, Shulman and Itskovitz-Eldor, 2004

Table 3.1 (Continued).

Gene	Primer sequence (5' ----> 3')	Annealing temp. (°C)	Product size (bp)	Reference
Insulin	F: CTACCTAGTGTGCGGGGAAC R: CACAATGCCACGCTTCTG	61	~150	Yu et al., 2007
Somatostatin	F: GTA CTT CTT GGC AGA GCT GCT G R: CAG AAG AAA TTC TTG CAG CCA G	55	179	Segev, Fishman, Ziskind, Shulman and Itskovitz-Eldor, 2004
Glucagon	F: CCC AAG ATT TTG TGC AGT GGT T R: GCG GCC AAG TTC TTC AAC AAT	60	221	Seeberger et al., 2006

3.1.5 Dithizone (DTZ) staining

A DTZ (Sigma) stock solution was prepared by dissolution 50 mg of DTZ in 5 ml of dimethylsulfoxide (DMSO) and stored briefly at -15 °C. DTZ staining was performed by adding 20 µl of the stock solution to 1 ml of culture medium. The cells were then incubated at 37°C for 15 minutes. After rinsing with Hank's balanced salt solution (HBSS), the stained cells were analyzed by a phase contrast microscope.

3.1.6 Measurement of insulin secretion of differentiated cells

The differentiated cells were rinsed twice in Krebs-Ringer bicarbonate HEPES (KRBH) buffer. The cells were then incubated in KRBH buffer containing 5, 20 or 50 mM glucose at 37°C for 1 h, respectively. Supernatant were collected for insulin secretion measurement. Insulin levels were determined by insulin enzyme-linked immunosorbent assay (ELISA) kit (Dako).

3.1.7 Alginate encapsulation of human embryonic stem cell-derived insulin-producing cells (hES-DIPCs)

Insulin-producing cells were suspended in a 1.5% alginate solution at a concentration of 40,000 cells/ml and extruded the cell-alginate mixture from a plastic syringe into a 200 ml bath of $CaCl_2$ (100 mM), containing 145 mM NaCl and 10 mM MOPS. After the alginate beads were formed, the $CaCl_2$ solution was removed and beads were washed with buffer solution. Finally, the encapsulated hES-DIPCs were then cultured overnight in complete medium at 37°C.

3.1.8 *In vitro* assessment of insulin secretion of encapsulated hES-DIPCs

The encapsulated hES-DIPCs were cultured at 37°C, 5% O_2, 4.5% CO_2 and 95% humidity. Cell culture media samples were collected at D0, D2, D4, D6, D8,

D10, D12 and D14 to assess the quantity of insulin secreted by the cells. Insulin levels were determined by insulin enzyme-linked immunosorbent assay (ELISA) kit (Dako).

3.1.9 Diabetic treatment by hES-DIPCs transplantation

Mice were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on mice in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT).

Eight-week old male mice weighing approximately 20 to 30 g bred were used. The animals were maintained under controlled temperature (23-25°C) with a 12 h light and 12 h dark lighting. They were fed with a water and standard laboratory diet *ad libitum* throughout the experiment.

To generate diabetic mice, non diabetic mice were fasted overnight (12 h) prior to intraperitoneal injection of streptozotocin (STZ, 40 mg/kg body weight). Diabetes was confirmed by the determination of fasting glucose concentration on the seventh day post administration of STZ. STZ-treated animals were considered diabetic when the blood glucose exceeded 250 mg/dl.

Diabetic mice were then divided into three groups as follows:

Group I: Diabetic control mice without cell transplantation. (n = 5)

Group II: Diabetic mice were subcutaneously (SC) transplanted with non-encapsulated hES-DIPCs. (n =5)

Group III: Diabetic mice were subcutaneously (SC) transplanted with encapsulated hES-DIPCs. (n =5)

The encapsulated hES-DIPCs were removed from the culture dishes, placed in a conical tube and washed three times with HBSS. Each animal received 1×10^5 cells corresponding to a capsule volume of 500 µl in a 1 ml syringe. Treated mice did not receive any immunosuppressive drug and the cells were transplanted at D0, D14, D28 and D42. The body weight was measured weekly during the experimental period.

3.1.10 Examination of hES-DIPCs transplantation recipients

After transplantation, mice were fasted overnight. Blood samples were obtained from the tail bleeding. Fasting blood glucose level of each mouse was then determined in every 14 days until 70 days of experiments. The blood glucose levels were measured by Accu-Chek Advantage II (Roche Diagnostic, Mannheim, Germany). The body weight of experimental animals was measured before and after transplantation. At the end of the experiment, the mice were sacrificed by cervical dislocation. Blood samples were collected and centrifuged at 1,500 g for 10 minutes. The serum was used for determination of blood urea nitrogen (BUN), creatinine, cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The atherogenic index (AI) and HDL/cholesterol ratio were calculated for all samples. AI was calculated from the ratio of (cholesterol-HDL)/HDL.

3.1.11 Detection of inflammatory mediator of transplanted mice

At the end of the experiments, serum IL-1β inflammatory cytokine was determined by an enzyme immunoassay (Mouse IL-1β/IL-1F2: Quantikine, R&D systems). The cytokine contents were expressed as pg/ml. The mean minimum detectable dose (MDD) of mouse IL-1β was 2.31 pg/ml. The test was performed following the instruction of the manufacturer.

3.1.12 Statistical analysis

Data were expressed as mean ± S.E.M. for each group. Statistical analysis was performed using SPSS software package Version 11.5. The intragroup comparisons were performed by using Paired-Samples T-test. The intergroup comparisons were performed by using a one-way analysis of variance (ANOVA) followed by Duncan test. Differences were considered to be significant when $P < 0.05$.

3.2 Combination of human Wharton's jelly derived mesenchymal stem cells (hWJ-MSCs) transplantation and GPE treatment

3.2.1 Mesenchymal stem cell culture

The hWJ-MSCs cell line, WJM-1 was generated previously from Dr. Wilairat Leeanansaksiri Laboratory. WJM-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-Invitrogen) containing 10% FBS (HyClone), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 µg/ml amphotericin B (Abbott). Cells were cultured in 5% CO_2 and 5% O_2 at 37ºC and were passaged every 3 days under the same culture condition.

3.2.2 Plant material and preparation of plant extract

Gynura procumbens leaves were obtained from Dr. Wilairat Leeanansaksiri and the identity of this plant was confirmed by comparison with a voucher specimen no. 82892 at the Herbarium of the Royal Forest Department of Thailand.

Fresh leaves of the plant were washed well three times by tap water and then washed once again by distilled water. The leaves were ground with 95% ethanol

by an electrical grinder and then filtered through a filter paper. The ethanol extract was concentrated under vacuum in a rotary evaporator (Büchi Vacuum Controller V-800, Switzerland) (Figure 3.1) to yield a semi-solid mass, which was further frozen and lyophilized by a freeze dryer system (Labconco, Kansus, USA) (Figure 3.2). The dried powder was stored at -80°C for further use. The percentage yield for ethanol extract was 0.9% (w/w).

Figure 3.1 Rotary evaporator used in the extraction process.

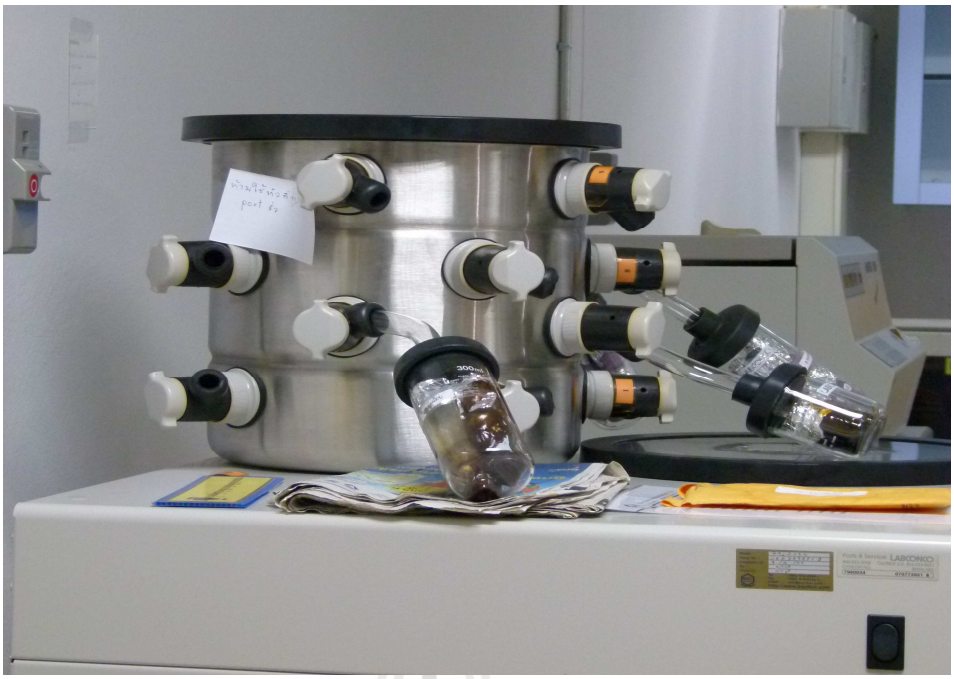

Figure 3.2 Lyophilization apparatus used in the freeze drying process.

3.2.3 Assessment of the effects of GPE alone or hWJ-MSCs transplantation alone or combination of treatments in diabetic mice

Mice were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on mice were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT).

Eight-week old non-diabetic male mice weighing approximately 25 to 35 g bred were used. The animals were maintained under controlled temperature (23-25°C) with a 12 h light and 12 h dark lighting. They were fed with a water and standard laboratory diet *ad libitum* throughout the experiment.

To generate diabetic mice, non-diabetic mice were fasted overnight (12 h) prior to intraperitoneal injection of streptozotocin (STZ, 40 mg/kg body weight). Diabetes was confirmed by the determination of fasting glucose concentration on the seventh day post administration of STZ. STZ-treated animals were considered diabetic when the blood glucose exceeded 250 mg/dl.

The animals were divided into four groups as follows:

Group I: Diabetic control mice were fed with distilled water. (n = 5)

Group II: Diabetic mice were fed once a day with GPE (500 mg/kg) in aqueous solution orally for 84 days. (n = 5)

Group III: Diabetic mice were intravenously (IV) transplanted with hWJ-MSCs. (n = 5)

Group IV: Diabetic mice were treated with the combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg orally for 84 days). (n = 5)

Each animal was transplanted with 1×10^5 cells by means of a 1 ml syringe. Treated mice did not receive any immunosuppressive drug and the cells were transplanted at D0, D7, D14 and D21. The body weight was measured weekly during until day 84 after transplantation.

3.2.4 Measurement of fasting blood glucose level

After overnight fasted, the blood glucose was assessed from a capillary blood sample from the tail tip of rats. Fasting blood glucose level of each mouse was then determined in every 14 days until 84 days of experiment. Fasting blood glucose level was measured by Accu-Chek Advantage II (Roche Diagnostic, Mannheim, Germany).

3.2.5 Biochemical analysis

At the end of the experiment, the mice were euthanized and the blood samples were collected from the heart and hepatic portal vein. The serum was used for the determination of blood urea nitrogen (BUN), creatinine, cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The atherogenic index (AI) and HDL/cholesterol ratio were calculated for all samples.

3.2.6 Detection of inflammatory mediator

At the end of experiment, serum IL-1β inflammatory cytokine was determined by an enzyme immunoassay (Mouse IL-1β/IL-1F2: Quantikine, R&D systems). The cytokine contents were expressed as pg/ml. The mean minimum detectable dose (MDD) of mouse IL-1β was 2.31 pg/ml. The test was performed following the instruction of the manufacturer.

3.2.7 *In vitro* study of the GPE effect on human pancreatic cell line (PANC-1)

A human pancreatic cell line **(**PANC-1) were cultured in a mixture of DMEM containing 10% FBS. The cells were passaged 3-4 days before the experiment and plated in 6-well plates at a density of 0.5×10^6 cells/wells. To induce islet-like cell aggregates (ICAs) formation, cell culture medium was removed, cells were exposed to 0.05% trypsin/EDTA at 25°C for 60 s (to loosen but not detach the cells). These cells were then cultured in KO-DMEM medium containing 1% BSA and insulin-transferin-selenium (GIBCO). The PANC-1 cells formed ICAs within the next 4 days. These ICAs stained positive for DTZ, and were then tested for their ability to secrete insulin in response to glucose. These cells were incubated for 1 h with different glucose concentration (5, 20 or 50 mM) in the presence of GPE

(25 or 50 μg/ml). Following incubation, the solutions in the wells were removed and stored at -80°C for insulin measurement. The insulin secretion level was measured by an insulin ELISA kit (Dako).

3.2.8 Statistical analysis

Data were expressed as mean ± S.E.M. for each group. Statistical analysis was performed using SPSS software package Version 11.5. The intragroup comparisons were performed by using Paired-Samples T-test. The intergroup comparisons were performed by using a one-way analysis of variance (ANOVA) followed by Duncan test. Differences were considered to be significant when $P < 0.05$.

3.3 Transplantation of hWJ-MSCs

3.3.1 Transplantation of hWJ-MSCs alone into STZ-induced diabetic rats

Rats were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology (SUT).

Eight-week old male Wistar rats weighing approximately 200 to 250 g bred were used. The animals were maintained under controlled temperature (23-25°C) with a 12 h light and 12 h dark lighting. They were fed with a water and standard laboratory diet *ad libitum* throughout the experiment. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, 60 mg/kg body weight) dissolved in 0.1 M citrate buffer (pH 4.5) immediately before use to adult rats fasted for 12 h.

Ten days after STZ injection, fasting blood glucose of each rat was measured to evaluate the diabetic status. The STZ-injected rats with fasting blood glucose levels higher than 250 mg/dl were considered as diabetic rats and were used in experiments.

The diabetic rats were divided into four groups as follows:

Group I: diabetic control rats without hWJ-MSCs transplantation. (n= 4)

Group II: diabetic rats were intraperitoneally (IP) transplanted with hWJ-MSCs. (n = 4)

Group III: diabetic rats were subcutaneously (SC) transplanted with hWJ-MSCs. (n = 4)

Group IV: diabetic rats were intravenously (IV) transplanted with hWJ-MSCs. (n = 4)

Each animal was transplanted with 1×10^6 cells by means of a 1 ml syringe. Treated rats did not receive any immunosuppressive drug and the cells were transplanted at D0, D7, D14 and D21. The body weight was measured weekly until day 91 after transplantation.

3.3.2 Measurement of fasting blood glucose level

The rats were fasted overnight, blood samples were collected from the tail tip of rats. Fasting blood glucose level of each rat was then determined in every 14 days until 91 days of experiment. The fasting blood glucose level was measured by Accu-Chek Advantage II (Roche Diagnostic, Mannheim, Germany).

3.3.3 Biochemical analysis

At the end of the experiment, the rats were euthanized and the blood samples were collected from the heart and hepatic portal vein. The serum was used

for the determination of blood urea nitrogen (BUN), creatinine, uric acid, cholesterol and triglyceride.

3.3.4 Statistical analysis

Data were expressed as mean ± S.E.M. for each group. Statistical analysis was performed using SPSS software package Version 11.5. The intergroup comparisons were performed by using a one-way analysis of variance (ANOVA) followed by Duncan test. The differences of fasting blood glucose level and body weight at day 0 and day 91 of treatment were analyzed by using Paired-Samples T-test. Differences were considered to be significant when $P < 0.05$.

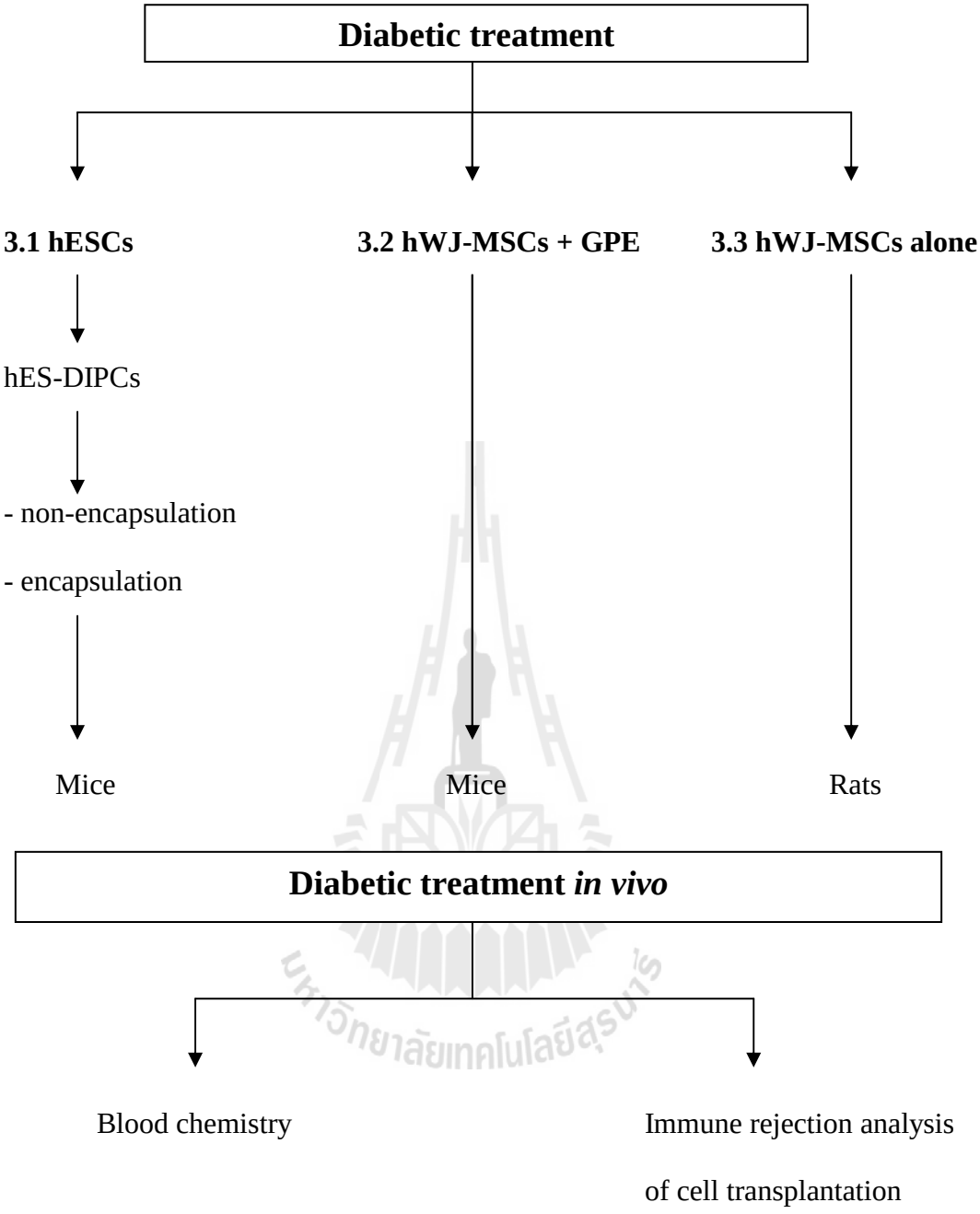

Figure 3.3 Schematic summarization of the study design.

CHAPTER IV

RESULTS

4.1 The potential capability of hES-DIPCs for diabetes treatment

4.1.1 Differentiation of hESCs into insulin-producing cells (hES-DIPCs)

To promote the differentiation of hESCs into insulin-producing cells, the procedure was performed according to a differentiation protocol as described in Materials and Methods Section. Differentiation of undifferentiated hESCs (Figure 4.1A) was initiated in the absence of feeder layers to promote the embryoid bodies (EBs) formation. After an induction period of 7 days, the EBs were found as three dimensional ball structure as shown in Figure 4.1B. After healthy EBs were obtained, the EBs were subjected to differentiation process, stage 1-3, as described in Materials and Methods Section. The medium was refreshed every 3 days. Stage 1 of differentiation, the EBs were attached and grown into a confluent monolayer by 8 days postdifferentiation. The cells were allowed to further mature in this cocktail for 6 days for pancreatic endoderm induction (Figure 4.1C). The cells were then cultured in stage 2 differentiation media for 7 days to generate pancreatic endocrine cells. The cell morphology was illustrated as Figure 4.1D. Cells from stage 2 were then cultured in stage 3 differentiation medium as described in Materials and Methods Section for 18 days to generate insulin-producing cells. The formations of small islet cell clusters were obtained as demonstrated in Figures 4.1E and 4.1F.

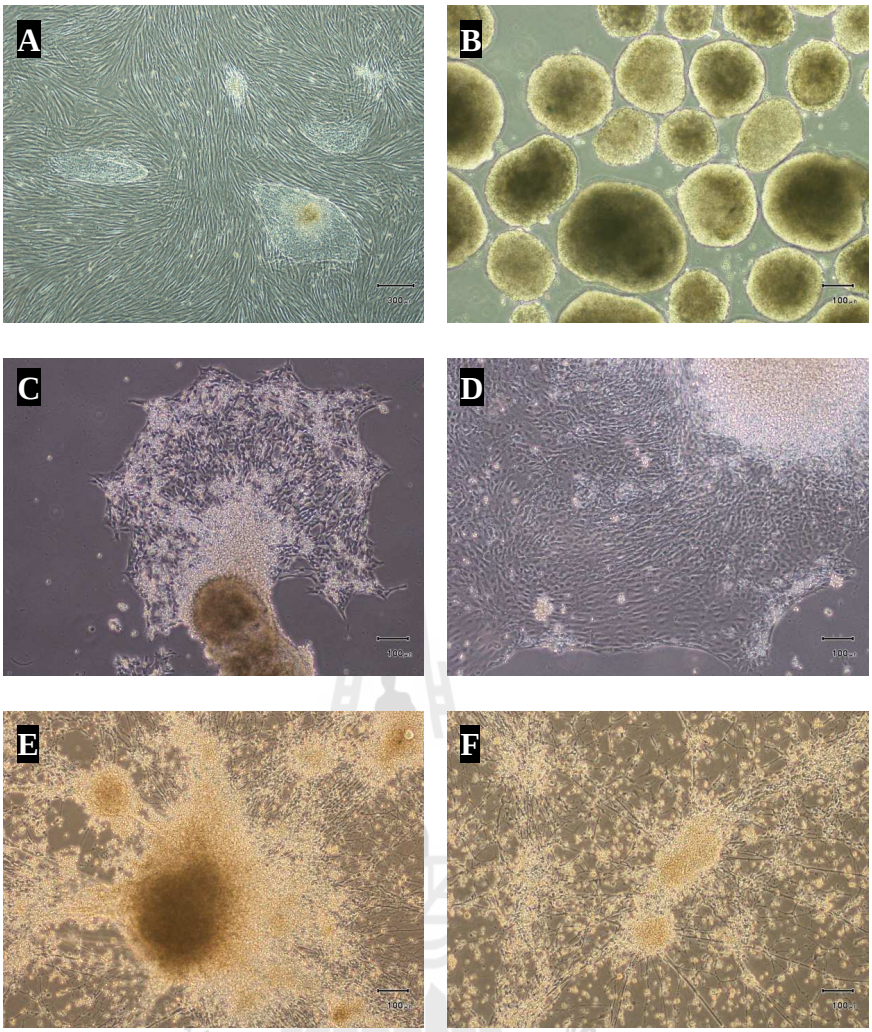

Figure 4.1 Differentiation of hESCs into insulin-producing cells. Phase contrast microscopy of hESCs colonies (A), Embryoid bodies; EBs (B) and differentiated cells at D21 (C), D28 (E) and D46 (E and F). Scale bars, 300 µm (A); 100 µm (B-F).

4.1.2 Characterization of hESC-derived insulin-producing cells (hES-DIPCs)

To assess whether the differentiation protocol used in this experiment is an optimized protocol for differentiation of hESCs into insulin-producing cells. Specific gene expressions of cells in each differentiation stage were determined by real-time PCR as demonstrated in Figures 4.2 and 4.3, respectively. The differentiation of the cells was examined throughout the processes, including cells at D7, D21, D28 and D46. Gene expression of transcription factor, pancreatic duodenal homeobox 1 (*Pdx1*), was detected mainly in induced cells (D28) and minimal decreased in 46 days postdifferentiation (D46). The cells at D21 were highly expressed *glucagon* and neurogenine 3 (*Ngn3*) and then reduced along the progression of differentiation stage. The expression of *Nestin*, *Hnf3β*, *Nkx6.1*, *Pax6* genes were detected starting at D7 and increased thereafter. Similarly, upregulation of *somatostatin* was increasingly observed from D21 until D46 of differentiation process. The expression of the gene encoding neurogenic differentiation factor 1 (*NeuroD1*) was upregulated only at D28. In addition, the expression of insulin gene was highly induced at later stage of differentiation (D46). The glucose transporter *GLUT2* was induced between D21 and D46 with a higher level at the end of the differentiation process (D46).

To confirm whether the hESCs had differentiated into islet-like clusters, protein expression analysis was carried out by immunofluorescence staining (Figures 4.4-4.6). The data indicated that expression of C-peptide, a byproduct of insulin production, was highly expressed in these cells (Figure 4.6A). In this stage, the glucagon-positive cells (Figures 4.5B and 4.6B), and somatostatin-positive cells

(Figure 4.5A) were also observed. However, the population of nestin-expressing cells was detected in day 46 of differentiation (Figure 4.4A).

Additionally, hESC-derived insulin-like clusters were further tested using a dithizone (DTZ) staining. DTZ, a zinc-chelating agent, is known to selectively stain pancreatic β-cells crimson red. The cells were stained positive for DTZ as shown in Figure 4.7, thus confirming that insulin-producing cells were present at various stages throughout differentiation.

To determine whether these cells can release insulin, we examined the insulin secretion in response to glucose by using 5 mM, 20 mM and 50 mM glucose. The insulin levels in response to glucose were 25.73 ± 2.41, 30.33 ± 1.14 and 63.47 ± 0.52 pmol/l, respectively. Significant insulin secretion was observed with 50 mM glucose as compared with 5 mM glucose (Figure 4.8).

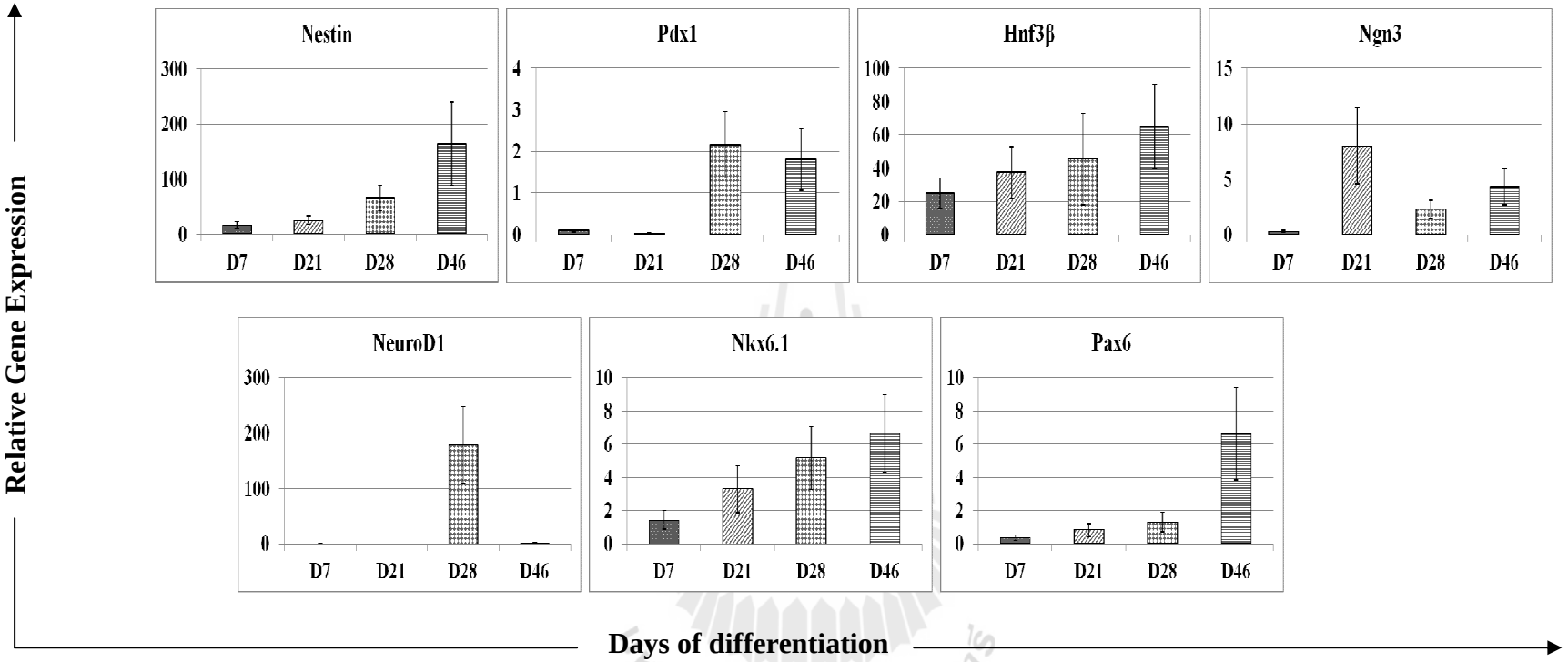

Figure 4.2 The expression pattern of pancreatic lineage genes. Real-time PCR was performed on D7, D21, D28 and D46 of differentiation. The levels of *Nestin*, *Pdx1*, *Hnf3β*, *Ngn3*, *NeuroD1*, *Nkx6.1* and *Pax6* gene expression were determined. The relative gene expression levels were normaized with undifferentiated hESCs levels.

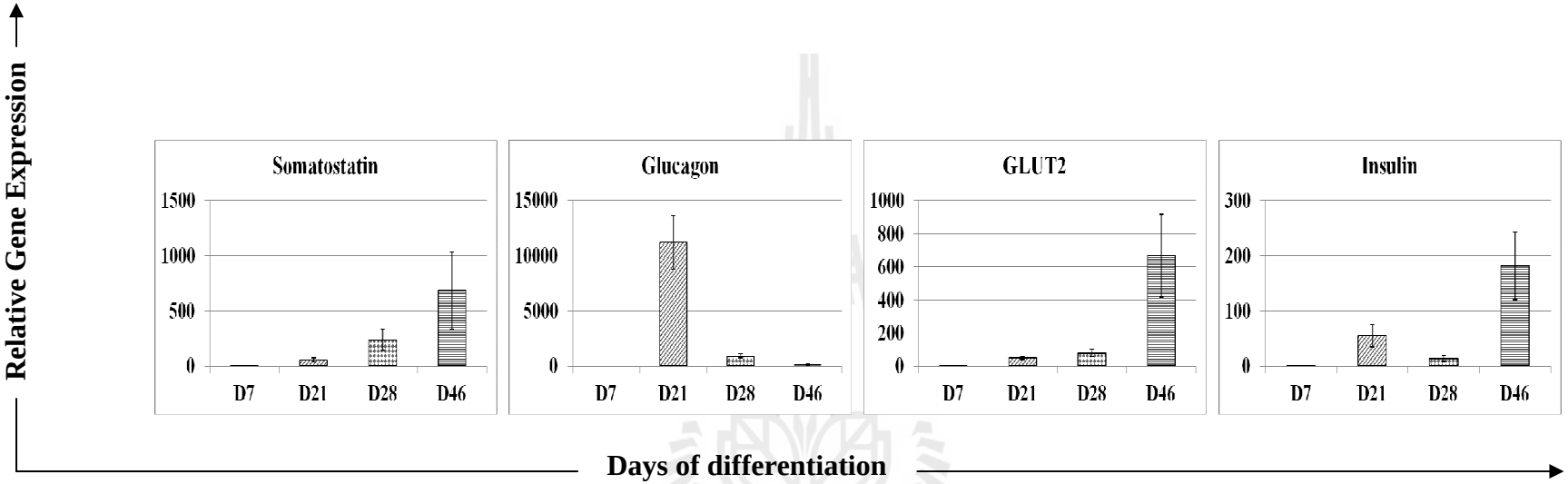

Figure 4.3 The gene expression patterns during insulin-producing cells derivation from hESCs. Real-time PCR was performed on D7, D21, D28 and D46 of differentiation. The levels of *somatostatin*, *glucagon*, *GLUT2* and *insulin* gene expression were determined. For each sample, the relative gene expression levels were normaized with undifferentiated hESCs levels.

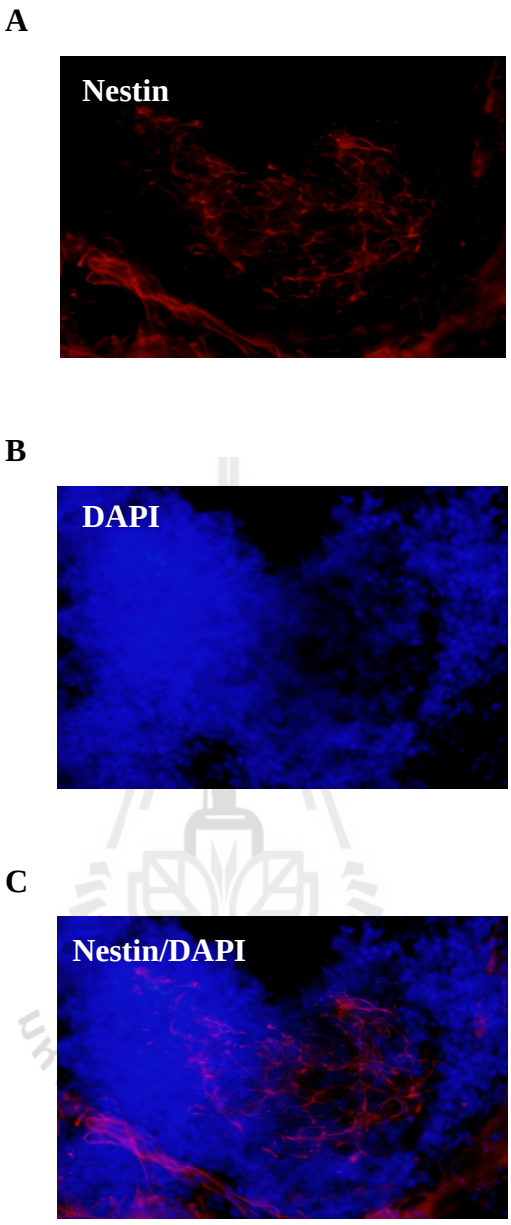

Figure 4.4 Fluorescence microscopic imaging of immunostaining for nestin. The cells after 46 days of differentiation were stained with antibodies against nestin (panel A, red = Cy3), followed by appropriate secondary antibodies conjugated to fluorochromes. Panel B is DAPI nuclear staining (blue color). A merge of nestin and DAPI images was shown in panel C. Scale bar = 50 µm.

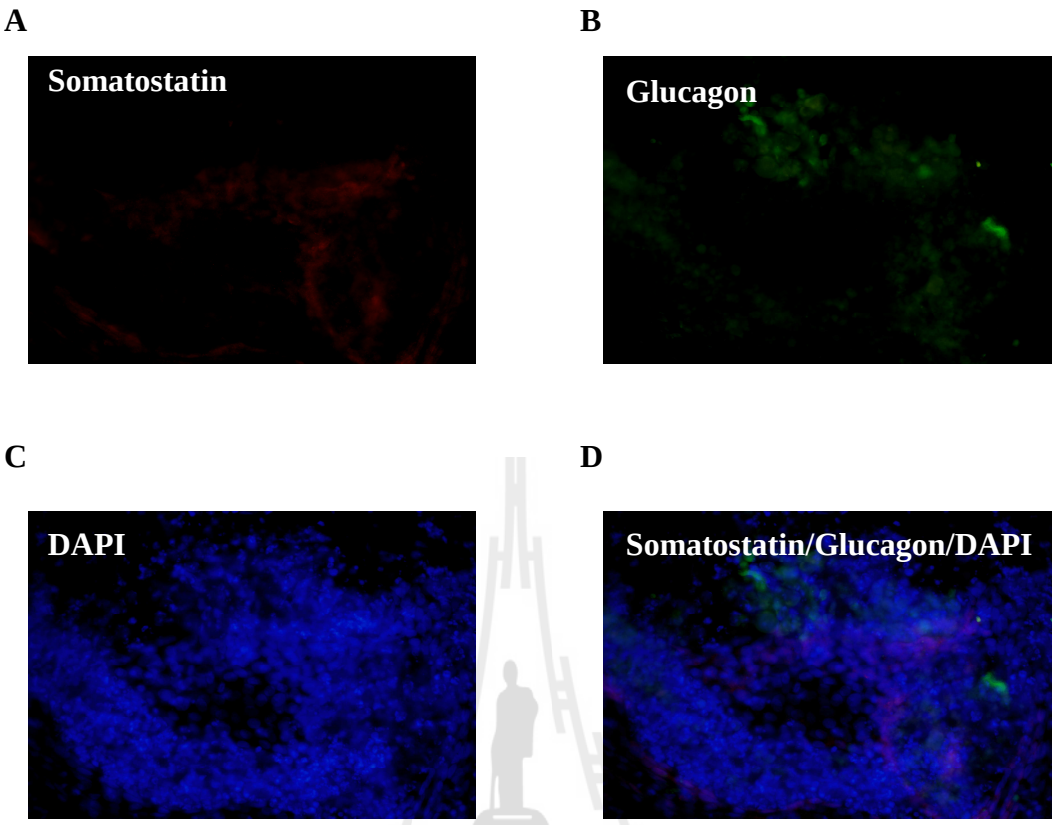

Figure 4.5 Fluorescence microscopic imaging of immunostaining for somatostatin and glucagon. The cells after 46 days of differentiation were stained with antibodies against somatostatin (panel A, red = Cy3) and glucagon (panel B, green = FITC), followed by appropriate secondary antibodies conjugated to fluorochromes. Panel C is DAPI nuclear staining (blue color). A merge of somatostatin, glucagon and DAPI images was shown in panel D. Scale bar = 50 µm.

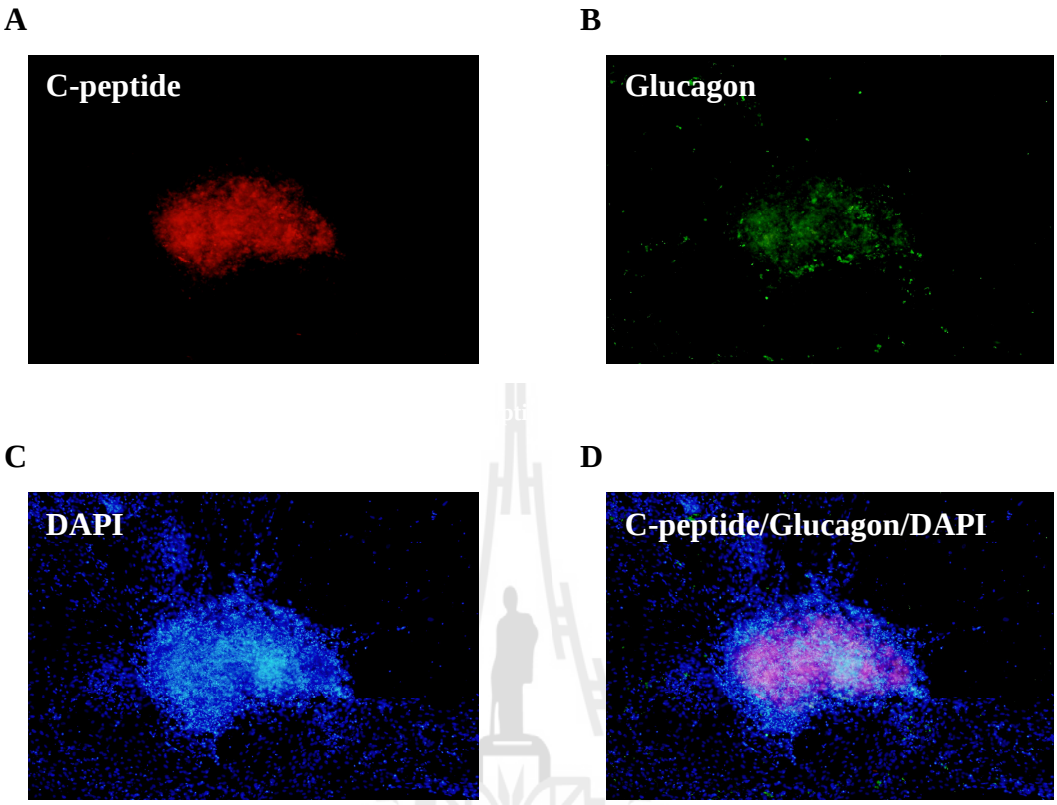

Figure 4.6 Fluorescence microscopic imaging of immunostaining for C-peptide and glucagon proteins. The cells after 46 days of differentiation were stained with antibodies against C-peptide (panel A, red = Cy3) and glucagon (panel B, green = FITC), followed by appropriate secondary antibodies conjugated to fluorochromes. Panel C is DAPI nuclear staining (blue color). A merge of C-peptide, glucagon and DAPI images was shown in panel D. Scale bar = 100 µm.

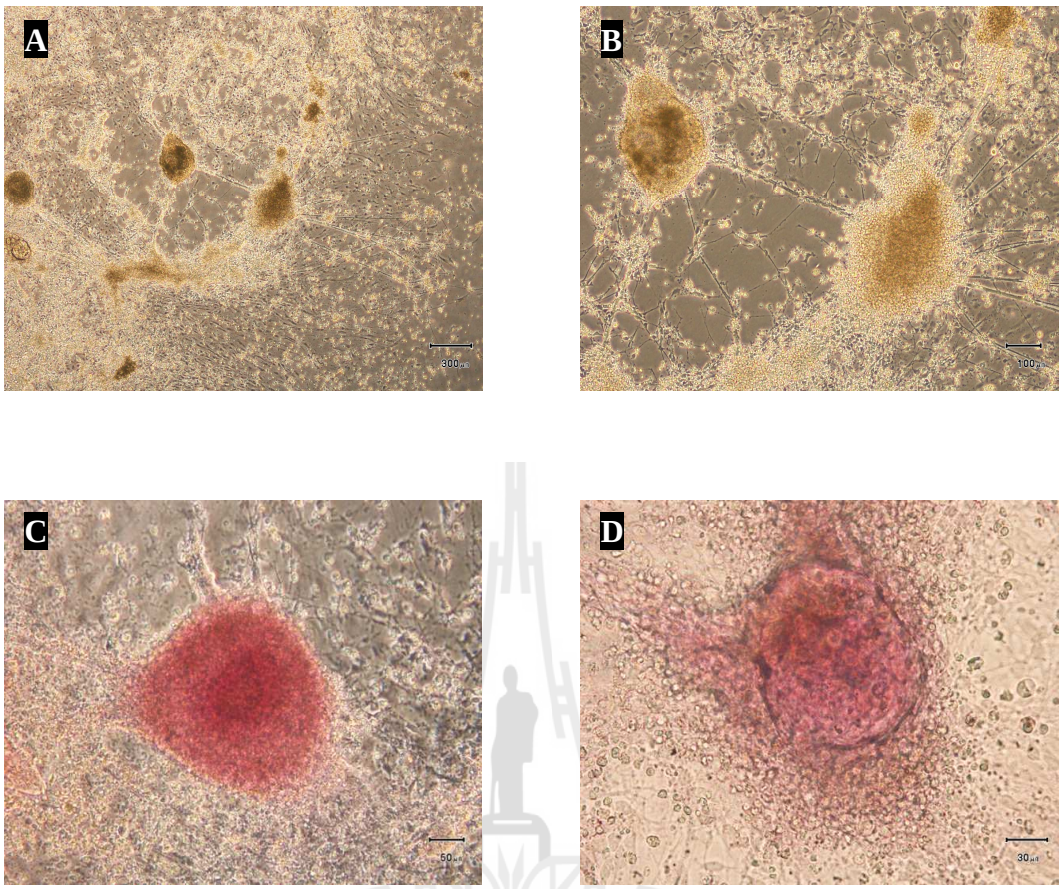

Figure 4.7 Dithizone (DTZ) staining. The cells after 46 days of differentiation were identified for insulin-producing cells within the differentiated hESC colonies (A and B). These clusters were DTZ positive (C and D). Scale bars, 300 µm (A); 100 µm (B); 50 µm (C); 30µm (D).

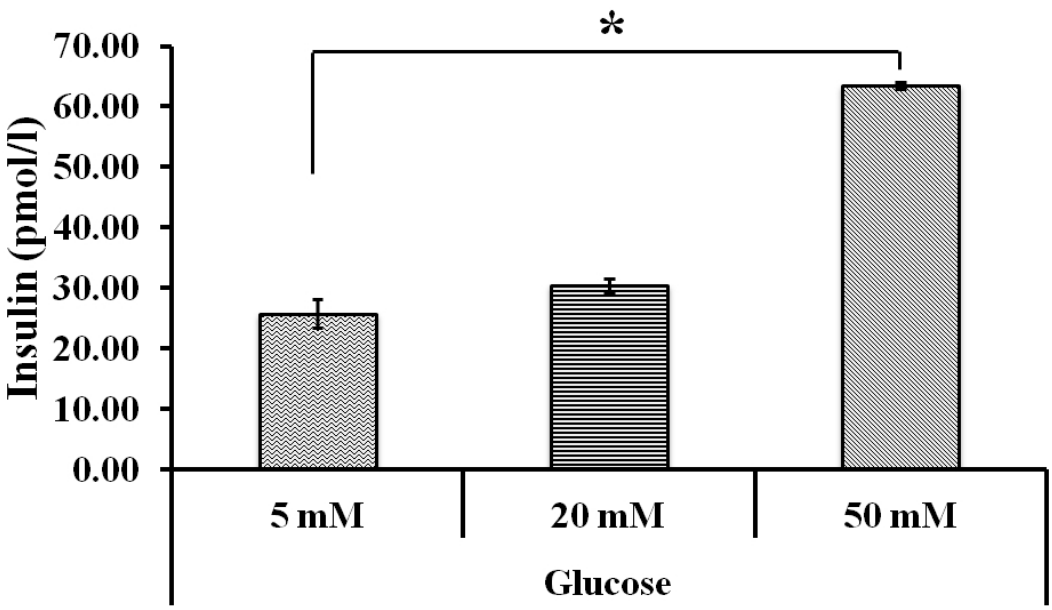

Figure 4.8 Insulin secretions at various glucose concentrations. Differentiated cells (D46) were examined for their insulin-secretion potential. The differences of secreted insulin concentrations were observed in 5 mM, 20 mM and 50 mM glucose stimulations. Significant insulin concentration was observed when incubated with 50 mM glucose. The data of experiment are expressed as the mean ± S.E.M of three independent experiments. *****Values deviate significantly from corresponding 5 mM glucose ($P < 0.05$).

4.1.3 Alginate encapsulation of hES-DIPCs

In order to generate alginate encapsulated hES-DIPCs, cells were mixed with alginate as described in Materials and Methods Section to form coated colony with diameter of approximately 2,000 μm (Figures 4.9A and 4.9B). The ability of encapsulated cells to release insulin into the culture medium was examined by using an ELISA assay. The amounts of insulin released from the encapsulated cells increased from D4 (16.90 ± 0.40 pmol/l) to D14 (127.92 ± 1.58 pmol/l) (Figure 4.9C).

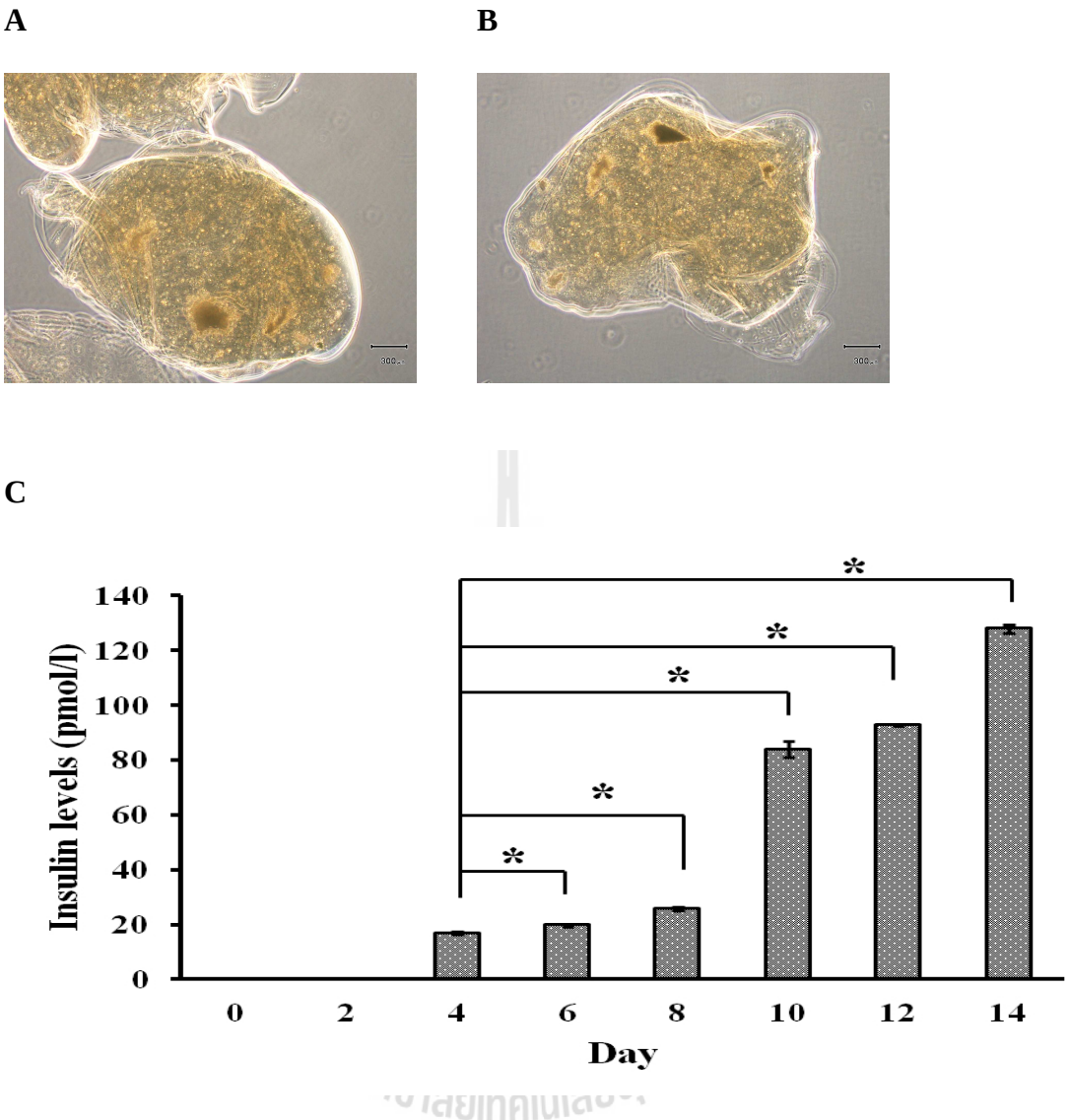

Figure 4.9 Encapsulated hES-DIPCs. Phase contrast microscopy of encapsulated hESC-derived insulin-producing cells (A and B). Insulin secretion was performed by ELISA assay. Representative results of insulin secretion levels were retrieved from the encapsulated hES-DIPCs cultured in differentiation medium stage 3 on D0, D2, D4, D6, D8, D10, D12 and D14, respectively (C). All values are mean ± S.E.M. for each group. *****Significantly different from D4 ($P < 0.05$). Scale bar = 300 μm.

4.1.4 Examination of body weight and fasting blood glucose levels of diabetic mice after hES-DIPCs transplantation

Body weight and fasting blood glucose levels of diabetic mice either transplanted with non-encapsulated or encapsulated hES-DIPCs were evaluated and shown in Figures 4.11 and 4.12, respectively. Untreated mice were included as controls. After non-encapsulated or encapsulated hES-DIPCs were transplanted into diabetic mice for 70 days, body weight of all mice was not significantly changed during the treatment, except encapsulated hES-DIPCs transplanted mice at D14 (Figure 4.11). In addition, the results demonstrated that fasting blood glucose levels were increased significantly in the untreated group throughout the study period. On the other hand, fasting blood glucose levels of transplanted mice were maintained. However, diabetic mice which received non-encapsulated hES-DIPCs transplantation showed significantly lower fasting blood glucose on D28 when compared with D14 posttransplantation (Figure 4.12).

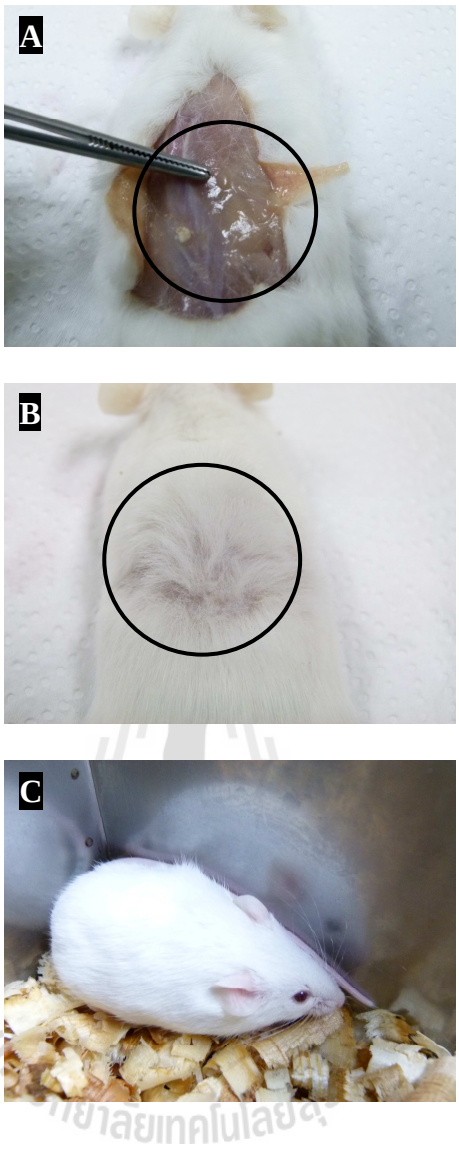

Figure 4.10 The observation of both non-encapsulated and encapsulated hES-DIPCs in diabetic mice groups. Gross observation after encapsulated hES-DIPCs transplanted into the subcutaneous site of diabetic mice (A). The appearance of diabetic mice after transplanted with encapsulated hES-DIPCs (B) and non-encapsulated hES-DIPCs (C).

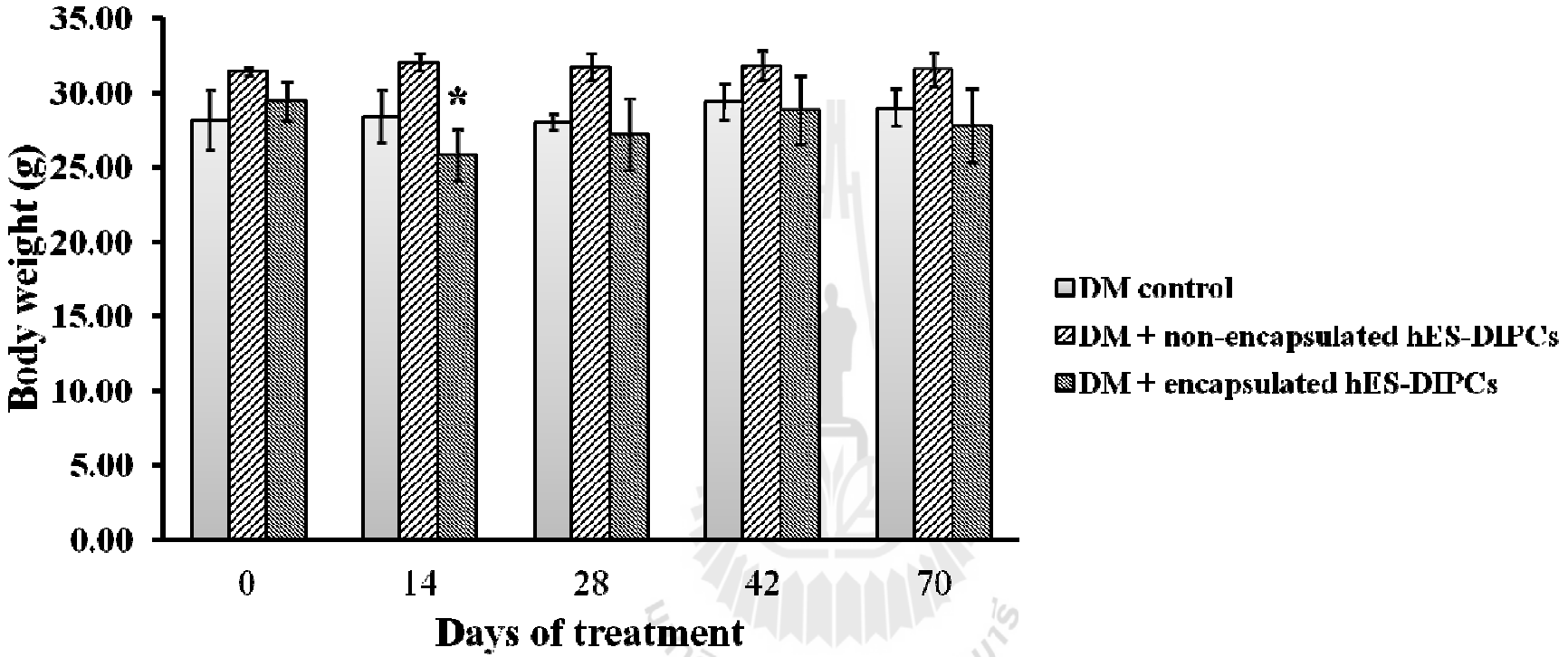

Figure 4.11 Body weight in diabetic mice either untreated or treated with non-encapsulated or encapsulated hESC-DIPCs. All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$).

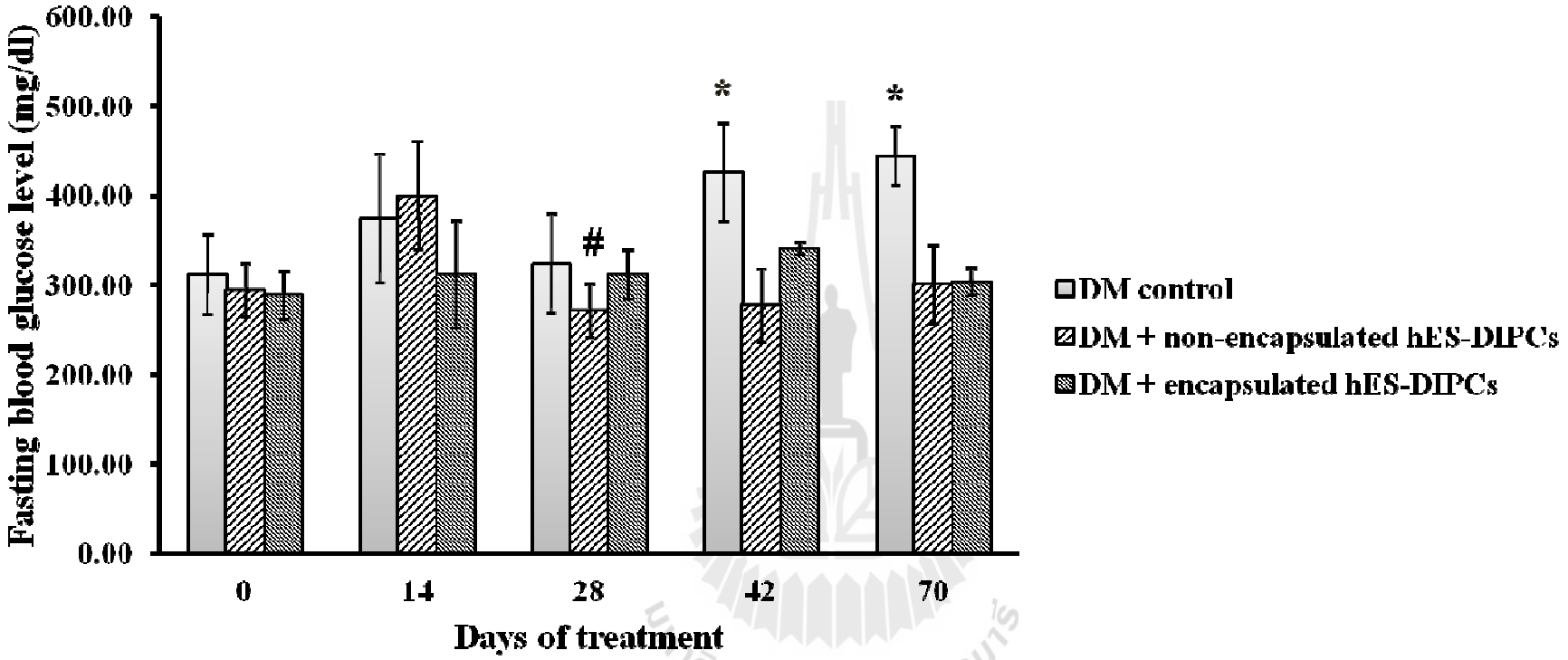

Figure 4.12 Fasting blood glucose levels in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$). #Significantly different from D14 ($P < 0.05$).

4.1.5 BUN and creatinine level of diabetic mice transplanted with hES-DIPCs

The levels of BUN and creatinine of diabetic mice transplanted with either non-encapsulated or encapsulated hES-DIPCs were determined after 70 days of transplantation. Untreated control group was included. The levels of BUN and creatinine of all three groups were not significantly differences as demonstrated in Figures 4.13 and 4.14, respectively.

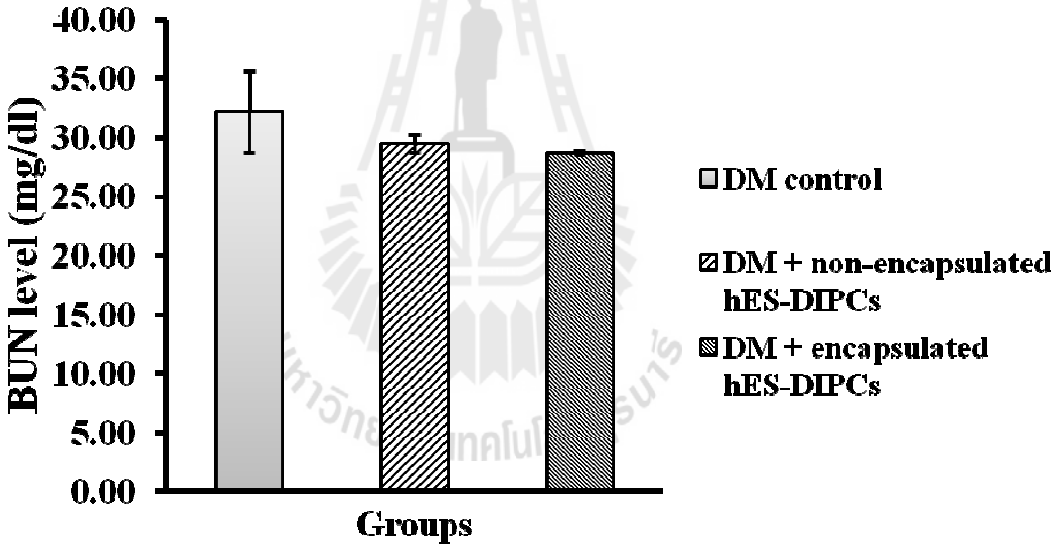

Figure 4.13 Levels of blood urea nitrogen (BUN) in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group.

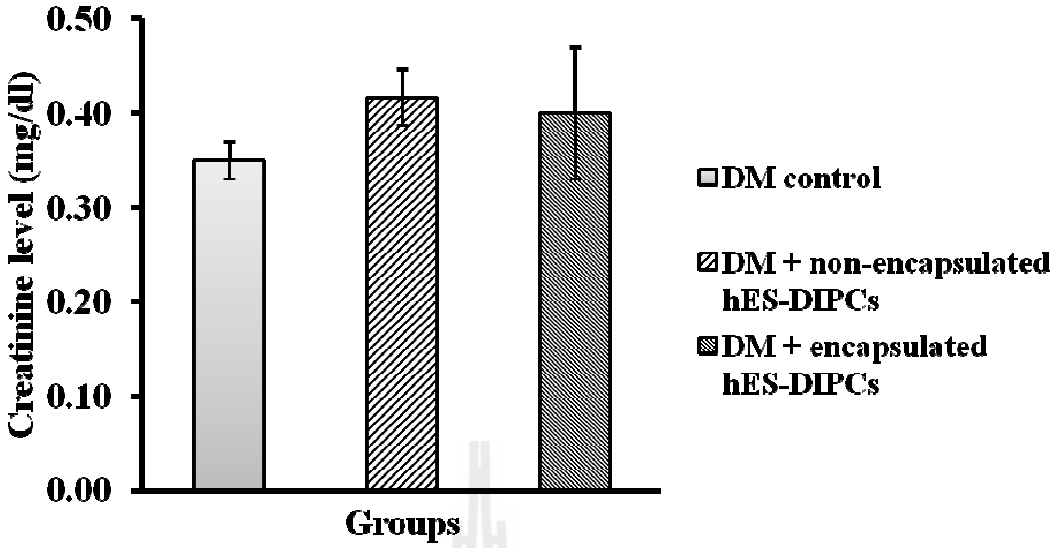

Figure 4.14 Levels of creatinine in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group.

4.1.6 Lipid profile of diabetic mice transplanted with hES-DIPCs

Blood samples of diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs were subjected to lipid profile analysis. The levels of LDL, AI and HDL/cholesterol ratio in these diabetic mice were shown in Figures 4.15-4.17, respectively. There was no significant difference in LDL levels between the non-encapsulated hES-DIPCs transplantation and diabetic control mice. However, a significant decrease in AI (Figure 4.16) and a significant increase in HDL/cholesterol ratio (Figure 4.17) were observed in non-encapsulated hES-DIPCs transplanted mice when compared to the diabetic control mice.

Transplantation of encapsulated hES-DIPCs did not produce any significant difference in LDL, AI and HDL/cholesterol ratio in these mice when compared to the control, as shown in Figures 4.15-4.17, respectively.

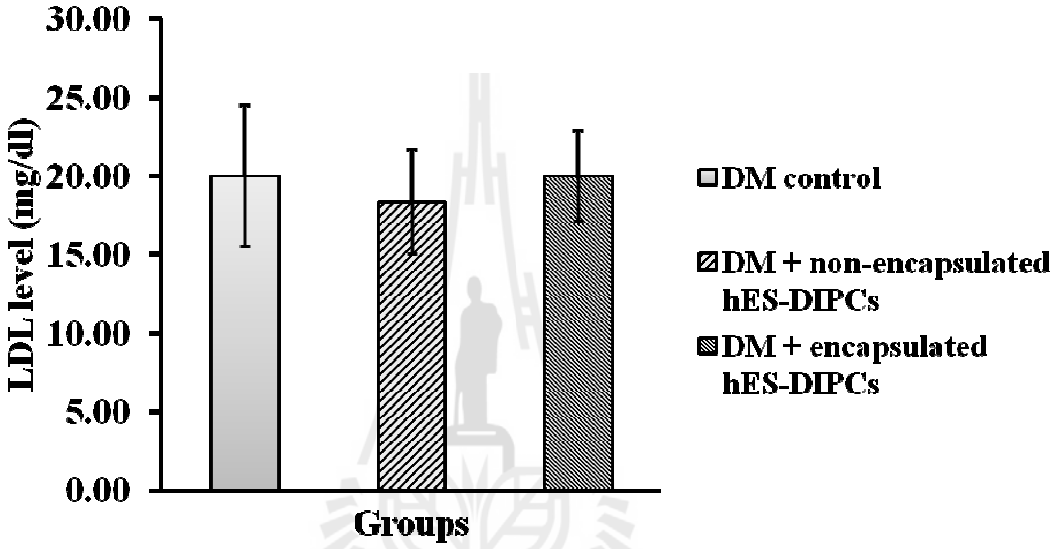

Figure 4.15 Levels of low-density lipoprotein (LDL) in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group.

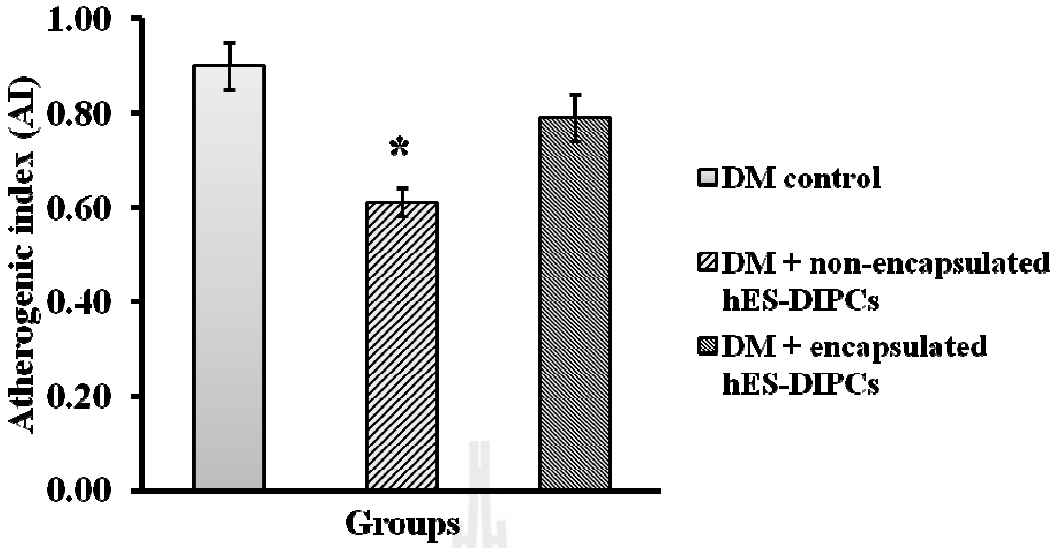

Figure 4.16 Atherogenic index (AI) in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group. *****Significantly different from the diabetic control mice ($P < 0.05$).

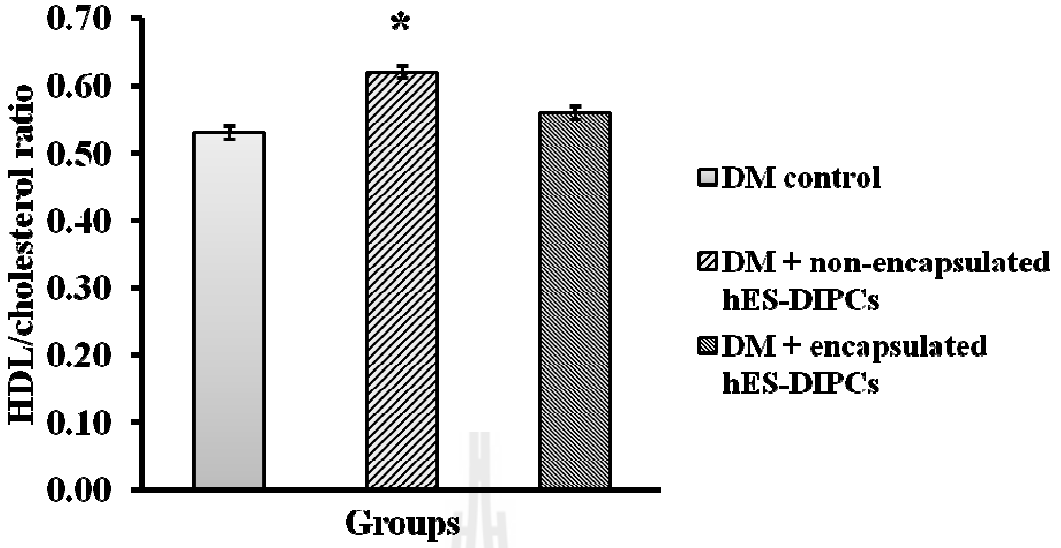

Figure 4.17 HDL/cholesterol ratio in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. All values are mean ± S.E.M. for each group. *****Significantly different from the diabetic control mice ($P < 0.05$).

4.1.7 Inflammatory evaluation of hES-DIPCs transplanted diabetic mice

The IL-1β inflammatory cytokine of non-encapsulated hES-DIPCs and encapsulated hES-DIPCs transplanted groups were evaluated by an ELISA assay. The results indicated significant decrease of IL-1β level in non-encapsulated and encapsulated hES-DIPCs transplanted groups compared to the diabetic control group, as shown in Figure 4.18.

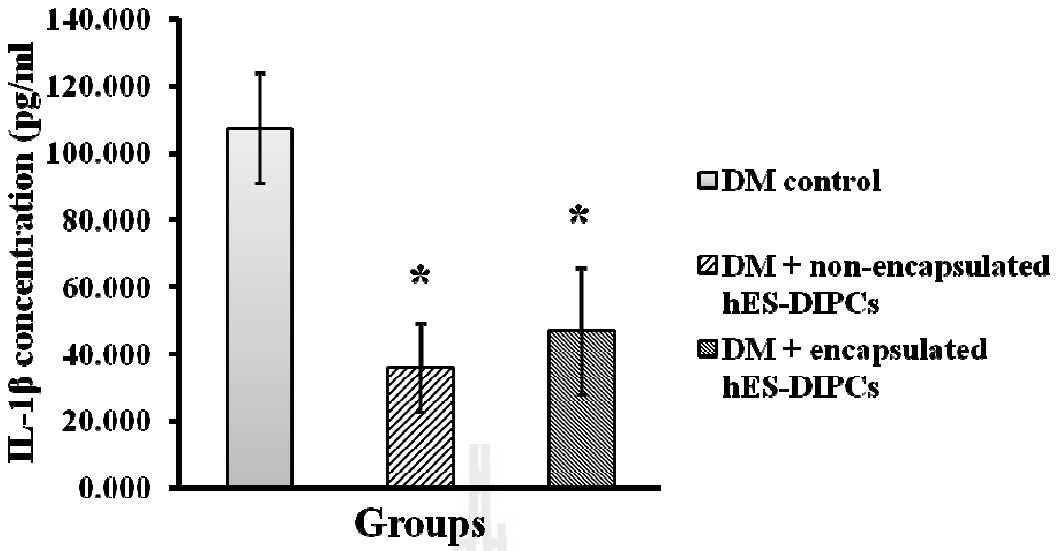

Figure 4.18 The IL-1β inflammatory cytokine concentration in diabetic mice either untreated or treated with non-encapsulated or encapsulated hES-DIPCs. The amounts of IL-1β (mean ± S.E.M.) are expressed as pg/ml. *****Values deviate significantly from corresponding diabetic control mice ($P < 0.05$).

4.2 The potential capability of the combination of hWJ-MSCs transplantation and GPE for diabetes treatment

4.2.1 Fasting blood glucose levels and body weight in diabetic mice

Fasting blood glucose levels and body weight in diabetic mice treatment by GPE alone, hWJ-MSCs transplantation alone or combination of both treatments were shown in Figures 4.19 and 4.20, respectively. The results showed that no significance difference was observed in the fasting blood glucose level of the group treated with GPE alone, hWJ-MSCs transplantation alone or combination of both treatments for 84 days of treatment. Moreover, no significant changes in the body weight were found in all treatment groups, compared with the diabetic control group. In contrast, diabetic mice control showed significant increase in fasting blood glucose on the D84.

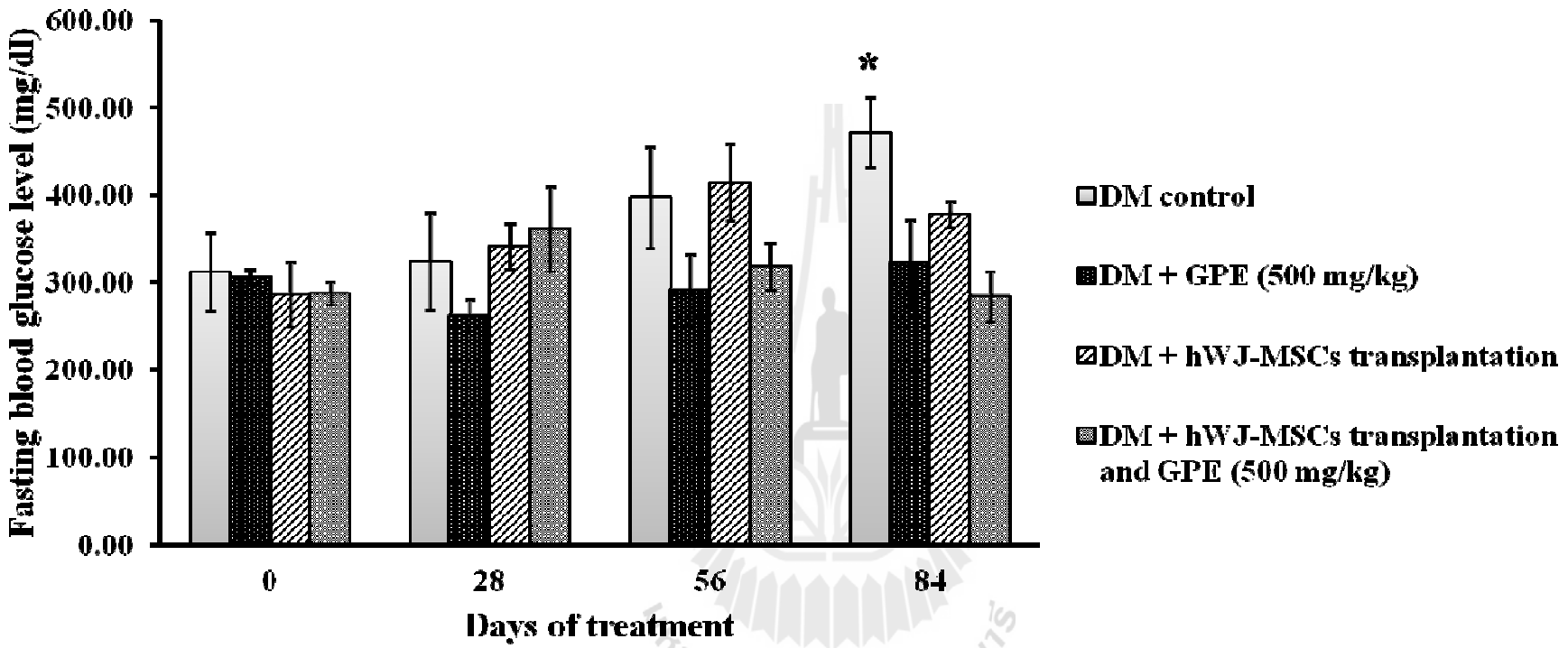

Figure 4.19 Fasting blood glucose levels in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$).

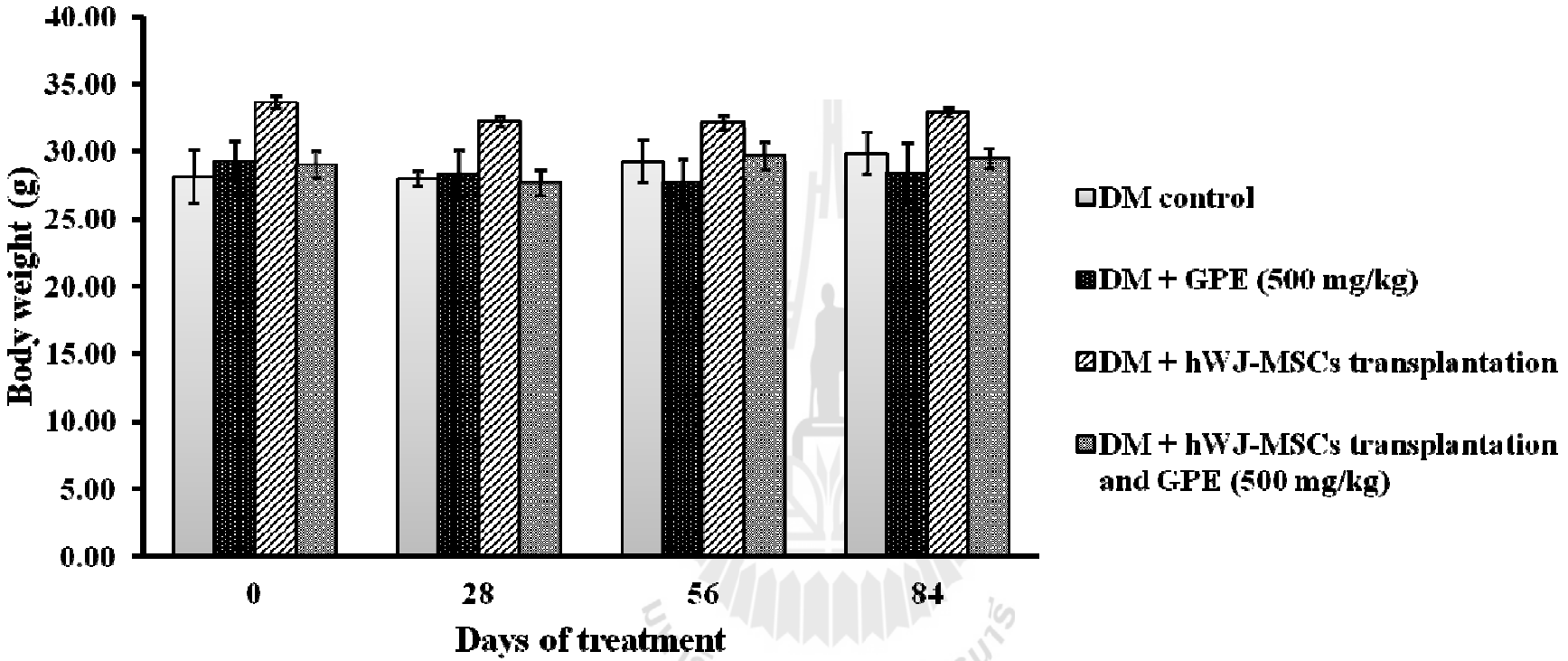

Figure 4.20 Body weight in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

4.2.2 Blood urea nitrogen (BUN) and creatinine in diabetic mice

The effects of GPE alone, hWJ-MSCs transplantation alone or combination of both treatments on BUN and creatinine in diabetic mice were shown in Figures 4.21 and 4.22, respectively. The treatment with GPE, hWJ-MSCs transplantation and combination of both treatments did not change BUN and creatinine levels in diabetic mice when compared with diabetic control mice.

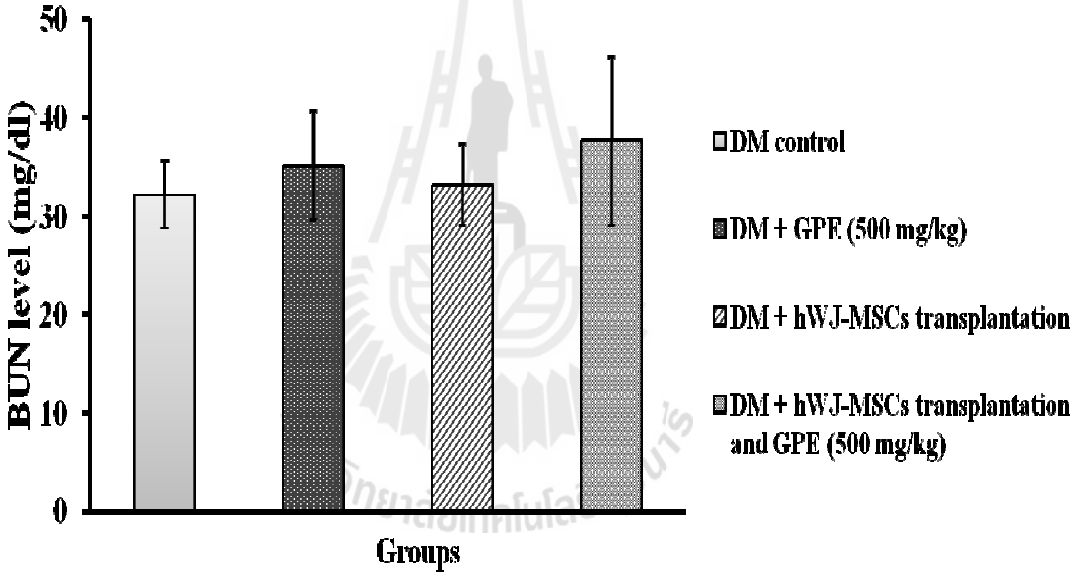

Figure 4.21 Levels of blood urea nitrogen (BUN) in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

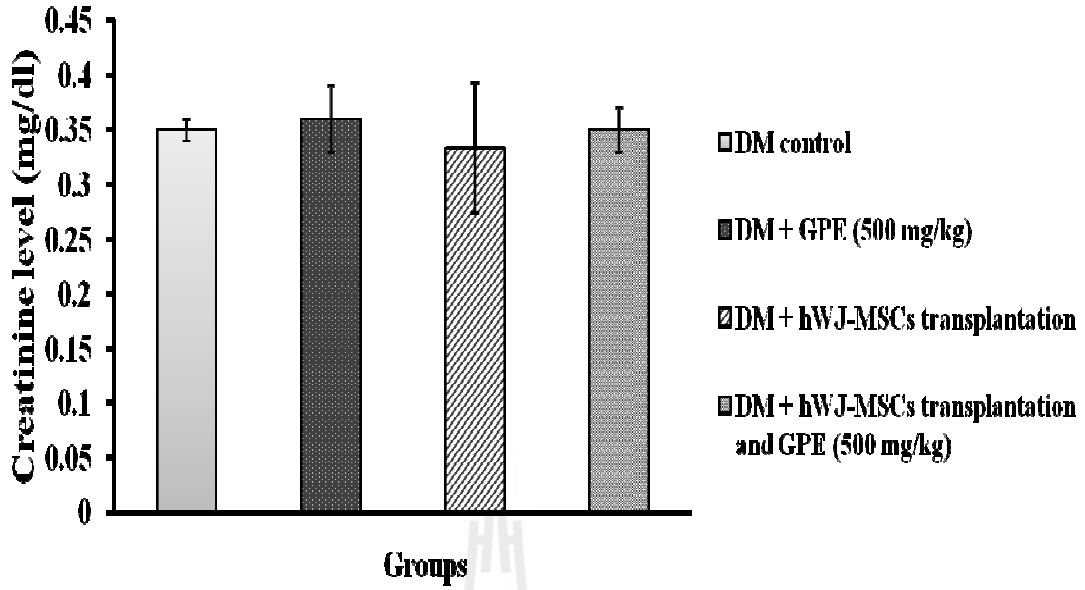

Figure 4.22 Levels of creatinine in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

4.2.3 Lipid profile in diabetic mice

The effects of GPE alone, hWJ-MSCs transplantation alone or combination of both treatments on cholesterol, triglyceride, HDL, LDL, AI and HDL/cholesterol ratio in diabetic mice were shown in Figures 4.23-4.31, respectively. There was a significant decrease in AI (Figure 4.27) and a significant in HDL (Figure 4.25) and HDL/cholesterol ratio (Figure 4.28) in the GPE-treated diabetic mice when compared to the diabetic control mice. However, GPE did not alter cholesterol, triglyceride and LDL significantly. In contrast, no significant changes in

the cholesterol, triglyceride, HDL, LDL, AI and HDL/cholesterol ratio were present between the control group and treated-diabetic mice with hWJ-MSCs transplantation or combination of both treatments.

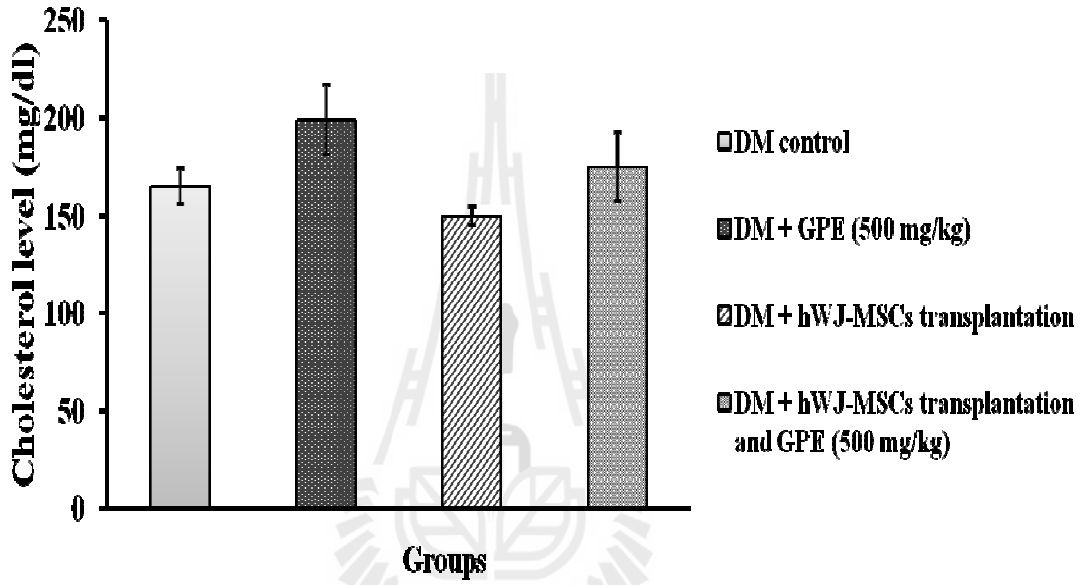

Figure 4.23 Levels of cholesterol in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

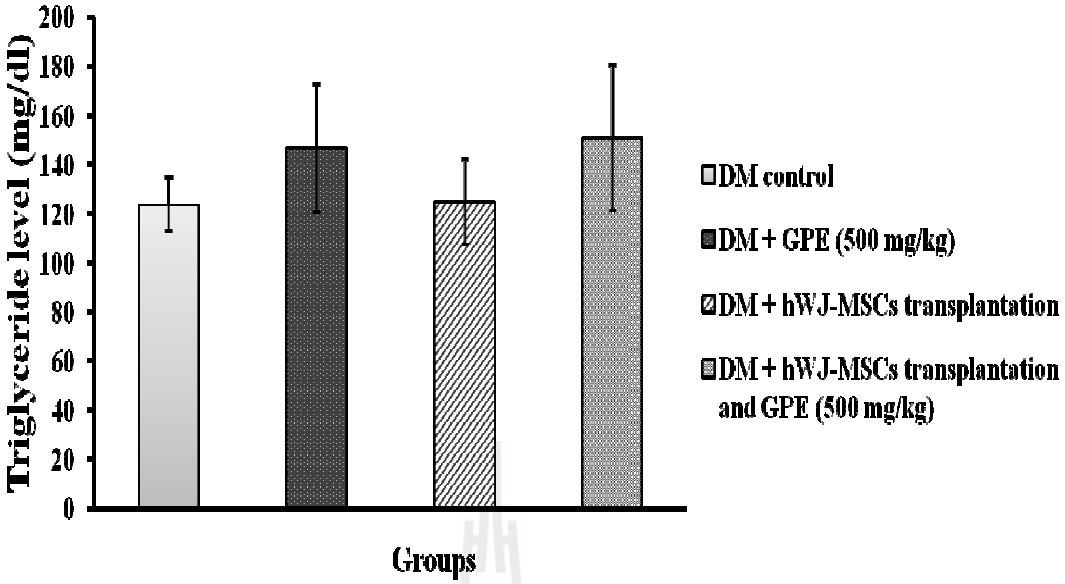

Figure 4.24 Levels of triglyceride in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

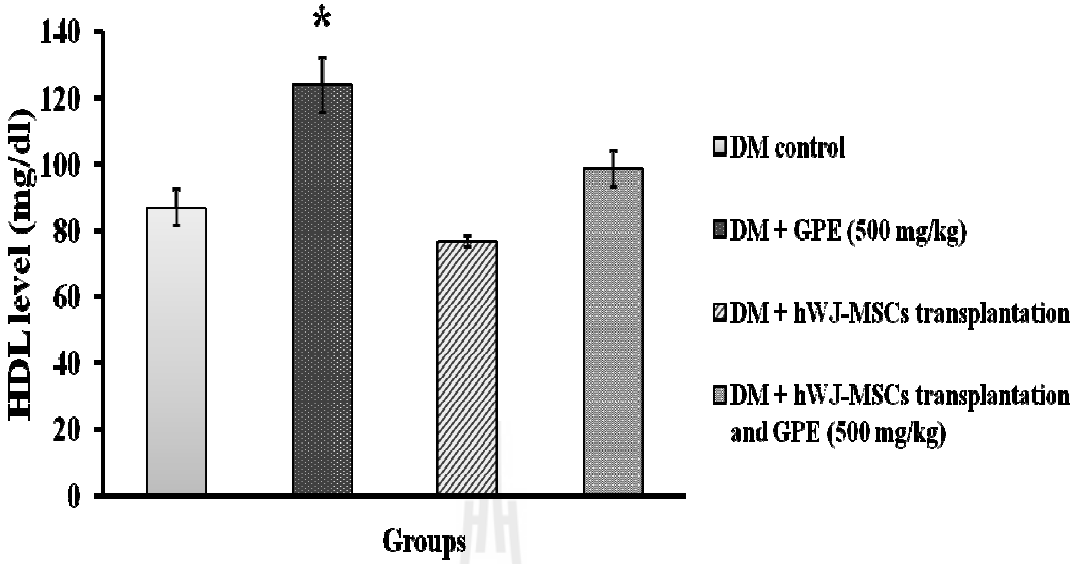

Figure 4.25 Levels of high-density lipoprotein (HDL) in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group. *****Significantly different from the diabetic control mice ($P < 0.05$).

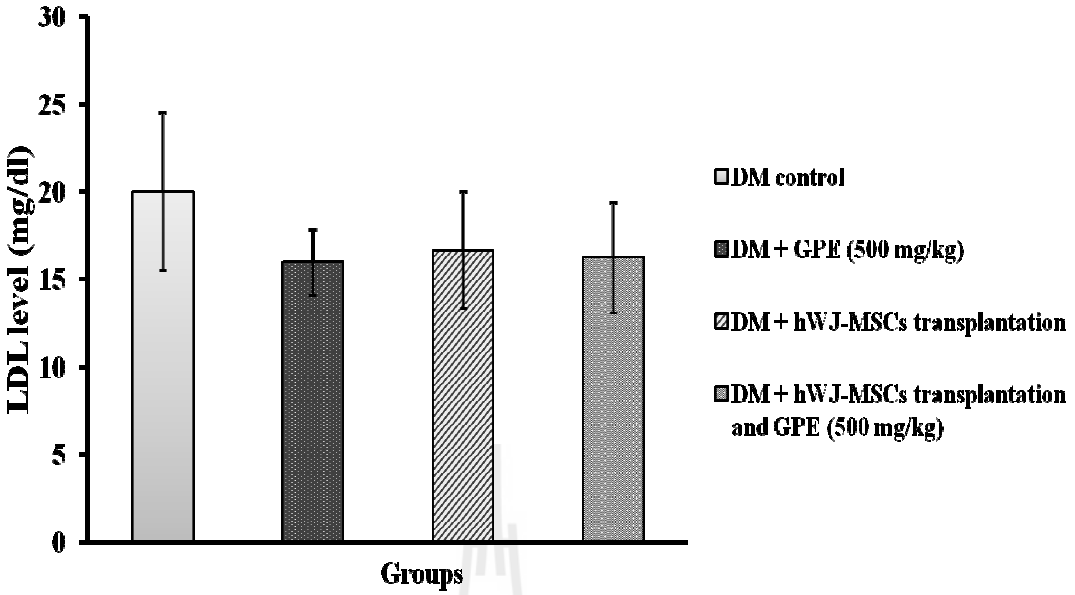

Figure 4.26 Levels of low-density lipoprotein (LDL) in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group.

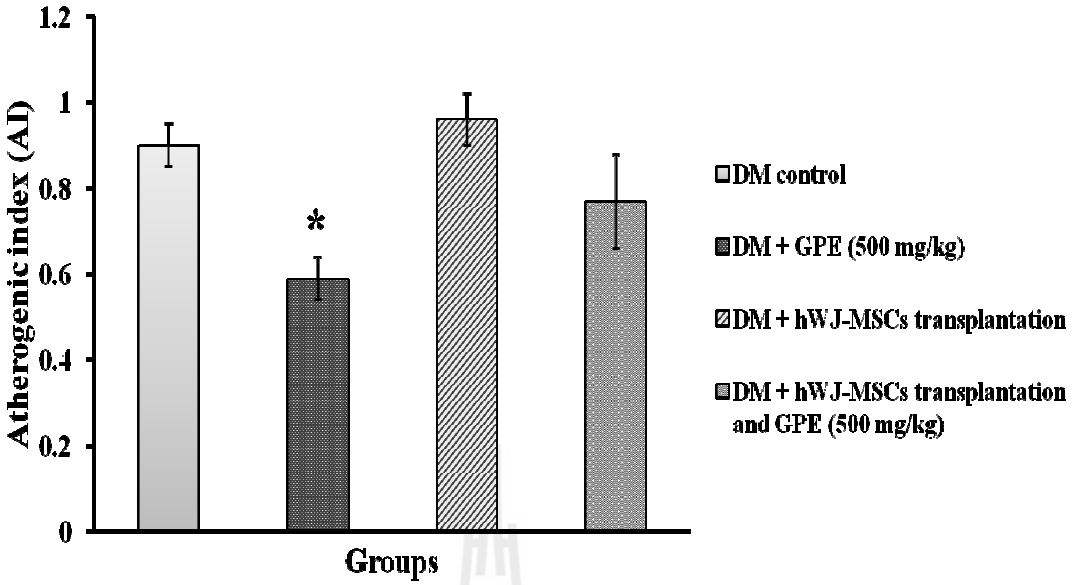

Figure 4.27 Atherogenic index (AI) in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group. *****Significantly different from the diabetic control mice ($P < 0.05$).

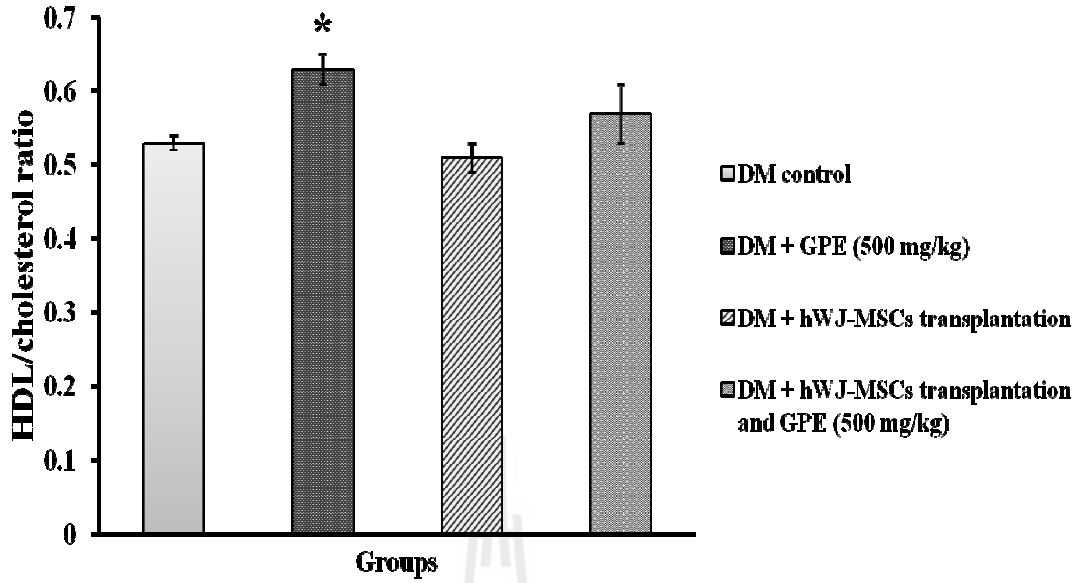

Figure 4.28 HDL/cholesterol ratio in diabetic mice either untreated or treated with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). All values are mean ± S.E.M. for each group. *****Significantly different from the diabetic control mice ($P < 0.05$).

4.2.4 Inflammatory evaluation of diabetic mice

The inflammatory activity of GPE alone, hWJ-MSCs transplantation alone or combination of both treatments in diabetic mice was evaluated by an ELISA assay. Diabetic mice treated with GPE alone and combination of both treatments had significantly lower IL-1β concentrations than the diabetic control mice. Although, the effect of hWJ-MSCs transplantation on IL-1β was also lower than diabetic control mice but there was no significant statistic difference in this aspect (Figure 4.29).

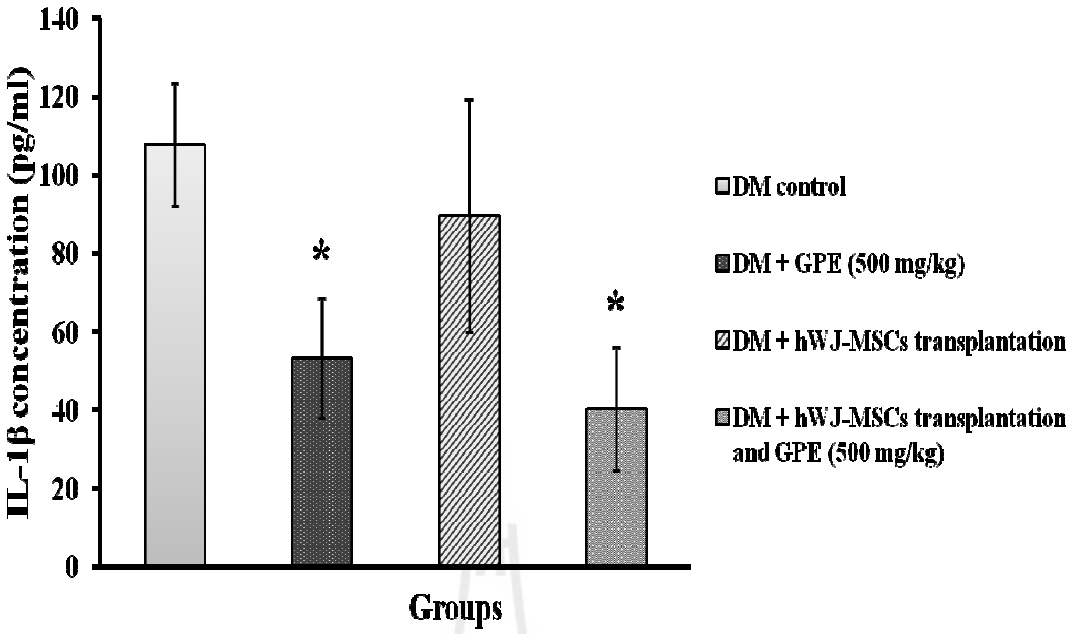

Figure 4.29 The IL-1β inflammatory cytokine concentration in diabetic mice treatment with GPE (500 mg/kg) or hWJ-MSCs transplantation or combination treatment (hWJ-MSCs transplantation and GPE 500 mg/kg). The amounts of IL-1β (mean ± S.E.M.) are expressed as pg/ml. *****Significant different from the diabetic control mice ($P < 0.05$).

4.2.5 Effects of GPE on insulin secretion by PANC-1 cells formed islet-like cell aggregates (ICAs)

The mechanism of action of GPE on PANC-1 formed ICAs was determined according to Materials and Methods Section. The data indicated that GPE at a dose of 50 µg/ml significantly increased insulin release by PANC-1 formed ICAs when stimulated with 5 mM, 20 mM and 50 mM glucose, compared with 25µg/ml of

GPE-treated cells as shown in Figure 4.31. The morphologies of PANC-1 formed ICAs were illustrated in Figure 4.30.

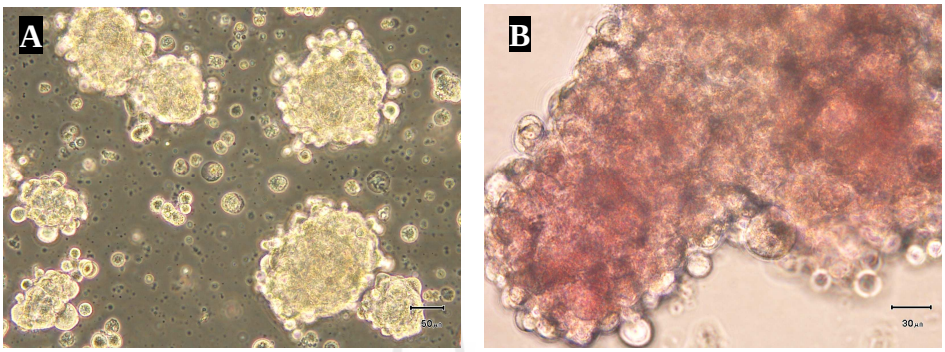

Figure 4.30 Morphologies of PANC-1 cells formed islet-like cell aggregates (ICAs). PANC-1 cells differentiated into islet-like cell aggregates (ICAs) (A). and these ICAs were DTZ positive (B). Scale bars, 50 µm (A); 30 µm (B).

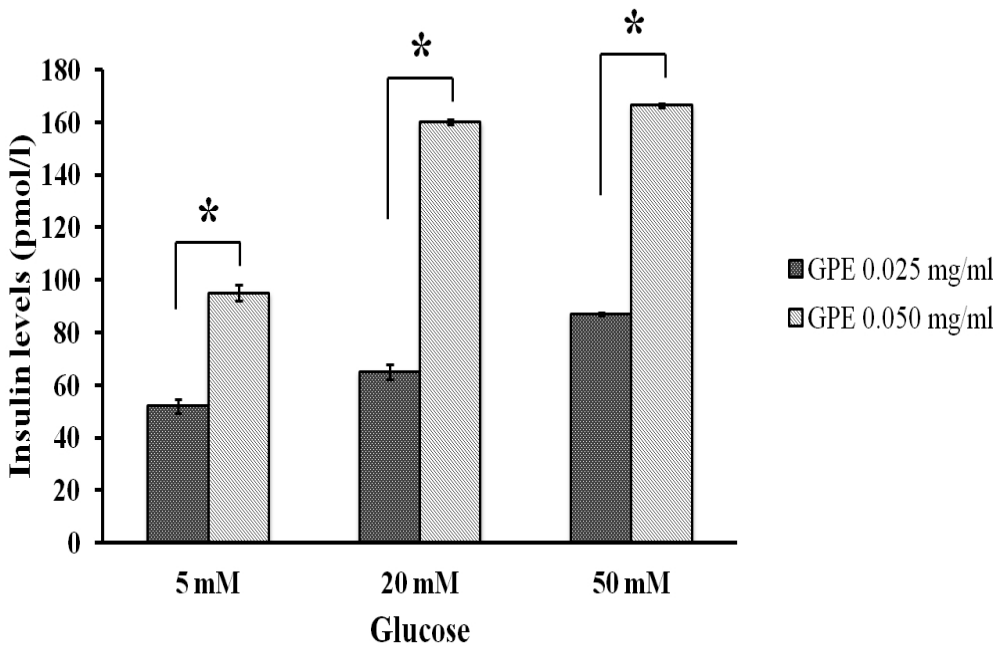

Figure 4.31 Effects of GPE (25 or 50 μg/ml) on insulin secretion by PANC-1 formed ICAs. All values are mean ± S.E.M. *****Values are statistically significant at $P < 0.05$.

4.3 The potential capability of hWJ-MSCs transplantation alone for diabetes treatment

4.3.1 Fasting blood glucose and body weight in diabetic rats

Fasting blood glucose levels and body weight in diabetic rats either untreated or treated with hWJ-MSCs at three sites of transplantations; intraperitoneal, subcutaneous and intravenous, were shown in Figures 4.32 and 4.33, respectively. After treatment for 56 days, the transplantation via intraperitoneal site had significant decrease in the level of fasting blood glucose as compared with the diabetic control rats. Although this group turned out to increase the level of fasting blood glucose at 84^{th} and 91^{st} day of the experiment but no significant statistic data was found. In addition, there was no significant difference in fasting blood glucose level after hWJ-MSCs transplantation at subcutaneous site. However, fasting blood glucose levels at the end of experiment were potent significantly reduced in intravenously transplantation group compared to the diabetic control group. The level of fasting blood glucose in diabetic rats significantly decreased after hWJ-MSCs transplantation at intravenous site from the 28^{th} day to the 91^{st} day (Figure 4.32). The reduction in blood glucose levels after treatment with hWJ-MSCs at intravenous site was 65.33%, subcutaneous site was 41.84% and intraperitoneal site was 9.78% (Table 4.1A). Intraperitoneal transplantation group showed minimum decrease in percentage blood glucose level with less than the diabetic control group (30.32%).

The body weight of hWJ-MSCs transplantations via three sites (intraperitoneal, subcutaneous and intravenous) was significantly greater than the diabetic control group. Percentage increase in body weight was 29.55% for subcutaneous transplantation, 25.81% for intravenous transplantation and 24.71% for

intraperitoneal transplantation (Table 4.1B). Moreover, there was no mortality in the animals after being transplanted with hWJ-MSCs.

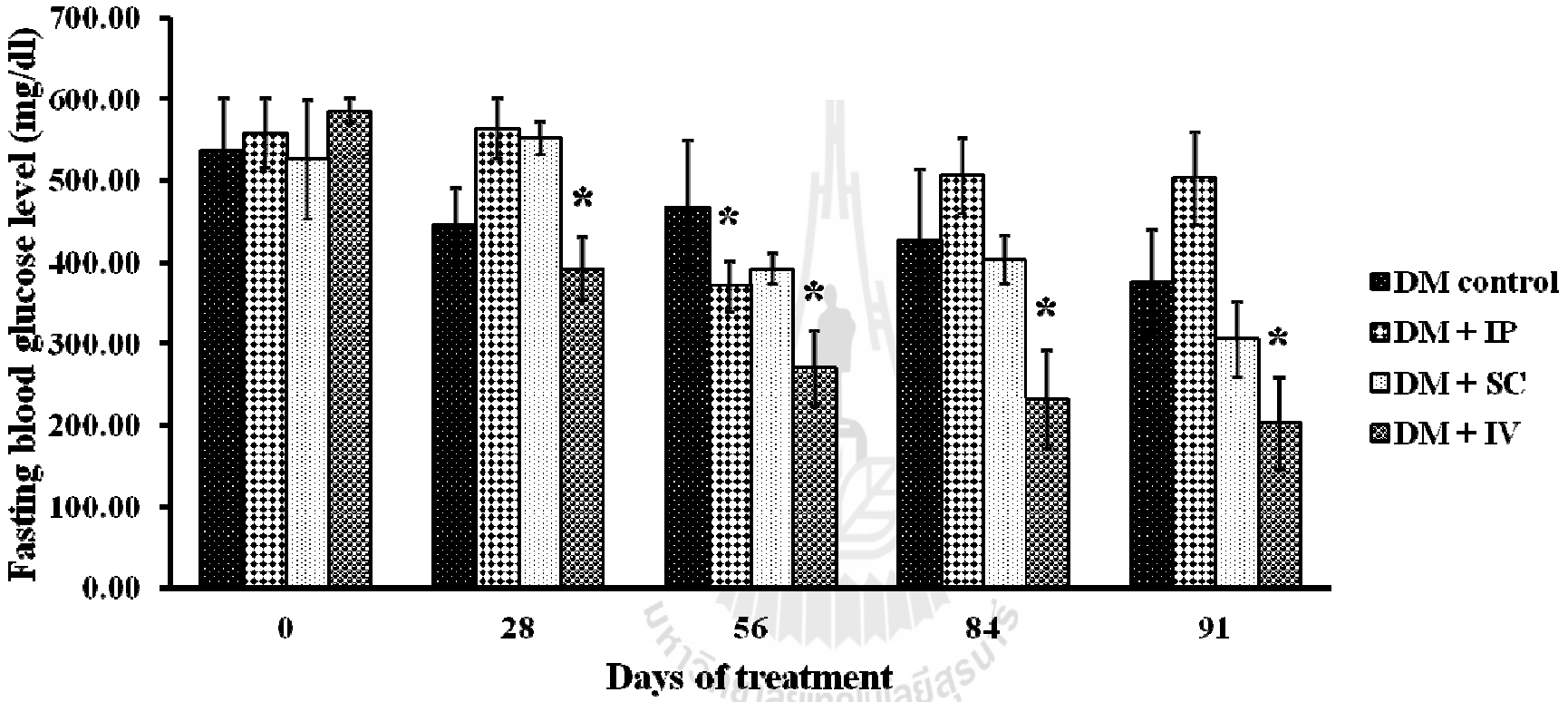

Figure 4.32 Fasting blood glucose levels in diabetic rats either untreated or treated with hWJ-MSCs transplantations alone. All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$).

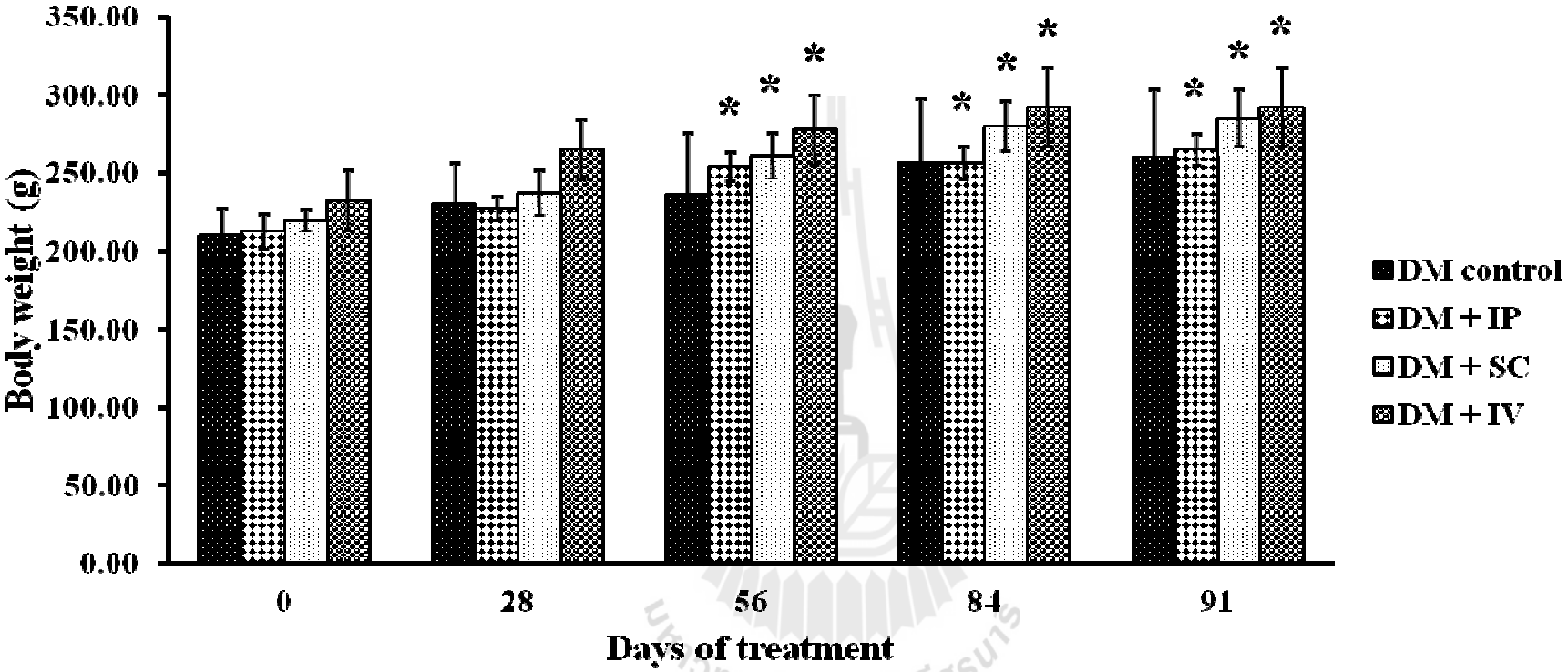

Figure 4.33 Body weight in diabetic rats either untreated or treated with hWJ-MSCs transplantations alone. All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$).

Table 4.1 Fasting blood glucose levels (A) and body weight (B) in diabetic rats before and after treatment with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group. *****Significantly different from the corresponding initial values ($P < 0.05$).

A

	Fasting blood glucose levels (mg/dl)		
Groups	**D0**	**D91**	**% different**
DM control	537.67 ± 62.33	374.67 ± 65.79	-30.32
DM + IP	557.50 ± 42.50	503.00 ± 57.08	-9.78
DM + SC	525.75 ± 73.92	305.75 ± 45.92	-41.84
DM + IV	584.75 ± 15.25	202.75 ± 57.49	-65.33*****

B

		Body weight (g)	
Groups	**D0**	**D91**	**% different**
DM control	210.00 ± 17.32	260.00 ± 43.59	23.81
DM + IP	212.50 ± 11.09	265.00 ± 10.41	24.71*****
DM + SC	220.00 ± 7.07	285.00 ± 18.48	29.55*****
DM + IV	232.50 ± 19.74	292.50 ± 24.96	25.81*****

4.3.2 Blood urea nitrogen (BUN), creatinine and uric acid in diabetic rats transplanted with hWJ-MSCs

After 91 days of hWJ-MSCs transplantation, blood samples were collected and subjected to BUN, creatinine and uric acid analysis. Untransplanted control rats were included. The levels of these results were shown in Figures 4.34-4.36, respectively. There was no significant difference in serum BUN, creatinine and uric acid after hWJ-MCs transplantations (intraperitoneal, subcutaneous and intravenous) when compared with diabetic control rats.

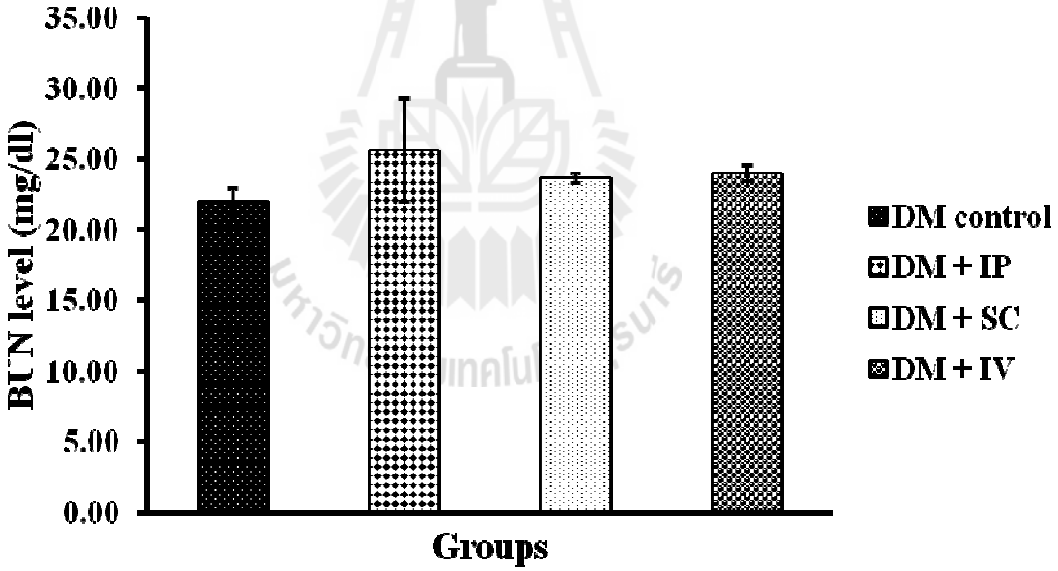

Figure 4.34 Levels of blood urea nitrogen (BUN) in diabetic rats either untreated or treated with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group.

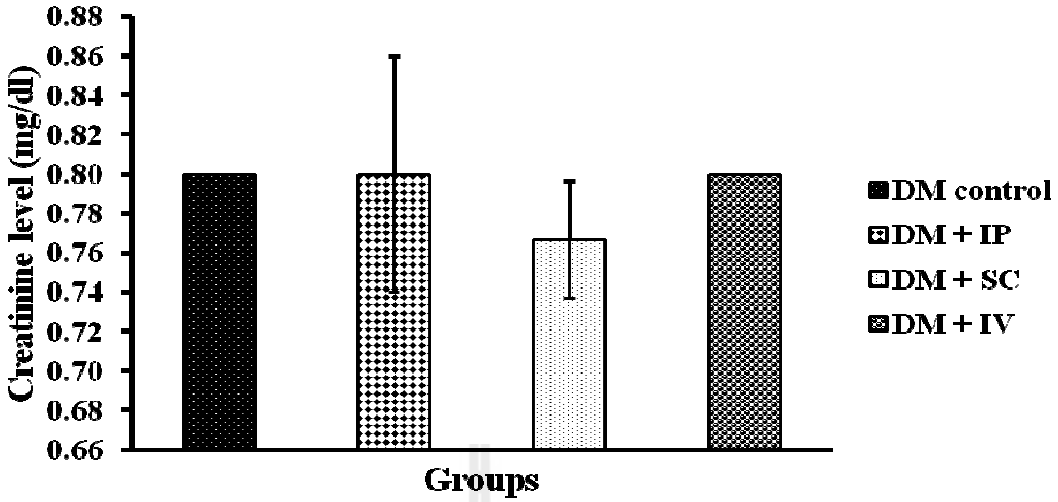

Figure 4.35 Levels of creatinine in diabetic rats either untreated or treated with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group.

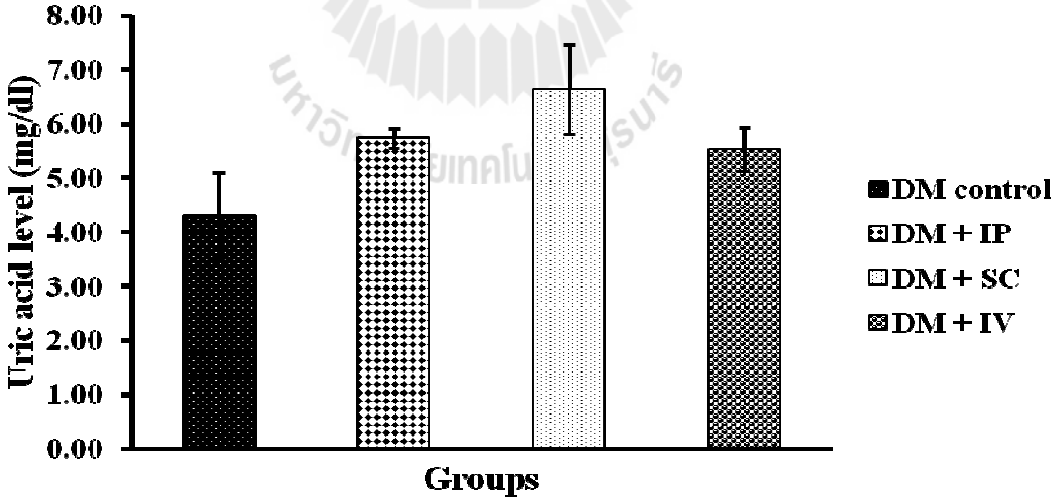

Figure 4.36 Levels of uric acid in diabetic rats either untreated or treated with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group.

4.3.3 Analysis of cholesterol and triglyceride levels in hWJ-MSCs transplanted diabetic rats

The levels of cholesterol and triglyceride of control and experimental groups of rats were shown in Figures 4.37 and 4.38, respectively. Treatment with hWJ-MSCs transplantation (intraperitoneal, subcutaneous and intravenous) had no effect on serum cholesterol and triglyceride levels when compared with diabetic control group.

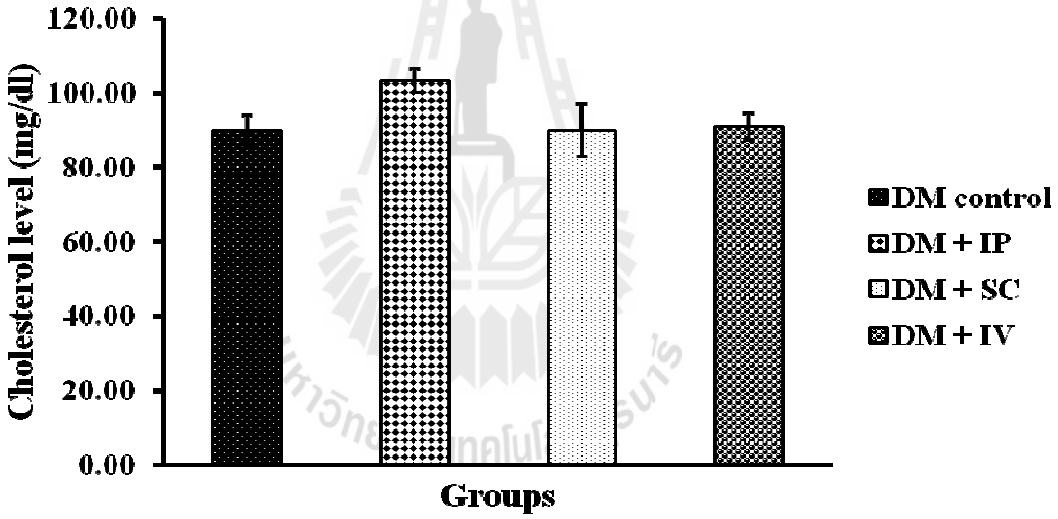

Figure 4.37 Levels of cholesterol in diabetic rats either untreated or treated with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group.

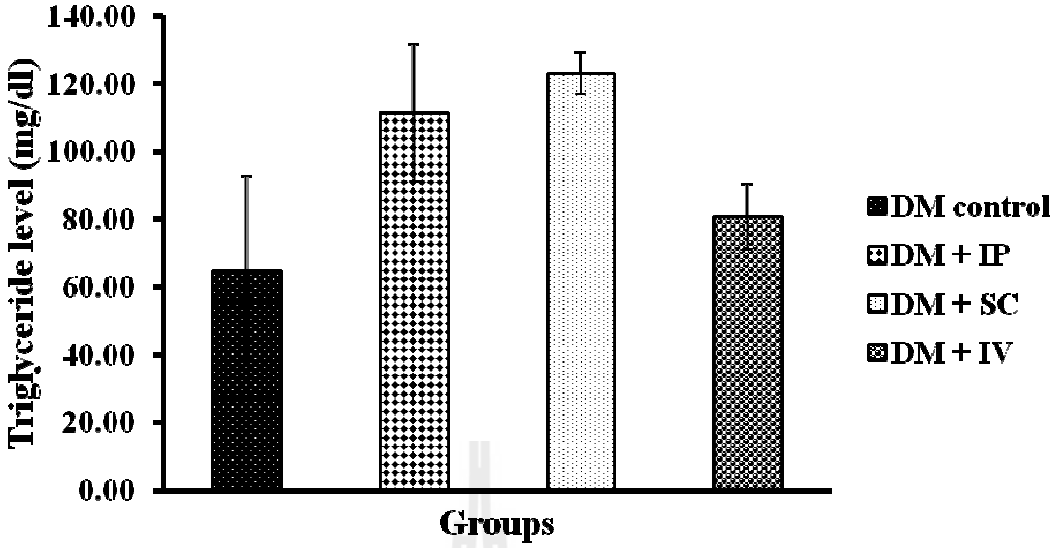

Figure 4.38 Levels of triglyceride in diabetic rats either untreated or treated with hWJ-MSCs alone at three sites of transplantations. All values are mean ± S.E.M. for each group.

CHAPTER V

DISCUSSION

5.1 The potential capability of hES-DIPCs for diabetes treatment

In order to derive insulin-producing cells from hESCs *in vitro*, hESCs were induced through a stage of differentiation protocol described previously with some modification (Jiang et al., 2007). The insulin-producing cells were induced to differentiate from hESCs in a manner that mimics differentiation *in vivo*. The differentiation stages range from starting with definitive endoderm (DE) induction, pancreatic endoderm formation, pancreatic endocrine induction and subsequently to islet-like clusters (ILC) maturation.

The DE is the embryonic germ layer which can give rise to the pancreas (Docherty, Bernardo and Vallier, 2007). In the present study, EBs were formed within hanging drops. These cells expressed *Hnf3β* which is a critical factor in the endodermal cell lineage development. In fact, *Hnf3β* is a transcriptional regulator of *Pdx1*, which is important in the regulation of insulin gene expression and also required for the differentiation of the mature pancreas (Soria, 2001). *Pdx1* is subsequently required during mid-pancreatic development for cellular differentiation. Depletion of *Pdx1* gene expression demonstrated that the next development phase, the onset of acinar and islet development are blocked (Hale et al., 2005). Another study reported that recombinant adenovirus containing a constitutively active mutant of *Pdx1* efficiently induced insulin production in hepatocytes, resulting in reversal of

STZ-induced hyperglycemia. However, these hepatocytes were preserved hepatocytic functions even when albumin and transferrin expressions were observed in insulin-producing cells. Thus, it indicated that endogenous *Pdx1* expression is likely to maintain the insulin-producing cell function (Imai et al., 2005).

During early stage of insulin-producing cells differentiation from hESCs, a marker of pancreatic endocrine progenitor and neural stem cells, *Nestin*, was observed in these cells (Figure 4.4). The expression of *Nestin* in these cells has been noted to play a role in the development of pancreatic endocrine cells. The nestin-expressing cells might represent the precursors for neogenesis of pancreatic endocrine cells (Hunziker and Stein, 2000). These data also supported by the works in mESCs and hESCs which could generate the insulin-producing cells from nestin-positive cells (Lumelsky et al., 2001; Mao, Chen, Bai, Song and Wang, 2009).

In addition, in our works, the differentiated cells were found to exhibit *Nkx6.1*, a homeobox gene presented in differentiated β-cells. Interestingly, *Nkx6.1* is also restricted to β-cells and some neurons (Docherty, Bernardo and Vallier, 2007). This finding suggests the presence of a common progenitor of neurons and insulin-positive cells during *in vitro* differentiation (Soria, 2001).

In the present study, the expression of *Nkx6.1* was detected starting at definitive endoderm formation stage and then increased until the later stages of differentiation. The results also revealed the *Pdx1* gene expression during the DE induction. Its expression level peaked in the pancreatic endocrine stage and slightly decreased at ILC maturation stage.

Moreover, the results demonstrated that the islet cell precursor, *Ngn3*, was highly expressed at 21 days of the differentiation process (pancreatic endoderm

induction). The expression of *Ngn3* is required for the specification of a common precursor for the four pancreatic endocrine cell types (Gradwohl, Dierich, LeMeur and Guillemot, 2000). Similarly, it has been demonstrated that *Ngn3* and *NeuroD1/BETA2* can drive the early differentiation of islet cells (Schwitzgebel et al., 2000). *Ngn3* has been shown to induce the expression of BETA2 (NeuroD), a transcription factor implicated in the insulin gene expression and in islet cells differentiation (Soria, 2001). It has been hypothesized that *Ngn3* is involved in activating the expression of *BETA2* at an early stage of islet cell differentiation through the E boxes in the *BETA2* promoter (Huang et al., 2000).

Our results also shown that *Ngn3* is downregulated during the transition of the endocrine cell precursor into complete islets, expression of *Nkx6.1* is upregulated. It has been demonstrated that ectopic *Nkx6.1* expression is induced in *Foxa2* (*Hnf3β*) positive endodermal cells that express exogenous *Pdx1* (Pedersen et al., 2005). Moreover, recent report has shown that synergistic expression of *NeuroD1* and *Pdx1* might be crucial for maintenance of the property of insulin-producing cells derived from ESCs (Saitoh, Yamato, Miyazaki and Miyazaki, 2007). This data indicates that the expression of *Nkx6.1*, *NeuroD1* and *Pdx1* play a role in the formation of pancreatic β-cells.

In order to promote the mature pancreatic β-cells, the expression of a key transcription factors should be achieved *in vitro.* Additionally, the candidate factors are required to drive the maturation of progenitors. As demonstrated in our study, after pancreatic endocrine cells were exposed to the combination of nicotinamide and IGF II, the induction of an important transcription factors could be acheived for ILCs development. The cells at the end of differentiation stage expressed pancreas-related

genes including *GLUT2*, *insulin, somatostatin* and *glucagon*. *Nestin* and *Hnf3β* were also highly expressed in these cells. According to the immunofluorescence staining, hESC-derived ILCs expressed islet-specific hormone insulin which represented by the presence of C-peptide; a genuine marker of *de novo* insulin production. The somatostatin- and glucagon-expressing cells were also detected in these cell clusters. Surprisingly, the expression of Nestin was also found in these populations. Thus, although the modified protocol succeeded to derive the ILCs but the small portions of insulin progenitors are still remained. Consequently, the current protocol should be improved to yield lower the progenitor cells.

To define the functional of hES-DIPCs, further experiments were carried out including the characterization of pancreatic β-cells, the ability to release insulin in response to glucose and the capacity of these cells for future clinical application. Our finding demonstrated that hES-DIPCs are DTZ-positive cells. The zinc-chelating agent DTZ is known to selectively stain pancreatic β-cells, which contain relatively high levels of zinc (Shiroi et al., 2005; Baharvand, Jafary, Massumi and Ashtiani, 2006). In this work, DTZ-positive cellular clusters were observed in the clusters at days 46 of differentiation. This finding supports the achievement of insulin-producing cell differentiation within the cellular clusters. In addition, the insulin secretion from the ILCs exhibited a glucose-dependent manner as shown by higher level of insulin release in 50 mM glucose stimulation than in 5 mM glucose activation (Figure 4.8). In addition, we succeeded to generate alginate encapsulated hES-DIPCs containing capacity of insulin release (Figure 4.9C). Therefore, both encapsulated and non-encapsulated hES-DIPCs could function as insulin-producing cells *in vitro*.

The encapsulation can be applied to protect implanted-cells against antibodies and cytotoxic cells of the host immune system. It has been shown that alginated-based microcapsules allow transplantation of cells in the absence of immunosuppression (de Vos, Faas, Strand and Calafiore, 2006). Moreover, the alginate technology has also been applied to the differentiation of mESCs into insulin-producing cells (Wang, Adams, Buttery, Falcone and Stolnik, 2009).

In order to determine whether the hES-DIPCs generated in this work could functions *in vivo*, both non-encapsulated and encapsulated hES-DIPCs were separately transplanted into STZ-induced diabetic mice. All cell-transplanted mice maintained their blood glucose level for 70 days after transplantation. In contrast, the control group which did not receive hES-DIPCs, the fasting blood glucose was significantly increased throughout the experiment. These results suggest that our hES-DIPCs are capable of reducing the elevated blood glucose which results in maintain glucose level and prevent the diabetic progression in diabetic mice. This rescue rate might be due to the quality of hES-DIPCs. Islet purity and insulin content have been previously reported to guarantee *in vivo* function in an animal model of allotransplantation (Migliavacca et al., 2004). In our study, the pancreatic exocrine cells are still present in the mixing clusters with other islet cells. Therefore, the improvement of high yield and high purity of the hES-DIPCs is needed to increase more successful transplantation.

Among the treated mice, the excellent health and pelage status were observed in the hES-DIPCs transplanted mice (Figure 4.10C). In the present study, as shown in Figure 4.18, the mice transplanted with either non encapsulated or encapsulated hES-DIPCs showed a significant decrease in serum IL-1β concentration when

compared to the control. These results suggest that the transplanted cells could suppress IL-1β production and also delay the diabetic progression in diabetic mice. The mechanism by which these cells decrease the production of IL-1β is not known. It has been noted that IL-1β was produced by activated macrophages and monocytes (Li et al., 2008). In T1D, islet infiltrating macrophages produce IL-1 which is cytotoxic specifically to β-cells *in vitro* (Sjoholm, 1998). It has also been suggested that the development and progression of diabetic complications are associated with inflammation which mediated by the inflammatory mediators, such as pro-inflammatory cytokines (Navarro and Mora, 2005). In addition, Garcia et al. (2010) indicated that the inflammation markers are increased in diabetic patients which serve as factor for cardiovascular risk prediction as they are associated with endothelial dysfunction. Nevertheless, insulin has been considered a regulator of inflammatory and immune responses in which it can attenuate inflammation and regulate immune reactions. Intensive insulin therapy is also maintained the inflammatory reaction balance, which significantly decreases pro-inflammatory cytokine levels and increases anti-inflammatory cytokine levels (Deng and Chai, 2009).

Besides the results on blood glucose and IL-1β levels from both hES-DIPCs and encapsulated hES-DIPCs transplantation, the other biochemical analysis are also determined. The present data indicated that all the treated mice did not exhibit any significant alterations in BUN and creatinine levels, when compared with diabetic control mice. Elevation of the serum urea and creatinine, key kidney function markers, is related to kidney dysfunction in diabetic hyperglycemia (Gatua et al.,

2011). It is also suggested that these transplanted cells did not effect on the kidney function.

Serum lipid profile including LDL, AI and HDL/cholesterol ratio are used in the evaluation of lipid abnormalities. The transplantation of non-encapsulated hES-DIPCs in STZ-diabetic mice caused a significant decrease in the AI and a significant increase in HDL/cholesterol ratio after 70 days of treatment. In fact, diabetes is associated with a high incidence of vascular disease (Wagner, Martijnez-Rubio, Ordonez-Llanos and Perez-Perez, 2002). In addition, the increase of HDL level has been previously shown to reduce risk for cardiovascular disease (Assmann and Gotto, 2004). Therefore, hES-DIPCs might reduce the risk of cardiovascular disease. However, the mechanisms by which hES-DIPCs evoke these effects need to be further clarified.

5.2 The potential capability of the combination of hWJ-MSCs transplantation and GPE for diabetes treatment

The present study examined the effects of orally administered GPE (500 mg/kg), hWJ-MSCs transplantation and the combination of both treatments on STZ-induced diabetic mice. These treatments were compared to the diabetic control mice. The results showed that the fasting blood glucose levels did not significantly changed in all treated diabetic mice after 84 days of treatments. However, there was significant increase in fasting blood glucose levels in the control diabetic mice. In addition, the treatment with either hWJ-MSCs transplantation or the combination of both treatments did not change BUN, creatinine, cholesterol, triglyceride and LDL in diabetic mice.

The daily administration of GPE (500 mg/kg) for 84 days significantly increased HDL and HDL/cholesterol ratio and significantly decreased in atherogenic index (AI). The results implied that GPE may have the potential to prevent the formation of cardiovascular disease. The components and the mechanisms by which GPE exerts these effects are not completely understood. However, the presence of flavonoids within the herb has been previously shown to increase HDL and reduce the AI when treated in hypercholesterolemic rabbits (Subramaniam et al., 2010).

According to the inflammatory process, activation of macrophages and monocytes resulted in the production of IL-1β (Li et al., 2008). In this study, the concentration of IL-1β was determined in the serum samples taken from the experimental animals. This study demonstrated that STZ-induced diabetic mice treated with GPE or combination treatment significantly reduced the serum IL-1β concentration. It is speculated that the anti-inflammatory action exerted by GPE could be attributed to its substances. In agreement with this data, the crude ethanolic extract of *G. procumbens* has been shown to inhibit mouse ear inflammation. In addition, the extracts and fractions have been shown to contain chemical constituents, such as essential oil, steroid/triterpenes, bitter principles, valepotriates and coumarins (Iskander, Song, Coupar and Jiratchariyakul, 2002). In contrast, the transplantation of hWJ-MSCs was found to have no significant difference in the serum IL-1β concentration. We hypothesized that the intravenous site of transplantation might be a problem in mice. The factor for cell loss during transplantation is a small vessel of the mouse tail vein. The cells that are transplanted into tail vein may accidentally introduce outside the vein or leak out of the vein after transplantation. In addition, the mechanical trauma from the transplantation process can lead to cell damage and death

(Brundin et al., 2000). Thus, it is important to be aware of the advantages and disadvantages of experimental model as this may influence the experimental outcome.

The treatment of PANC-1 formed ICAs with various concentrations of GPE (25 and 50 µg/ml), also elicited a significant increase in the insulin secretion levels *in vitro*. GPE promoted insulin release from ICAs in glucose-dependent manner, when exposed the cells with 5 mM, 20 mM or 50 mM glucose. This result may accounting for the role of GPE in maintaining blood glucose level through activation of insulin release *in vivo*. However, the additional mechanisms of GPE are required to elucidate.

5.3 The potential capability of hWJ-MSCs transplantation alone for diabetes treatment

We used hWJ-MSCs in this study due to several reasons; 1) the hMSCs has several advantages in therapeutic applications (Meirelles Lda, Fontes, Covas and Caplan, 2009; Xu et al., 2008); 2) hWJ-MSCs is an easily accessible source of human MSCs isolation ethical controversy (Nekanti et al., 2010; Secco et al., 2008); 3) these cells are found to improve a variety of disease conditions in animal models (Barry and Murphy, 2004; Brooke et al., 2007; Deans and Moseley, 2000; Fiorina et al., 2009); and 4) The hWJ-MSCs has been serve as an alternative source for MSCs isolation. It retains the differentiation potential and immunosuppressive capacity.

Although, there is a report of successful hWJ-MSCs transplantation (Prasanna and Jahnavi, 2011), the optimal transplantation site has not to be determined. The impact of the transplantation site for cell engraftment and function has been reported (Carlsson, 2011; Lau et al., 2007; Troppmann, Papalois, Gruessner, Nakhleh and

Gruessner, 1997). The present study examined the effect of three different sites (intraperitoneal, subcutaneous and intravenous) on the potential of hWJ-MSCs transplantation. Our data demonstrated the influence of hWJ-MSCs transplantation on fasting blood glucose levels as well as on body weight in STZ-induced diabetic rats. Intravenous transplantation of hWJ-MSCs had shown a significant hypoglycemic effect in STZ-induced diabetic rats after 91 days treatment. The increase in body weight in STZ-induced diabetic rats was observed after transplantation with hWJ-MSCs. Although the subcutaneously transplanted site had greater increase in body weight than those other groups, the improvement in body weight was also shown in intraperitoneal and intravenous sites of transplantation. Thus, transplantation of hWJ-MSCs into diabetic rats may improve their health condition from diabetes. Moreover, there was no mortality in the animals transplanted with hWJ-MSCs. These results suggest that the efficacy of hWJ-MSCs to correct hyperglycemia condition may be due to the generation of new β-cells by mechanisms through secreting trophic cytokines that promote endogenous pancreatic stem cells in the ductal epithelium differentiate into new β-cells. Furthermore, they also produce a variety of cytokines and growth factors that have an effect on the survival of surrounding cells, and improve the microenvironment of the pancreas (Si, Zhao, Hao, Fu and Han, 2011). The reversal of hyperglycemia has also been reported in NOD mice by allogeneic MSCs transplantation that may be due to its immunosuppressive effect (Fiorina et al., 2009). The hMSCs have been shown to have beneficial effects in animal models including (i) the selective homing of hMSCs to both pancreatic islets and renal glomeruli of diabetic mice and (ii) the ability of the cells to repair the tissues (Lee et al., 2006). As previously described by Xu et al. (2009), the administration of

conditioned rat pancreatic extract (RPE)-treated MSCs media significantly improved the blood glucose levels of STZ-induced diabetic rats. It must also be noted that the therapeutic benefits of MSCs in tissue protection and repair may be attributable primarily to paracrine mechanisms and not to transdifferentiation into insulin-producing cells. Nevertheless, the precise mechanism of hMSCs in preventing T1D remains controversial (Vija et al., 2009).

In this current study, the findings exhibited significant decrease in blood glucose level and increase in body weight of rats after 91 days post-transplantation via intravenous injection. However, the results demonstrated that there is no significant difference in serum BUN, creatinine, cholesterol and triglyceride levels in those untreated and treated diabetes rats. Interestingly, the diabetic rats in the intravenous transplantation group did not demonstrate any evidence of adverse effects from hWJ-MSCs. The intravascular site has been previously shown to allow better functional graft survival in STZ-induced diabetic rats (Troppman, Papalois, Gruessner, Nakhleh and Gruessner, 1997). In addition, the intravenous site has been shown to provide oxygen and nutrient supply to the grafted tissue (Hirshberg et al., 2002). Interestingly, intravenously transplanted MSCs have been reported to migrate into multiple tissues with circulation of blood flow (Li et al., 2011). In this study, the intraperitoneal site appears to have less potential than another site for transplantation. Dufrane et al. (2006) also reported the intraperitoneal site seems not appropriate for cell transplantation. Our study demonstrated, as does the previously reported, that the transplantation site may influence the cell function for diabetes treatment.

CHAPTER VI

CONCLUSION

This study aimed at investigating new approaches for the treatment of diabetes. There were three major objectives set out for this investigation; 1) to produce insulin-producing cells from undifferentiated hESCs (hES-DIPCs) and evaluate the capability of hES-DIPCs for diabetic treatment *in vivo*; 2) to examine the antidiabetic effect of the combination of *Gynura procumbens* extract and Wharton's jelly derived mesenchymal stem cells (hWJ-MSCs) in diabetic animal model; and 3) to investigate whether hWJ-MSCs can be used as a possible source for diabetes treatment.

6.1 The potential capability of hES-DIPCs for diabetes treatment

The results of this study revealed the achievement of hESCs differentiation into functional insulin-producing cells (hES-DIPCs) *in vitro* by our new four-stage differentiation protocol. This new procedure provides suitable environment for hESCs transitioned through definitive endoderm (DE), pancreatic endoderm and pancreatic endocrine stage, until mature islet-like clusters (ILC). An analysis of the gene expression patterns revealed that these differentiated cells were very similar in their development to pancreatic islets. In addition, the hES-DIPCs exhibited the characteristic of pancreatic β-cells including C-peptide and DTZ-positive cellular clusters, the ability to release insulin in response to glucose. Furthermore, the transplantation of hES-DIPCs could control blood glucose levels and prevent

atherosclerosis in diabetic animals. Altogether, we succeeded to generate hES-DIPCs which exhibit the characteristics insulin-producing cells both in cellular and molecular levels. In addition, this hES-DIPCs has therapeutic value in blood glucose regulation and prevention of diabetic progression *in vivo*.

6.2 The potential capability of the combination of hWJ-MSCs transplantation and GPE treatment

The study has demonstrated that oral administration of GPE evokes a beneficial effect on the atherogenesis- and inflammation-related diabetes. In addition, the combination of hWJ-MSCs transplantation and GPE treatment could reduce inflammatory cytokine in diabetic animals. The findings of the study support traditional medicinal use of *G. procumbens* for diabetic treatment. Moreover, this work also suggests therapeutic value of the combination treatment of hWJ-MSCs transplantation and GPE treatment in blood glucose maintaining and prevention medicine for diabetic progression *in vivo*.

6.3 The potential capability of hWJ-MSCs alone for diabetes treatment

This study has explored the effect of various sites of hWJ-MSCs injections for diabetic treatment *in vivo*. To this end, the hWJ-MSCs were transplanted into the animals by the intraperitoneal, subcutaneous and intravenous routes. The animals that received an intravenous transplantation exhibited significant decrease in the blood glucose levels. In addition, hWJ-MSCs treated diabetic animals showed a marked increase in body weight following the intraperitoneal, subcutaneous and intravenous

transplantation. This study indicates that hWJ-MSCs may serve as a promising cell source for the treatment of diabetes. Furthermore, the intravenous is also considered the best site for hWJ-MSCs transplantation. There is also a need to further investigate the molecular and signaling mechanisms underlying the therapeutic effects of hWJ-MSCs.

REFERENCES

REFERENCES

Aekplakorn, W., Stolk, R. P., Neal, B., Suriyawongpaisal, P., Chongsuvivatwong, V., Cheepudomwit, S. and Woodward, M. (2003). The prevalence and management of diabetes in Thai adults: the international collaborative study of cardiovascular disease in Asia. **Diabetes Care.** 26(10): 2758-2763.

Afzali, B., Al-Khoury, S., Shah, N., Mikhail, A., Covic, A. and Goldsmith, D. (2006). Anemia after renal transplantation. **American Journal of Kidney Diseases.** 48(4): 519-536.

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K.L. and Tzukerman, M. (2001). Insulin production by human embryonic stem cells. **Diabetes.** 50(8): 1691-1697.

Assmann, G. and Gotto, A. M., Jr. (2004). HDL cholesterol and protective factors in atherosclerosis. **Circulation.** 109(23 Suppl 1): III8-14.

Auttachoat, W., Chitsomboon, B., Peachee, V. L., Guo, T. L. and White, K. L., Jr. (2004). Immunomodulation by Dok Din Daeng (*Aeginetia indica* Roxb.) extracts in female B6C3F1 mice: II. Humoral immunity, innate immunity and hematology. **International Immunopharmacology.** 4(10-11): 1381-1390.

Baharvand, H., Hashemi, S. M., Ashtiani, S. K. and Farrokhi, A. (2006a). Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems *in vitro*. **The International Journal of Developmental Biology.** 50(7): 645-652.

Baharvand, H., Jafary, H., Massumi, M. and Ashtiani, S. K. (2006b). Generation of insulin-secreting cells from human embryonic stem cells. **Development Growth & Differentiation.** 48(5): 323-332.

Barry, F. P. and Murphy, J. M. (2004). Mesenchymal stem cells: clinical applications and biological characterization. **The International Journal of Biochemistry & Cell Biology.** 36(4): 568-584.

Beattie, G. M. and Hayek, A. (Winter 2004). Human embryonic stem cells and type I diabetes: How far to the clinic? **The Permanente Journal.** 8(1): 11-14.

Bernroider, E., Brehm, A., Krssak, M., Anderwald, C., Trajanoski, Z., Cline, G., Shulman, G. I. and Roden, M. (2005). The role of intramyocellular lipids during hypoglycemia in patients with intensively treated type 1 diabetes. **The Journal of Clinical Endocrinology & Metabolism.** 90(10): 5559-5565.

Bethel, M. A. and Feinglos, M. N. (2005). Basal insulin therapy in type 2 diabetes. **Journal of the American Board of Family Practice.** 18(3): 199-204.

Bieback, K., Schallmoser, K., Kluter, H. and Strunk, D. (2008). Clinical protocols for the isolation and expansion of mesenchymal stromal cells. **Transfusion Medicine and Hemotherapy.** 35(4): 286-294.

Blosser, C. D. and Bloom, R. D. (2010). Posttransplant anemia in solid organ recipients. **Transplantation Reviews (Orlando).** 24(2): 89-98.

Botanica sistemztica. (2011). ***Gynura procumbens (Lour.) Merr.*** [On-line]. Available: http://www.homolaicus.com/scienza/erbario/utility/botanica_sistematica/hypertext/0791.html.

Boyd, A. S., Higashi, Y. and Wood, K. J. (2005). Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation. **Advanced Drug Delivery Reviews.** 57(13): 1944-1969.

Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M., Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T. and Atkinson, K. (2007). Therapeutic applications of mesenchymal stromal cells. **Seminars in Cell & Developmental Biology.** 18(6): 846-858.

Brundin, P., Karlsson, J., Emgard, M., Schierle, G. S., Hansson, O., Petersen, A. and Castilho, R. F. (2000). Improving the survival of grafted dopaminergic neurons: a review over current approaches. **Cell Transplantation.** 9(2): 179-195.

Buchanan, T. A. (1995). **Pregnancy in preexisting diabetes.** [On-line]. Available: http://diabetes.niddk.nih.gov/dm/pubs/America/pdf/chapter36.pdf.

Buhler, D. R. and Miranda, C. (2000). **Antioxidant activities of flavonoids.** [On-line]. Available: http://lpi.oregonstate.edu/f-w00/flavonoid.html.

Bunnell, B. A., Flaat, M., Gagliardi, C., Patel, B. and Ripoll, C. (2008). Adipose-derived stem cells: isolation, expansion and differentiation. **Methods.** 45(2): 115-120.

Burant, C. F. and Simeone, D. M. (2002). Connecting islet development and developing useful islets. **Trends in Endocrinology & Metabolism.** 13(2): 48-49.

Cameron, A. (2010). The metabolic syndrome: validity and utility of clinical definitions for cardiovascular disease and diabetes risk prediction. **Maturitas.** 65(2): 117-121.

Caplan, A. I. and Dennis, J. E. (2006). Mesenchymal stem cells as trophic mediators. **Journal of Cellular Biochemistry.** 98(5): 1076-1084.

Carlsson, P. O. (2011). Influence of microenvironment on engraftment of transplanted beta-cells. **Upsala Journal of Medical Sciences.** 116(1): 1-7.

Chakrabarti, S. K. and Mirmira, R. G. (2003). Transcription factors direct the development and function of pancreatic beta cells. **Trends in Endocrinology & Metabolism.** 14(2): 78-84.

Chan, K. (2003). Some aspects of toxic contaminants in herbal medicines. **Chemosphere.** 52: 1361-1371.

Chao, C. S., Loomis, Z. L., Lee, J. E. and Sussel, L. (2007). Genetic identification of a novel NeuroD1 function in the early differentiation of islet alpha, PP and epsilon cells. **Developmental Biology.** 312(2): 523-532.

Chayosumrit, M., Tuch, B. and Sidhu, K. (2010). Alginate microcapsule for propagation and directed differentiation of hESCs to definitive endoderm. **Biomaterials.** 31(3): 505-514.

Chen, Y., Shao, J. Z., Xiang, L. X., Dong, X. J. and Zhang, G. R. (2008). Mesenchymal stem cells: a promising candidate in regenerative medicine. **The International Journal of Biochemistry & Cell Biology.** 40(5): 815-820.

Chen, V. C., Couture, S. M., Ye, J., Lin, Z., Hua, G., Huang, H. I., Wu, J., Hsu, D., Carpenter, M. K. and Couture, L. A. (2012). Scalable GMP compliant suspension culture system for human ES cells. **Stem Cell Research.** 8(3): 388-402.

Cheng, L., Hammond, H., Ye, Z., Zhan, X. and Dravid, G. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. **Stem Cells.** 21(2): 131-142.

Cherdshewasart, W., Sutjit, W., Pulcharoen, K., Panriansaen, R. and Chulasiri, M. (2008). Antimutagenic potential of the Thai herb, *Mucuna collettii* Lace. **Journal of Ethnopharmacology.** 115(1): 96-103.

Cheung, N. and Wong, T. Y. (2008). Diabetic retinopathy and systemic vascular complications. **Progress in Retinal and Eye Research.** 27(2): 161-176.

Chiang, M. K. and Melton, D. A. (2003). Single-cell transcript analysis of pancreas development. **Developmental Cell.** 4(3): 383-393.

Cohen, C. B., Brandhorst, B., Nagy, A., Leader, A., Dickens, B., Isasi, R. M., Evans, D. and Knoppers, B. M. (2008). The use of fresh embryos in stem cell research: Ethical and policy issues. **Cell Stem Cell.** 2: 416-421.

Collombat, P., Hecksher-Sorensen, J., Serup, P. and Mansouri, A. (2006). Specifying pancreatic endocrine cell fates. **Mechanisms of Development.** 123(7): 501-512.

da Silva Meirelles, L., Caplan, A. I. and Nardi, N. B. (2008). In search of the *in vivo* identity of mesenchymal stem cells. **Stem Cells.** 26(9): 2287-2299.

da Silva Meirelles, L., Fontes, A. M., Covas, D. T. and Caplan, A. I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. **Cytokine & Growth Factor Reviews.** 20: 419-427.

Dabla, P. K. (2010). Renal function in diabetic nephropathy. **The World Journal of Diabetes.** 1(2): 48-56.

de Vos, P., Faas, M. M., Strand, B. and Calafiore, R. (2006). Alginate-based microcapsules for immunoisolation of pancreatic islets. **Biomaterials.** 27(32): 5603-5617.

Deans, R. J. and Moseley, A. B. (2000). Mesenchymal stem cells: biology and potential clinical uses. **Experimental Hematology.** 28(8): 875-884.

Deedwania, P. C. and Fonseca, V. A. (2005). Diabetes, prediabetes, and cardiovascular risk: shifting the paradigm. **American Journal of Medicine.** 118(9): 939-947.

Delcourt, C., Massin, P. and Rosilio, M. (2009). Epidemiology of diabetic retinopathy: expected vs reported prevalence of cases in the French population. **Diabetes & Metabolism.** 35(6): 431-438.

Deng, H. P. and Chai, J. K. (2009). The effects and mechanisms of insulin on systemic inflammatory response and immune cells in severe trauma, burn injury, and sepsis. **International Immunopharmacology.** 9(11): 1251-1259.

Desbaillets, I., Ziegler, U., Groscurth, P. and Gassmann, M. (2000). Embryoid bodies: an *in vitro* model of mouse embryogenesis. **Experimental Physiology.** 85(6): 645-651.

Dhawan, S., Georgia, S. and Bhushan, A. (2007). Formation and regeneration of the endocrine pancreas. **Current Opinion in Cell Biology.** 19(6): 634-645.

Diagnosis and classification of diabetes mellitus. (2012). **Diabetes Care.** 35 Suppl 1: S64-71.

Docherty, K., Bernardo, A. S. and Vallier, L. (2007). Embryonic stem cell therapy for diabetes mellitus. **Seminars in Cell & Developmental Biology.** 18(6): 827-838.

Dohrmann, C., Gruss, P. and Lemaire, L. (2000). Pax genes and the differentiation of hormone-producing endocrine cells in the pancreas. **Mechanisms of Development.** 92(1): 47-54.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D. and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy.** 8(4): 315-317.

Draper, J. S., Pigott, C., Thomson, J. A. and Andrews, P. W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. **Journal of Anatomy.** 200: 249-258.

Drukker, M. and Benvenisty, N. (2004). The immunogenicity of human embryonic stem-derived cells. **Trends in Biotechnology.** 22(3): 136-141.

Drukker, M., Katz, G., Urbach, A., Schuldiner, M., Markel, G., Itskovitz-Eldor, J., Reubinoff, B., Mandelboim, O. and Benvenisty, N. (2002). Characterization of the expression of MHC proteins in human embryonic stem cells. **Proceedings of the National Academy of Sciences of the United States of America.** 99(15): 9864-9869.

Dufrane, D., Steenberghe, M., Goebbels, R. M., Saliez, A., Guiot, Y. and Gianello, P. (2006). The influence of implantation site on the biocompatibility and survival of alginate encapsulated pig islets in rats. **Biomaterials.** 27(17): 3201-3208.

Edwards, J. L., Vincent, A. M., Cheng, H. T. and Feldman, E. L. (2008). Diabetic neuropathy: mechanisms to management. **Pharmacology & Therapeutics.** 120(1): 1-34.

Eidi, A., Eidi, M. and Esmaeili, E. (2006). Antidiabetic effect of garlic (*Allium sativum* L.) in normal and streptozotocin-induced diabetic rats. **Phytomedicine.** 13(9-10): 624-629.

Eldor, R., Stern, E., Milicevic, Z. and Raz, I. (2005). Early use of insulin in type 2 diabetes. **Diabetes Research and Clinical Practice.** 68 Suppl 1: S30-35.

Ellis, H. (2006). Anatomy of the pancreas. **Surgery.** 25(2): 72-73.

Elvin-Lewis, M. (2001). Should we be concerned about herbal remedies. **Journal of Ethnopharmacology.** 75(2-3): 141-164.

Eurich, D. T., McAlister, F. A., Blackburn, D. F., Majumdar, S. R., Tsuyuki, R. T., Varney, J. and Johnson, J. A. (2007). Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: systematic review. **British Medical Journal.** 335(7618): 497.

Fiorina, P., Jurewicz, M., Augello, A., Vergani, A., Dada, S., La Rosa, S., Selig, M., Godwin, J., Law, K., Placidi, C., Smith, R. N., Capella, C., Rodig, S., Adra, C. N., Atkinson, M., Sayegh, M. H. and Abdi, R. (2009). Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. **The Journal of Immunology.** 183(2): 993-1004.

Fong, C. Y., Gauthaman, K. and Bongso, A. (2010a). Teratomas from pluripotent stem cells: A clinical hurdle. **Journal of Cellular Biochemistry.** 111(4): 769-781.

Fong, C. Y., Subramanian, A., Biswas, A., Gauthaman, K., Srikanth, P., Hande, M. P. and Bongso, A. (2010b). Derivation efficiency, cell proliferation, freeze-thaw survival, stem-cell properties and differentiation of human Wharton's jelly stem cells. **Reproductive Biomedicine Online.** 21(3): 391-401.

Fowler, M. J. (2007). Diabetes treatment, part 2: Oral agents for glycemic management. **Clinical Diabetes.** 25(4): 131-134.

Fujikawa, T., Oh, S. H., Pi, L., Hatch, H. M., Shupe, T. and Petersen, B. E. (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. **The American Journal of Pathology.** 166(6): 1781-1791.

Funnell, M. M. (2008). Quality of life and insulin therapy in type 2 diabetes mellitus. **Insulin.** 3: 31-36.

Gangaram-Panday, S. T., Faas, M. M. and de Vos, P. (2007). Towards stem-cell therapy in the endocrine pancreas. **Trends in Molecular Medicine.** 13(4): 164-173.

Gao, F., Wu, D. Q., Hu, Y. H., Jin, G. X., Li, G. D., Sun, T. W. and Li, F. J. (2008). *In vitro* cultivation of islet-like cell clusters from human umbilical cord blood-derived mesenchymal stem cells. **Translational Research.** 151(6): 293-302.

Gao, N., White, P., Doliba, N., Golson, M. L., Matschinsky, F. M. and Kaestner, K. H. (2007). Foxa2 controls vesicle docking and insulin secretion in mature β cells. **Cell Metabolism.** 6(4): 267-279.

Garcia, C., Feve, B., Ferre, P., Halimi, S., Baizri, H., Bordier, L., Guiu, G., Dupuy, O., Bauduceau, B. and Mayaudon, H. (2010). Diabetes and inflammation: fundamental aspects and clinical implications. **Diabetes & Metabolism.** 36(5): 327-338.

Gardner, T. W., Antonetti, D. A., Barber, A. J., LaNoue, K. F. and Levison, S. W. (2002). Diabetic retinopathy: more than meets the eye. **Survey of Ophthalmology.** 47 Suppl 2: S253-262.

Gasa, R., Mrejen, C., Lynn, F. C., Skewes-Cox, P., Sanchez, L., Yang, K. Y., Lin, C. H., Gomis, R. and German, M. S. (2008). Induction of pancreatic islet cell differentiation by the neurogenin-neuroD cascade. **Differentiation.** 76(4): 381-391.

Gatua, W. K., Makumi, J. N., Njagi, E. M., Kigondu, C. S., Mcligeyo, S. O. and Waithaka, S. K. (2011). Evaluation of urinary tubular enzymes as screening markers of renal dysfunction in patients suffering from diabetes mellitus. **Asian Journal of Medical Sciences.** 3(3): 84-90.

Gauthier, B. R., Wiederkehr, A., Baquie, M., Dai, C., Powers, A. C., Kerr-Conte, J., Pattou, F., MacDonald, R. J., Ferrer, J. and Wollheim, C. B. (2009). PDX1 deficiency causes mitochondrial dysfunction and defective insulin secretion through TFAM suppression. **Cell Metabolism.** 10(2): 110-118.

Gavin, J. R., 3rd (1998). New classification and diagnostic criteria for diabetes mellitus. **Clinical Cornerstone.** 1(3): 1-12.

Ghazavi, M. K. and Johnston, G. A. (2011). Insulin allergy. **Clinics in Dermatology.** 29(3): 300-305.

Girach, A. and Vignati, L. (2006). Diabetic microvascular complications--can the presence of one predict the development of another? **Journal of Diabetes and its Complications.** 20(4): 228-237.

Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F. (2000). *Neurogenin3* is required for the development of the four endocrine cell lineages of the pancreas. **Proceedings of the National Academy of Sciences of the United States of America.** 97(4): 1607-1611.

Gunasekaran, S. (2003). Human pancreatic islet transplantation. **International Journal of Diabetes in Developing Countries.** 23: 55-57.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. **Molecular Aspects of Medicine.** 27(1): 1-93.

Hale, M. A., Kagami, H., Shi, L., Holland, A. M., Elsasser, H. P., Hammer, R. E. and MacDonald, R. J. (2005). The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. **Developmental Biology.** 286(1): 225-237.

Hall, A. P. and Davies, M. J. (2008). Assessment and management of diabetes mellitus. **The Foundation Years.** 4(6): 224-229.

Hassan, Z., Yam, M. F., Ahmad, M. and Yusof, A. P. (2010). Antidiabetic properties and mechanism of action of Gynura procumbens water extract in streptozotocin-induced diabetic rats. **Molecules.** 15(12): 9008-9023.

Hentze, H., Soong, P. L., Wang, S. T., Phillips, B. W., Putti, T. C. and Dunn, N. R. (2009). Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. **Stem Cell Research.** 2(3): 198-210.

Herzog, E. L., Chai, L. and Krause, D. S. (2003). Plasticity of marrow-derived stem cells. **Blood.** 102(10): 3483-3493.

Hirshberg, B., Montgomery, S., Wysoki, M. G., Xu, H., Tadaki, D., Lee, J., Hines, K., Gaglia, J., Patterson, N., Leconte, J., Hale, D., Chang, R., Kirk, A. D. and Harlan, D. M. (2002). Pancreatic islet transplantation using the nonhuman primate (Rhesus) model predicts that the portal vein is superior to the celiac artery as the islet infusion site. **Diabetes.** 51: 2135-2140.

Hocking, A. M. and Gibran, N. S. (2010). Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. **Experimental Cell Research.** 316(14): 2213-2219.

Hoe, S. Z., Lee, C. N., Mok, S. L., Kamaruddin, M. Y. and Lam, S. K. (2011). *Gynura procumbens* Merr. decreases blood pressure in rats by vasodilatation via inhibition of calcium channels. **Clinics (Sao Paulo).** 66(1): 143-150.

Hoogduijn, M. J., Popp, F., Verbeek, R., Masoodi, M., Nicolaou, A., Baan, C. and Dahlke, M. H. (2010). The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. **International Immunopharmacology.** 10(12): 1496-1500.

Hsu, F. L., Liu, I. M., Kuo, D. H., Chen, W. C., Su, H. C. and Cheng, J. T. (2003). Antihyperglycemic effect of puerarin in streptozotocin-induced diabetic rats. **Journal of Natural Products.** 66(6): 788-792.

Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene *BETA2* (*neuroD*) by neurogenin 3. **Molecular and Cellular Biology.** 20(9): 3292-3307.

Hunziker, E. and Stein, M. (2000). Nestin-expressing cells in the pancreatic islets of Langerhans. **Biochemical and Biophysical Research Community.** 271(1): 116-119.

Hwang, N. S., Varghese, S. and Elisseeff, J. (2008). Controlled differentiation of stem cells. **Advanced Drug Delivery Reviews.** 60(2): 199-214.

Imai, J., Katagiri, H., Yamada, T., Ishigaki, Y., Ogihara, T., Uno, K., Hasegawa, Y., Gao, J., Ishihara, H., Sasano, H., Mizuguchi, H., Asano, T. and Oka, Y. (2005). Constitutively active PDX1 induced efficient insulin production in adult murine liver. **Biochemical and Biophysical Research Community.** 326(2): 402-409.

Iskander, M. N., Song, Y., Coupar, I. M. and Jiratchariyakul, W. (2002). Antiinflammatory screening of the medicinal plant *Gynura procumbens*. **Plant Foods for Human Nutrition.** 57(3-4): 233-244.

Jacobsen, I. B., Henriksen, J. E., Hother-Nielsen, O., Vach, W. and Beck-Nielsen, H. (2009). Evidence-based insulin treatment in type 1 diabetes mellitus. **Diabetes Research and Clinical Practice.** 86(1): 1-10.

Jacquemin, P., Durviaux, S. M., Jensen, J., Godfraind, C., Gradwohl, G., Guillemot, F., Madsen, O. D., Carmeliet, P., Dewerchin, M., Collen, D., Rousseau, G. G. and Lemaigre, F. P. (2000). Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene *ngn3*. **Molecular and Cellular Biology.** 20(12): 4445-4454.

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G. and Majumdar, A. S. (2007). Generation of insulin-producing islet-like clusters from human embryonic stem cells. **Stem Cells.** 25(8): 1940-1953.

Jones, M. P., Burns, C. J., and Persaud, S. (2004). Beta-cell replacement technologies: the potential of stem cells. **Drug Discovery Today: Therapeutic Strategies.** 1(2): 213-217.

Kahn, C. R., Weir, G. C., King, G. L., Jacobson, A. M., Moses, A. C., and Smith, R. J. (2005). **Joslin's Diabetes Mellitus** (14th ed.). Philadelphia: Lippincott Williams & Wilkins.

Kandaswamy, R. and Sutherland, D. E. (2006). Pancreas versus islet transplantation in diabetes mellitus: How to allocate deceased donor pancreata? **Transplantation Proceedings.** 38(2): 365-367.

Karp, J. M., Yeh, J., Eng, G., Fukuda, J., Blumling, J., Suh, K. Y., Cheng, J., Mahdavi, A., Borenstein, J., Langer, R. and Khademhosseini, A. (2007). Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells. **Lab on a Chip.** 7(6): 786-794.

Kastenberg, Z. J. and Odorico, J. S. (2008). Alternative sources of pluripotency: science, ethics, and stem cells. **Transplantation Reviews (Orlando).** 22(3): 215-222.

Kengne, A. P., Turnbull, F. and MacMahon, S. (2010). The Framingham Study, diabetes mellitus and cardiovascular disease: turning back the clock. **Progress in Cardiovascular Diseases.** 53(1): 45-51.

Khoo, M. L., McQuade, L. R., Smith, M. S., Lees, J. G., Sidhu, K. S. and Tuch, B. E. (2005). Growth and differentiation of embryoid bodies derived from human embryonic stem cells: effect of glucose and basic fibroblast growth factor. **Biology of Reproduction.** 73(6): 1147-1156.

Kiatpongsan, S., Tannirandorn, Y. and Virutamasen, P. (2006). Introduction to stem cell medicine. **Journal of the Medical Association of Thailand.** 89(1): 111-117.

Kibschull, M., Mileikovsky, M., Michael, I. P., Lye, S. J. and Nagy, A. (2011). Human embryonic fibroblasts support single cell enzymatic expansion of human embryonic stem cells in xeno-free cultures. **Stem Cell Research.** 6(1): 70-82.

Kim, H. S., Oh, S. K., Park, Y. B., Ahn, H. J., Sung, K. C., Kang, M. J., Lee, L. A., Suh, C. S., Kim, S. H., Kim, D. W. and Moon, S. Y. (2005). Methods for derivation of human embryonic stem cells. **Stem Cells.** 23(9): 1228-1233.

Kim, J. Y., Jeon, H. B., Yang, Y. S., Oh, W. and Chang, J. W. (2010). Application of human umbilical cord blood-derived mesenchymal stem cells in disease models. **World Journal of Stem Cells.** 2(2): 34-38.

Kim, M. J., Lee, H. J., Wiryowidagdo, S. and Kim, H. K. (2006). Antihypertensive effects of *Gynura procumbens* extract in spontaneously hypertensive rats. **Journal of Medical Food.** 9(4): 587-590.

Kim, S. K. and MacDonald, R. J. (2002). Signaling and transcriptional control of pancreatic organogenesis. **Current Opinion in Genetics & Development.** 12: 540-547.

King, F. W., Ritner, C., Liszewski, W., Kwan, H. C., Pedersen, A., Leavitt, A. D. and Bernstein, H. S. (2009). Subpopulations of human embryonic stem cells with distinct tissue-specific fates can be selected from pluripotent cultures. **Stem Cells and Development.** 18(10): 1441-1450.

King, H., Aubert, R. E. and Herman, W. H. (1998). Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. **Diabetes Care.** 21(9): 1414-1431.

Klonoff, D. C. and Schwartz, D. M. (2000). An economic analysis of interventions for diabetes. **Diabetes Care.** 23(3): 390-404.

Knip, M. and Siljander, H. (2008). Autoimmune mechanisms in type 1 diabetes. **Autoimmunity Reviews.** 7(7): 550-557.

Krolewski, A. S., Fogarty, D. G. and Warram, J. H. (1998). Hypertension and nephropathy in diabetes mellitus: what is inherited and what is acquired? **Diabetes Research and Clinical Practice.** 39 Suppl 1: S1-14.

Kuroe, A., Taniuguchi, A., Fukushima, M., Nakai, Y., Ohgushi, M., Ohya, M. and Seino, Y. (2007). Early and late onset side effects of short-acting insulin analogue in seven Japanese diabetic patients. **Diabetes Research and Clinical Practice.** 77(3): 412-413.

Kurosawa, H. (2007). Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. **Journal of Bioscience and Bioengineering.** 103(5): 389-398.

Kuzuya, T., Nakagawa, S., Satoh, J., Kanazawa, Y., Iwamoto, Y., Kobayashi, M., Nanjo, K., Sasaki, A., Seino, Y., Ito, C., Shima, K., Nonaka, K. and Kadowaki, T. (2002). Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. **Diabetes Research and Clinical Practice.** 55(1): 65-85.

Laco, F., Kun, M., Weber, H. J., Ramakrishna, S. and Chan, C. K. (2009). The dose effect of human bone marrow-derived mesenchymal stem cells on epidermal development in organotypic co-culture. **Journal of Dermatological Science.** 55(3): 150-160.

Lantz, K. A., Vatamaniuk, M. Z., Brestelli, J. E., Friedman, J. R., Matschinsky, F. M. and Kaestner, K. H. (2004). Foxa2 regulates multiple pathways of insulin secretion. **The Journal of Clinical Investigation.** 114(4): 512-520.

Lau, J., Mattsson, G., Carlsson, C., Nyqvist, D., Kohler, M., Berggren, P. O., Jansson, L. and Carlsson, P. O. (2007). Implantation site-dependent dysfunction of transplanted pancreatic islets. **Diabetes.** 56(6): 1544-1550.

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E. and Ringden, O. (2003). HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. **Experimental Hematology.** 31(10): 890-896.

Lee, M. K. and Bae, Y. H. (2000). Cell transplantation for endocrine disorders. **Advanced Drug Delivery Reviews.** 42(1-2): 103-120.

Lee, J. B., Song, J. M., Lee, J. E., Park, J. H., Kim, S. J., Kang, S. M., Kwon, J. N., Kim, M. K., Roh, S. I. and Yoon, H. S. (2004a). Available human feeder cells for the maintenance of human embryonic stem cells. **Reproduction.** 128(6): 727-735.

Lee, M. W., Choi, J., Yang, M. S., Moon, Y. J., Park, J. S., Kim, H. C. and Kim, Y. J. (2004b). Mesenchymal stem cells from cryopreserved human umbilical cord blood. **Biochemical and Biophysical Research Communications.** 320(1): 273-278.

Lee, R. H., Seo, M. J., Reger, R. L., Spees, J. L., Pulin, A. A., Olson, S. D. and Prockop, D. J. (2006). Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/*scid* mice. **Proceedings of the National Academy of Sciences of the United States of America.** 103(46): 17438-17443.

Li, L., Fei, Z., Ren, J., Sun, R., Liu, Z., Sheng, Z., Wang, L., Sun, X., Yu, J., Wang, Z. and Fei, J. (2008). Functional imaging of interleukin 1 beta expression in inflammatory process using bioluminescence imaging in transgenic mice. **BMC Immunology.** 9: 49.

Li, Z., Leung, M., Hopper, R., Ellenbogen, R. and Zhang, M. (2010). Feeder-free self-renewal of human embryonic stem cells in 3D porous natural polymer scaffolds. **Biomaterials.** 31(3): 404-412.

Li, Z. H., Liao, W., Cui, X. L., Zhao, Q., Liu, M., Chen, Y. H., Liu, T. S., Liu, N. L., Wang, F., Yi, Y. and Shao, N. S. (2011). Intravenous transplantation of allogeneic bone marrow mesenchymal stem cells and its directional migration to the necrotic femoral head. **International Journal of Medical Sciences.** 8(1): 74-83.

Lincoln, J. and Shotton, H. R. (2008). Diabetic autonomic neuropathy. **Tzu Chi Medical Journal.** 20(3): 161-168.

Lipipun, V. (2003). Efficacy of Thai medicinal plant extracts against herpes simplex virus type 1 infection *in vitro* and *in vivo*. **Antiviral Research.** 60(3): 175-180.

Lorenzati, B., Zucco, C., Miglietta, S., Lamberti, F. and Bruno, G. (2010). Oral hypoglycemic drugs: Pathophysiological basis of their mechanism of action. **Pharmaceuticals.** 3: 3005-3020.

Lu, H. F., Narayanan, K., Lim, S. X., Gao, S., Leong, M. F. and Wan, A. C. (2012). A 3D microfibrous scaffold for long-term human pluripotent stem cell self-renewal under chemically defined conditions. **Biomaterials.** 33(8): 2419-2430.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. and McKay, R. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. **Science.** 292(5520): 1389-1394.

Magenheim, J., Klein, A. M., Stanger, B. Z., Ashery-Padan, R., Sosa-Pineda, B., Gu, G. and Dor, Y. (2011). Ngn3(+) endocrine progenitor cells control the fate and morphogenesis of pancreatic ductal epithelium. **Developmental Biology.** 359(1): 26-36.

Migliavacca, B., Nano, R., Antonioli, B., Marzorati, S., Davalli, A. M., Di Carlo, V. and Bertuzzi, F. (2004). Identification of *in vitro* parameters predictive of graft function: a study in an animal model of islet transplantation. **Transplantation Proceedings.** 36(3): 612-613.

Mahmood, A. A., Mariod, A. A., Al-Bayaty, F. and Abdel-Wahab, S. I. (2010). Anti-ulcerogenic activity of *Gynura procumbens* leaf extract against experimentally-induced gastric lesions in rats. **Journal of Medicinal Plants Research.** 4(8): 685-691.

Mallon, B. S., Park, K. Y., Chen, K. G., Hamilton, R. S. and McKay, R. D. (2006). Toward xeno-free culture of human embryonic stem cells. **The International Journal of Biochemical & Cell Biology.** 38(7): 1063-1075.

Manosroi, J., Moses, Z. Z., Manosroi, W. and Manosroi, A. (2011). Hypoglycemic activity of Thai medicinal plants selected from the Thai/Lanna Medicinal Recipe Database MANOSROI II. **Journal of Ethnopharmacology.** 138(1): 92-98.

Mao, G. H., Chen, G. A., Bai, H. Y., Song, T. R. and Wang, Y. X. (2009). The reversal of hyperglycaemia in diabetic mice using PLGA scaffolds seeded with islet-like cells derived from human embryonic stem cells. **Biomaterials.** 30(9): 1706-1714.

Marks, J. B. and Raskin, P. (2000). Cardiovascular risk in diabetes: a brief review. **Journal of Diabetes and its Complications.** 14(2): 108-115.

Maw, S. S., Mon, M. M. and Oo, Z. K. (2011). Study on antioxidant and antitumor activities of some herbal extracts. **World Academy of Science, Engineering and Technology.** 75: 450-455.

Meirelles Lda, S., Fontes, A. M., Covas, D. T. and Caplan, A. I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. **Cytokine & Growth Factor Reviews.** 20(5-6): 419-427.

Melikian, C., White, T. J., Vanderplas, A., Dezii, C. M. and Chang, E. (2002). Adherence to oral antidiabetic therapy in a managed care organization: a comparison of monotherapy, combination therapy, and fixed-dose combination therapy. **Clinical Therapeutics.** 24(3): 460-467.

Morsanutto, A., Berto, P., Lopatriello, S., Gelisio, R., Voinovich, D., Cippo, P. P. and Mantovani, L. G. (2006). Major complications have an impact on total annual medical cost of diabetes: results of a database analysis. **Journal of Diabetes and its Complications.** 20(3): 163-169.

Mustafa R. A., Abdul Hamed, A., Mohamed, S. and Abu Baker, F. (2009). Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. **Journal of Food Science.** 75(1): C28-C35.

Muthu, C., Ayyanar, M., Raja, N. and Ignacimuthu, S. (2006). Medicinal plants used by traditional healers in Kancheepuram district of Tamil Nadu, India. **Journal of Ethnobiology and Ethnomedicine.** 2: 43.

Naftanel, M. A. and Harlan, D. M. (2004). Pancreatic islet transplantation. **PLoS Medicine.** 1(3): (e58); quiz e75.

Nam, S., Chesla, C., Stotts, N. A., Kroon, L. and Janson, S. L. (2011). Barriers to diabetes management: patient and provider factors. **Diabetes Research and Clinical Practice.** 93(1): 1-9.

National Diabetes Information Clearinghouse. (2008). **Diabetes overview.** [On-line]. Available: http://diabetes.niddk.nih.gov/dm/pubs/overview/ index.aspx.

National Diabetes Information Clearinghouse. (2012). **Complications of diabetes.** [On-line]. Available: http://diabetes.niddk.nih.gov/complications/index.aspx.

Nauta, A. J. and Fibbe, W. E. (2007). Immunomodulatory properties of mesenchymal stromal cells. **Blood.** 110(10): 3499-3506.

Navarro, J. F. and Mora, C. (2005). Role of inflammation in diabetic complications. **Nephrology Dialysis Transplantation.** 20(12): 2601-2604.

Nekanti, U., Mohanty, L., Venugopal, P., Balasubramanian, S., Totey, S. and Ta, M. (2010). Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. **Stem Cell Research.** 5(3): 244-254.

Noor, A., Gunasekaran, S., Soosai Manickam, A. and Vijayalakshmi, M. A. (2008). Antidiabetic activity of *Aloe vera* and histology of organs in streptozotocin induced diabetic rats. **Current Science.** 94(8): 1072-1076.

Norris, S. L., Nichols, P. J., Caspersen, C. J., Glasgow, R. E., Engelgau, M. M., Jack, L., Isham, G., Snyder, S. R., Carande-Kulis, V. G., Garfield, S., Briss, P. and McCulloch, D. (2002). The effectiveness of disease and case management for people with diabetes. A systematic review. **American Journal of Preventive Medicine.** 22(4 Suppl): 15-38.

Pedersen, J. K., Nelson, S. B., Jorgensen, M. C., Henseleit, K. D., Fujitani, Y., Wright, C. V., Sander, M. and Serup, P. (2005). Endodermal expression of Nkx6 genes depends differentially on Pdx1. **Developmental Biology.** 288(2): 487-501.

Peng, J., Wang, Y., Zhang, L., Zhao, B., Zhao, Z., Chen, J., Guo, Q., Liu, S., Sui, X., Xu, W. and Lu, S. (2011). Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth *in vitro*. **Brain Research Bulletin.** 84(3): 235-243.

Pountos, I. and Giannoudis, P. V. (2005). Biology of mesenchymal stem cells. **Injury.** 36 Suppl 3: S8-S12.

Prasanna, S. J., Gopalakrishnan, D., Shankar, S. R. and Vasandan, A. B. (2010). Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. **PLoS ONE.** 5(2): e9016.

Prasanna, S. J. and Jahnavi, V. S. (2011). Wharton’s jelly mesenchymal stem cells as off-the-shelf cellular therapeutics: A closer look into their regenerative and immunomodulatory properties. **The Open Tissue Engineering and Regenerative Medicine Journal.** 4: 28-38.

Qin, H., Yu, T., Qing, T., Liu, Y., Zhao, Y., Cai, J., Li, J., Song, Z., Qu, X., Zhou, P., Wu, J., Ding, M. and Deng, H. (2007). Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. **Journal of Biological Chemistry.** 282(8): 5842-5852.

Raikwar, S. P., Mueller, T. and Zavazava, N. (2006). Strategies for developing therapeutic application of human embryonic stem cells. **Physiology (Bethesda).** 21: 19-28.

Rajagopal, J., Anderson, W. J., Kume, S., Martinez, O. I. and Melton, D. A. (2003). Insulin staining of ES cell progeny from insulin uptake. **Science.** 299(5605): 363.

Rao, M. U., Sreenivasulu, M., Chengaiah, B., Reddy, K. J. and Chetty, C. M. (2010). Herbal medicines for diabetes mellitus: A review. **International Journal of PharmTech Research.** 2(3): 1883-1892.

Richards, M., Tan, S., Fong, C. Y., Biswas, A., Chan, W. K. and Bongso, A. (2003). Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. **Stem Cells.** 21(5): 546-556.

Rivellese, A. A., Riccardi, G. and Vaccaro, O. (2010). Cardiovascular risk in women with diabetes. **Nutrition, Metabolism & Cardiovascular Diseases.** 20(6): 474-480.

Rojas, J. J., Ochoa, V. J., Ocampo, S. A. and Munoz, J. F. (2006). Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. **BMC Complementary and Alternative Medicine.** 6: 2.

Sahoo, N., Manchikanti, P. and Dey, S. (2010). Herbal drugs: standards and regulation. **Fitoterapia.** 81(6): 462-471.

Saitoh, K., Yamato, E., Miyazaki, S. and Miyazaki, J. (2007). Both Pdx-1 and NeuroD1 genes are requisite for the maintenance of insulin gene expression in ES-derived differentiated cells. **Diabetes Research and Clinical Practice.** 77 Suppl 1: S138-142.

Sakunpak, A. and Panichayupakaranant, P. (2012). Antibacterial activity of Thai edible plants against gastrointestinal pathogenic bacteria and isolation of a new broad spectrum antibacterial polyisoprenylated benzophenone, chamuangone. **Food Chemistry.** 130: 826-831.

Salahuddin, M. and Jalalpure, S. S. (2010). Antidiabetic activity of aqueous fruit extract of *Cucumis trigonus* Roxb. in streptozotocin-induced-diabetic rats. **Journal of Ethnopharmacology.** 127: 565-567.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A. and German, M. (2000). Homeobox gene *Nkx6.1* lies downstream of *Nkx2.2* in the major pathway of beta-cell formation in the pancreas. **Development.** 127(2): 5533-5540.

Sattley, M. (2008). **The history of diabetes.** [On-line]. Available: http://www.diabeteshealth.com/read/2008/12/17/715/the-history-of-diabetes/.

Schisler, J. C., Jensen, P. B., Taylor, D. G., Becker, T. C., Knop, F. K., Takekawa, S., German, M., Weir, G. C., Lu, D., Mirmira, R. G. and Newgard, C. B. (2005). The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. **Proceedings of the National Academy of Sciences of the United States of America.** 102(20): 7297-7302.

Schneider, S., Feilen, P. J., Brunnenmeier, F., Minnemann, T., Zimmermann, H., Zimmermann, U. and Weber, M. M. (2005). Long-term graft function of adult rat and human islets encapsulated in novel alginate-based microcapsules after transplantation in immunocompetent diabetic mice. **Diabetes.** 54(3): 687-693.

Schwitzgebel, V.M. (2001). Programming of the pancreas. **Molecular and Cellular Endocrinology.** 185(1-2): 99-108.

Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and German, M. S. (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. **Development.** 127(16): 3533-3542.

Secco, M., Zucconi, E., Vieira, N. M., Fogaca, L. L., Cerqueira, A., Carvalho, M. D., Jazedje, T., Okamoto, O. K., Muotri, A. R. and Zatz, M. (2008). Mesenchymal stem cells from umbilical cord: do not discard the cord! **Neuromuscular Disorders.** 18(1): 17-18.

Seeberger, K. L., Dufour, J. M., Shapiro, A. M., Lakey, J. R., Rajotte, R. V. and Korbutt, G. S. (2006). Expansion of mesenchymal stem cells from human pancreatic ductal epithelium. **Laboratory Investigation.** 86(2): 141-153.

Segev, H., Fishman, B., Ziskind, A., Shulman, M. and Itskovitz-Eldor, J. (2004). Differentiation of human embryonic stem cells into insulin-producing clusters. **Stem Cells**. 22(3): 265-274.

Serra, M., Correia, C., Malpique, R., Brito, C., Jensen, J., Bjorquist, P., Carrondo, M. J. and Alves, P. M. (2011). Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells. **PLoS ONE.** 6(8): e23212.

Shapiro, A. M., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., Secchi, A., Brendel, M. D., Berney, T., Brennan, D. C., Cagliero, E., Alejandro, R., Ryan, E. A., DiMercurio, B., Morel, P., Polonsky, K. S., Reems, J. A., Bretzel, R. G., Bertuzzi, F., Froud, T., Kandaswamy, R., Sutherland, D. E., Eisenbarth, G., Segal, M., Preiksaitis, J., Korbutt, G. S., Barton, F. B., Viviano, L., Seyfert-Margolis, V., Bluestone, J. and Lakey, J. R. (2006). International trial of the Edmonton protocol for islet transplantation. **The New England Journal of Medicine.** 355(13): 1318-1330.

Shi, M., Liu, Z. W. and Wang, F. S. (2011). Immunomodulatory properties and therapeutic application of mesenchymal stem cells. **Clinical and Experimental Immunology.** 164: 1-8.

Shim, J. H., Kim, S. E., Woo, D. H., Kim, S. K., Oh, C. H., McKay, R. and Kim, J. H. (2007). Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. **Diabetologia.** 50(6): 1228-1238.

Shiroi, A., Ueda, S., Ouji, Y., Saito, K., Moriya, K., Sugie, Y., Fukui, H., Ishizaka, S. and Yoshikawa, M. (2005). Differentiation of embryonic stem cells into insulin-producing cells promoted by Nkx2.2 gene transfer. **World Journal of Gastroenterology.** 11(27): 4161-4166.

Shui, G. and Leong, L. P. (2002). Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography. **Journal of Chromatography A.** 977(1): 89-96.

Shyr, Y. M. (2009). Pancreas transplantation. **Journal of the Chinese Medical Association.** 72(1): 4-9.

Si, Y. L., Zhao, Y. L., Hao, H. J., Fu, X. B. and Han, W. D. (2011). MSCs: Biological characteristics, clinical applications and their outstanding concerns. **Ageing Research Reviews.** 10(1): 93-103.

Singh, S. K., Kesari, A. N., Gupta, R. K., Jaiswal, D. and Watal, G. (2007). Assessment of antidiabetic potential of *Cynodon dactylon* extract in streptozotocin diabetic rats. **Journal of Ethnopharmacology.** 114(2): 174-179.

Siti-Ismail, N., Bishop, A. E., Polak, J. M. and Mantalaris, A. (2008). The benefit of human embryonic stem cell encapsulation for prolonged feeder-free maintenance. **Biomaterials.** 29(29): 3946-3952.

Sjoholm, A. (1998). Aspects of the involvement of interleukin-1 and nitric oxide in the pathogenesis of insulin-dependent diabetes mellitus. **Cell Death and Differentiation.** 5(6): 461-468.

Skottman, H. and Hovatta, O. (2006). Culture conditions for human embryonic stem cells. **Reproduction.** 132(5): 691-698.

Soria, B. (2001). In-vitro differentiation of pancreatic beta-cells. **Differentiation.** 68(4-5): 205-219.

Sripanidkulchai, B., Wongpanich, V., Laupattarakasem, P., Suwansaksri, J. and Jirakulsomchok, D. (2001). Diuretic effects of selected Thai indigenous medicinal plants in rats. **Journal of Ethnopharmacology.** 75(2-3): 185-190.

Stewart, M. J., Moar, J. J., Steenkamp, P. and Kokot, M. (1999). Findings in fatal cases of poisoning attributed to traditional remedies in South Africa. **Forensic Science International.** 101(3): 177-183.

Stojkovic, M., Lako, M., Strachan, T. and Murdoch, A. (2004). Derivation, growth and applications of human embryonic stem cells. **Reproduction.** 128(3): 259-267.

Stojkovic, P., Lako, M., Stewart, R., Przyborski, S., Armstrong, L., Evans, J., Murdoch, A., Strachan, T. and Stojkovic, M. (2005). An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. **Stem Cells.** 23(3): 306-314.

Strulovici, Y., Leopold, P. L., O'Connor, T. P., Pergolizzi, R. G. and Crystal, R. G. (2007). Human embryonic stem cells and gene therapy. **Molecular Therapy.** 15(5): 850-866.

Subramaniam, S., Subramaniam, R., Rajapandian, S., Uthrapathi, S., Gnanamanickam, V. R. and Dubey, G. P. (2010). Anti-atherogenic activity of ethanolic fraction of *Terminalia arjuna* bark on hypercholesterolemic rabbits. **Evid-Based Complementary and Alternative Medicine.** 2011, 487916.

Suksomboon, N., Poolsup, N., Boonkaew, S. and Suthisisang, C. C. (2011). Meta-analysis of the effect of herbal supplement on glycemic control in type 2 diabetes. **Journal of Ethnopharmacology.** 137(3): 1328-1333.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L. and German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. **Development.** 125(12): 2213-2221.

Taegtmeyer, A. B., Rogers, P., Breen, J. B., Barton, P. J., Banner, N. R. and Yacoub, M. H. (2008). The effects of pre- and post-transplant anemia on 1-year survival after cardiac transplantation. **The Journal of Heart and Lung Transplantation.** 27(4): 394-399.

Taghizadeh, R. R., Cetrulo, K. J. and Cetrulo, C. L. (2011). Wharton's Jelly stem cells: future clinical applications. **Placenta.** 32 Suppl 4: S311-315.

Tan, G. D. (2005). The pancreas. **Anaesthesia and Intensive Care Medicine.** 6(10): 329-332.

Tesfaye, S. (2010). Neuropathy in diabetes. **Medicine.** 38(12): 649-655.

Thomas, S. and Viberti, G. (2006). Diabetic nephropathy. **Medicine.** 34(3): 83-86.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. **Science.** 282(5391): 1145-1147.

Tovar, R. T. and Petzel, R. M. (2009). Herbal toxicity. **Disease-a-Month.** 55: 592-641.

Troppmann, C., Papalois, B. E., Gruessner, A. C., Nakhleh, R. E. and Gruessner, R. W. (1997). Major impact of engraftment site on early functional outcome of discordant xenoislet grafts from a large animal donor. **Transplantation Proceedings.** 29(4): 2107-2108.

Trounson, A. (2006). The production and directed differentiation of human embryonic stem cells. **Endocrine Reviews.** 27(2): 208-219.

Verma, S. and Singh, S. P. (2008). Current and future status of herbal medicines. **Veterinary World.** 1(11): 347-350.

Vestra, M. D. and Fioretto, P. (2003). Diabetic nephropathy: renal structural studies in type 1 and type 2 diabetic patients. **International Congress Series.** 1253: 163-169.

Vija, L., Farge, D., Gautier, J. F., Vexiau, P., Dumitrache, C., Bourgarit, A., Verrecchia, F. and Larghero, J. (2009). Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. **Diabetes & Metabolism.** 35(2): 85-93.

Vinik, A. I. (2006). Benefits of early initiation of insulin therapy to long-term goals in type 2 diabetes mellitus. **Insulin.** 1: 2-12.

Vinik, A. I. and Mehrabyan, A. (2004). Diabetic neuropathies. **Medical Clinics of North America.** 88 (4): 947-999, xi.

Vogel, T. and Friend, P. (2011). Pancreas transplantation. **Surgery.** 29(7): 348-352.

Wagner, A. M., Martijnez-Rubio, A., Ordonez-Llanos, J. and Perez-Perez, A (2002). Diabetes mellitus and cardiovascular disease. **European Journal of Internal Medicine.** 13(1): 15-30.

Wakdikar, S. (2004). Global health care challenge: Indian experiences and new prescriptions. **Electronic Journal of Biotechnology.** 7(3): 214-220.

Wang, N., Adams, G., Buttery, L., Falcone, F. H. and Stolnik, S. (2009). Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells. **Journal of Biotechnology.** 144(4): 304-312.

Wells, J. M. (2003). Genes expressed in the developing endocrine pancreas and their importance for stem cell and diabetes research. **Diabetes/Metabolism Research and Reviews.** 19: 191-201.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. **Diabetes Care.** 27(5): 1047-1053.

Wilson, M. E., Scheel, D. and German, M. S. (2003). Gene expression cascades in pancreatic development. **Mechanisms of Development.** 120(1): 65-80.

Woradulayapinij, W., Soonthornchareonnon, N. and Wiwat, C. (2005). *In vitro* HIV type 1 reverse transcriptase inhibitory activities of Thai medicinal plants and Canna indica L. rhizomes. **Journal of Ethnopharmacology.** 101(1-3): 84-89.

Xie, Q. P., Huang, H., Xu, B., Dong, X., Gao, S. L., Zhang, B. and Wu, Y. L. (2009). Human bone marrow mesenchymal stem cells differentiate into insulin-producing cells upon microenvironmental manipulation *in vitro*. **Differentiation.** 77(5): 483-491.

Xu, Y. X., Chen, L., Hou, W. K., Lin, P., Sun, L., Sun, Y., Dong, Q. Y., Liu, J.B. and Fu, Y. L. (2009). Mesenchymal stem cells treated with rat pancreatic extract secrete cytokines that improve the glycometabolism of diabetic rats. **Transplantation Proceedings.** 41(5): 1878-1884.

Xu, Y. X., Chen, L., Wang, R., Hou, W. K., Lin, P., Sun, L., Sun, Y. and Dong, Q. Y. (2008). Mesenchymal stem cell therapy for diabetes through paracrine mechanisms. **Medical Hypotheses.** 71: 390-393.

Yale Medical Group. (2012). **The pancreas: Anatomy and functions.** [On-line]. Available: http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW023328.

Yao, S., Chen, S., Clark, J., Hao, E., Beattie, G. M., Hayek, A. and Ding, S. (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. **Proceedings of the National Academy of Sciences of the United States of America.** 103(18): 6907-6912.

Yu, S., Li, C., Xin-guo, H., Wei-kai, H., Jian-jun, D., Lei, S., Kuan-xiao, T., Bin, W., Jun, S., Hui, L. and Ke-xin, W. (2007). Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells *in vitro*. **Chinese Medical Journal.** 120(9): 771-776.

Zahra, A. A., Kadir, F. A., Mahmood, A. A., Al hadi, A. A., Suzy, S. M., Sabri, S. Z., Latif, I. I. and Ketuly, K. A. (2011). Acute toxicity study and wound healing potential of *Gynura procumbens* leaf extract in rats. **Journal of Medicinal Plants Research.** 5(12): 2551-2558.

Zhang, X. F. and Tan, B. K. H. (2000). Effects of an ethanolic extract of *Gynura procumbens* on serum glucose, cholesterol and triglyceride levels in normal and streptozotocin-induced diabetic rats. **Singapore Medical Journal.** 41(1): 9-13.

Zimmet, P., Shaw, J. and Alberti, K. G. (2003). Preventing type 2 diabetes and the dysmetabolic syndrome in the real world: a realistic view. **Diabetic Medicine.** 20(9): 693-702.

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Abstracts

1. **Establishment of insulin-producing cells from human embryonic stem cells (hESCs).** *The 4th World Congress on Regenerative Medicine*, March 12-14, 2009 (Oral presentation)
2. **Effect of ethanolic extract of *Gynura procumbens* in streptozotocin-induced diabetic rats.** *The 3rd International Conference on Natural Products for Health & Beauty (NATPRO3)*, March 16-18, 2011 (Poster presentation)