UNIVERSITI PUTRA MALAYSIA

COMPARATIVE STUDY OF THE BIOLOGICAL PROPERTIES OF FOETAL AND PLACENTAL DISTAL ENDS OF HUMAN UMBILICAL CORD WHARTON'S JELLY MESENCHYMAL STEM CELLS

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By

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Mesenchymal stem cells (MSC) are defined as a non-haematopoietic multipotent stem cells with the ability to self-renew; capable of differentiating into various mature cells and exert potent immunomodulatory activities on immune cells. These unique characteristics and properties of MSC had enabled them to be recognised as an attractive tool in stem cell based immunotherapy, gene therapy and regenerative medicine especially for fatal or incurable diseases. To date, many studies have demonstrated that MSC can be generated from various organs and tissues, such as birth-associated tissues including placenta, amnion and umbilical cord. Umbilical cord derived MSC (UC-MSC) has long been considered as an alternative to MSC that derived from bone marrow (BM-MSC) as they are regarded as medical waste with little ethical concern for research and easily culture expanded. The human umbilical cord (UC) comprises of various compartments such as two arteries, one vein, amnion, and Wharton's jelly (WJ). Cells isolated from these compartments were found to be plastic adherent and able to differentiate into many cell types such as osteoblasts, adipocytes, chondrocytes, hepatocytes, neural and cardiac cells. Since MSC are migrating from foetal liver to the bone marrow at last trimester of pregnancy, thus the chances of MSC to be deposited at various compartments of umbilical cord will principally different too. Thus, the present study is aimed to determine a better anatomical part of umbilical cord which potentially yield a greater number and quality of MSC. In this present study, two distinguished anatomical parts of human umbilical cord Wharton's jelly were consumed to generate MSC. These are foetal distal end (FE) that connected to the new-born baby and the placental distal end (PE) which is adjacent to the placenta. Mesenchymal stem cells were generated from the explants method where Wharton's jelly of umbilical cord were minced up to paste-like state and cultured in the same condition. The generated WJ-MSC were characterised using a standard panel of surface markers and their ability to differentiate into osteoblasts and adipocytes followed by quantification of PCR gene expression. The immunomodulatory effect of MSC that generated from both ends were tested with mitogen activated T cells along with Jurkat cells through tritiated thymidine assay, cell cycle assay and apoptosis assay. The

possibility of generating WJ-MSC from both distal ends of umbilical cord was about 37-50%. The adherent cells generated from FE and PE distal ends have exhibited similar morphological characteristics, presented as spindle-shaped fibroblast-like cells. As for immunophenotyping, comparable data were observed where MSC from both ends are positive for common MSC markers (CD29, CD73, CD90, CD106 and MHC-class I), negative expression for haematopoietic and immune markers (CD14, CD34, CD45, CD80, CD86 and MHC-class II). However, colony-forming unit assay of FE-WJMSC showed more number of colonies and bigger in size as compared to the PE-WJMSC. Moreover, FE-WJMSC showed rapid growth kinetics and shorter doubling time with an average of 34 hr. The predominant measurements in osteogenic and adipogenic induction assays via deposition of calcium and accumulation of lipid granules respectively demonstrated both MSC possessed an equivalent differentiation potency. Real time qPCR analysis on adipocytes and osteoblast differentiated from both ends showed up-regulation and down-regulation of specific set of genes, thus further confirmed the lineage specific differentiations. In term of immunomodulatory activity, MSC generated from both ends were able to suppress the proliferation of activated T cells in dose dependant manner and preserved cell viability simultaneously. Moreover, detention of T cells in G_0/G_1 phase of the cell cycle and arresting cells from entering into S phase were evident when FE-WJMSC were co-cultured with T cells. Furthermore, co-culture of FE-WJMSC with Jurkat cells has significantly increased the number of viable cells and rescued the cells from undergoing apoptosis. The current study displays the efficiency of generating MSC from different anatomical parts of human umbilical cord where FE-WJMSC can be regarded as a better source of MSC as compared to PE as they showed superior proliferation capacity, greater anti-proliferative effect on immune cells and cell preservation ability from apoptosis. The higher expansion potential and enhanced functionality of FE-WJMSC at *in vitro* promise a quick and uninterrupted supply of MSC to encounter the clinical demand and research purposes.

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

KAJIAN PERBANDINGAN TERHADAP CIRI-CIRI BIOLOGI SEL MESENKIMA YANG DIPEROLEHI DARIPADA HUJUNG JANIN DAN HUJUNG PLASENTA DARIPADA WHARTON'S JELLY TALI PUSAT MANUSIA

Oleh

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Sel induk mesenkima (MSC) didefinasikan sebagai sel bukan hematopoisis (*non-haematopoietic*) multipotensi yang berkebolehan untuk membezakan dirinya menjadi pelbagai jenis sel matang yang lain dan menunjukkan kegiatan immunomodulatori ke atas pelbagai sel imun. Ciri-ciri yang unik ini telah menjadikan MSC diiktiraf sebagai agen yang menarik untuk rawatan berdasar kanser dalam bidang imunoterapi, gen terapi dan regenerasi terutama sekali untuk penyakit yang tidak boleh dirawat atau yang boleh menyebabkan kematian. Sehingga kini, banyak kajian telah menunjukkan bahawa MSC boleh dijana daripada pelbagai jenis organ dan tisu termasuklah tisu yang berkaitan dengan kelahiran seperti uri, amnion dan tali pusat. Sel induk mesenkima yang dijana daripada tali pusat (UC-MSC) sudah sekian lama dikenali sebagai altenatif kepada MSC yang dijana daripada sum-sum tulang (BM-MSC) kerana ianya dianggap sebagai sisa klinikal yang bebas dari etika perubatan dan senang diperkembangkan dalam kultur. Tali pusat manusia terdiri daripada beberapa bahagian iaitu dua arteri, satu vena, amnion dan *Wharton's jelly* (WJ). Sel yang diasingkan dari berlainan jenis bahagian tali pusat ini didapati boleh melekat pada permukaan plastik dan mampu membezakan diri menjadi sel lain seperti tulang, lemak, rawan, hati, neural dan jantung. Oleh kerana MSC semasa kehamilan bergerak dari hati janin ke sum-sum tulang pada *trimester* terakhir, ini bermakna secara prinsipnya, kebarangkalian untuk MSC terserap ke dalam pelbagai bahagian tali pusat juga berbeza. Oleh itu, kajian ini bertujuan untuk menentukan keberkesanan bahagian tali pusat yang berlainan anatomi untuk menjana MSC yang baik dari segi kuantiti dan kualiti. *Wharton's jelly* dari dua hujung tali pusat yang berbeza telah digunakan dalam kajian ini iaitu hujung janin (FE), bahagian yang bersambung dengan janin dan hujung plasenta (PE) iaitu bahagian yang bersambung kepada uri. Penjanaan MSC secara *explants* telah digunakan dimana WJ dipotong menjadi cebisan tisu yang kecil dan dikultur. Selepas penjanaan dan perkembangan WJ-MSC, ianya dicirikan mengikut *immunophenotyping* dan keupayaan pembezaan kepada osteosit dan adiposit, diikuti dengan kuantifikasi ekspresi gen

melalui analisa PCR. Kesan imunomodulatori MSC yang telah dijana dari kedua-dua hujung tali pusat diuji dengan sel T yang diaktifkan dengan mitogen melalui ujian proliferasi, kitaran sel dan apoptosis. Kebarangkalian untuk menjana WJ-MSC dari kedua-dua hujung tali pusat adalah sebanyak 37-50%. Penjanaan sel melekat dari FE dan PE menunjukkan morfologi fibroblas berbentuk gelondong yang sama cirinya. Bagi ujian penanda permukaan, kedua-dua hujung menunjukkan ekspresi positif untuk penanda yang biasa untuk MSC (CD29, CD73, CD90, CD106 and MHC-class I) ekspresi negatif untuk haematopoisis (CD14, CD34, CD45, CD80, CD86 and MHC-class II). Walaubagaimanapun, ujian *colony-forming unit* FE-WJMSC menunjukkan jumlah koloni yang lebih banyak dan saiz yang lebih besar dari PE. Tambahan lagi, FE menunjukkan kinetik pertumbuhan sel dan penggandaan populasi lebih pantas iaitu purata 34 jam. Induksi osteogenesis dan adipogenesis pula menunjukkan pengumpulan mineral kalsium dan granul lipid dimana kedua-duanya mempunyai potensi pembezaan yang sama. Analisa qPCR ke atas adiposit dan osteosit yang berjaya dibezakan dari kedua-dua hujung menunjukkan peningkatan dan penurunan regulasi bagi gen spesifik. Berhubung dengan aktiviti immunomodulatori, MSC dari kedua-dua hujung boleh merencat proliferasi sel T, berdasarkan nisbah MSC. Tambahan lagi, kehadhiran FE-WJMSC dalam kultur sel T telah memerangkap sel-sel imun ini pada fasa G_0/G_1 pada kitaran sel dan menahan sel dari memasuki fasa S. Penemuan ini telah menunjukkan penjanaan MSC dari tisu yang sama tetapi bahagian anatomi yang berlainan mempunyai kecekapan yang berbeza dimana FE-WJMSC boleh dianggap sebagai sumber MSC yang lebih baik berbanding PE kerana telah menunjukkan kapasiti pertumbuhan lebih baik, kesan anti-pertumbuhan terhadap sel-sel imun yang lebih berkesan dan lebih cekap untuk memelihara sel dari apoptosis. Potensi perkembangan yang lebih tinggi dan peningkatan fungsi yang dipamerkan oleh FE-WJMSC secara *in vitro* boleh menjanjikan pembekalan MSC yang lebih pantas dan tanpa ganguan bagi memenuhi permintaan untuk kegunaan klinikal mahupun kajian.

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I certify that a Thesis Examination Committee has met on (07 August 2015) to conduct the final examination of Zuraidah Bt. Yusoff on her thesis entitled "Comparative Study of the Biological Properties of Foetal and Placental Distal Ends of Human Umbilical Cord Wharton's Jelly Mesenchymal Stem Cells" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

µl	Micro litre
^{3}H-TdR	Tritiated thymidine
ABB	Annexin binding buffer
ALP	Alkaline phosphatase
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
APC	Allophycoerythrin
B7-1	CD80
B7-2	CD86
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
bp	Base pair
BSP	Bone sialoprotein
c/EBP	CCAAT-enhancer-binding protein
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CII	Type II collagen
CDKI	Cyclin-dependant kinase inhibitor
CFU	Colony forming unit
cm^2	Centimeter square
CO_2	Carbon dioxide
Col	Collagen type II
colla-1	Collagen type 1 alpha-1
cpm	Count per minute
CSCs	Cancer stem cells
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DD	Death domain
DN	Double negative
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMEMF12	Dulbecco's Modified Eagle's Medium Ham's F-12
DMSO	Dymethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DP	Double positive
E2F	Transcription factor E2F
EAA	Essential amino acid
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FADD	Fas-Associated protein with Death Domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate

Flt-3	Fms-like tyrosine kinase 3
FoxP3	Forkhead box P3
G_0	Quiescence phase
G_1	Gap phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GvHD	Graft versus host disease
HDMEM	High glucose Dulbecco's Modified Eagle's Medium
HLA-G	Human leucocyte antigen-G
HSC	Haematopoietic stem cells
ICAM-1	Intercelllular adhesion molecule-1
IDO	Indoleamine-2,3-dioxygenase
IGF	Insulin growth factor
IMDM	Iscove'c modified Dulbecco's medium
IFN-γ	Interferon gamma
IL	Interleukin
iPSC	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
LIF	Leukaemia Inhibitory factor
LPL	Lipoprotein lipase
M	Mitotic phase
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MI	Myocardial infarction
ml	Mililiter
MLR	Mixed leukocyte reaction
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
ng	Nanogram
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD	Programmed death
PDT	Population doubling time
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PHA-L	Phytohaemagglutinin-Leukocytes
PI	Propidium iodide
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	phosphatidylserine
rBM-MSC	Rat bone marrow mesenchymal stem cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
scf	Stem cell factor
SCID	Severe combined immunodeficient
SD	Standard deviation
SDF-1	Stromal cell derived factor 1
SLE	Systemic lupus erythematosus

TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th 1	T helper 1
Th 2	T helper 2
TNF-α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
Tregs	T regulatory cells
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
α	Alpha
β	Beta
γ	Gamma
δ	Delta

CHAPTER 1

INTRODUCTION

A substantial amount of research over the past two decades has resulted in greater understanding of the human adult stem cell biology not only in the basic sciences but also their implication in therapeutic usage. Following the initial enthusiasm, it is now becoming clear that the use of stem cell transplantation is an important tool in the treatment of various malignancies, tissue repair and tissue regeneration. Although embryonic stem cell and inducible pluripotent stem cells (iPS) hold great potentials toward an unlimited differentiation, yet their ethical cues and high risk of cancer formation hindered their further application in clinical settings. This has allowed further exploration for a safe adult stem cells to be used in regenerative medicine. Adult stem cells are multipotent stem cells, which can be isolated from postnatal tissues, most commonly from bone marrow. Examples of adult stem cells are haematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and other tissue resident stem cell types such as neural, epithelial, oval and satellite cells. Among them, mesenchymal stem cells appear to be an outstanding candidate of adult stem cells that possess multipotential differentiation ability and immuno-modulative activity.

Mesenchymal stem cell were first identified by a Russian scientist Friedenstein in 1970 as adherent cells that form fibroblasts-like colonies (Friedenstein *et al*. 1970). Since MSC were first discovered in bone marrow and co-exist with resident haematopoietic stem cells (HSC), it often address as non-haematopoietic stem cells. Besides that, MSC also known as well as mesenchymal stromal cells due to its contribution for stromal compartment to facilitate haematopoiesis. As per classical definition of adult stem cells, MSC comprise of a very rare population of stem cells that capable of self-renewal and lineage specific differentiation potential. It has been shown that MSC give rise to cells of mesodermal lineages such as bone, adipose, cartilage, tendon and skeletal muscle (Reyes *et al*. 2001; Pittenger *et al*. 1999; Fridenstein *et al*. 1974a). As a matter of fact, several reports have been shown that MSC also potentially differentiate into various non-mesodermal lineage tissues including pancreatic islet cell (Dang *et al*. 2015), cardiac muscle (Potdar and Prasannan 2013), hepatocyte (Kazemnejad *et al*. 2009) and neural cell (Kopen *et al*. 1999).

Various *in vitro* and preclinical studies based on normal and disease models had conferred the significant biological functions of MSC. Unlike other tissue specific adult stem cells, MSC hold a great potential to be considered as therapeutic agent due to its targeted homing capacity to the site of injuries and ability to differentiate into many different mesenchymal and parenchymal cell types thus, regenerate the damaged tissues or organs (Nagamura and He 2014; Kim *et al*. 2013). Mesenchymal stem cells considered as a 'magic healing cells' that secrete large amounts of pro-angiogenic, anti-inflammatory and anti-apoptotic cytokines/factors that exerts remarkable regenerative, reparative and immunosuppressive properties, which may play a role in the induction of transplantation tolerance and control of autoimmunity.

Historically, MSC derived from the bone marrow (BM) is considered as the most common source of MSC and designated as a gold standard as they have been used in most of the research and development for clinical therapies. However, the use of BM-MSC is not always acceptable or feasible due to many reasons such as high degree of viral exposures during BM aspiration, low yield of cell numbers and a significant reduction of proliferative and differentiation capacity with increasing age of the donor. Moreover, BM aspiration involves an invasive procedure and it may cause pain and discomfort to donors which may lead to infection, excessive bleeding and other complications. Undoubtedly, stem cell derived from adult bone marrow has very high successful rate for expansion, nonetheless many conditions may limit the accessibility. For instance, in some clinical cases such as bone marrow failure, aplastic anaemia, leukaemias and post-myeloblative irradiation or chemotherapy, patients often encounter complications of inadequate cellular fractions in their bone marrow aspiration. For these patients, the only option for alternative sources of MSC will be a third party donor and most of the time it is difficult to find the right donor especially if they are volunteers. Furthermore, the number of cells required for clinical scale is large, which requires longer time period for *in vitro* culture expansion to obtain the adequate number of cells. These technical hitches can delay the treatment process and further affect the outcome of the treatment. This predicament has initiated an urge for finding the alternative source of MSC and umbilical cord MSC certainly could serve as an ideal candidate.

The human umbilical cord (UC) comprises of two arteries, one vein, cord lining and Wharton's jelly (WJ). Previous reports have described that each of these compartments can give rise to MSC (Conconi *et al.* 2011). Cells isolated from these compartments were found to be plastic adherent and able to differentiate into many cell types such as osteoblasts, adipocytes, chondrocytes, hepatocytes, neural and cardiac cells (Prasanna and Jahnavi 2011). In fact, UC can be considered as a best alternative to BM as they are regarded as medical waste with little ethical concern for research (Hass *et al*. 2011). Moreover, a comparison study done on MSC derived from human umbilical cord revealed that they were better with respect to their proliferation capacity, rapid osteogenic differentiation and superior in transfection capacities when compared to the "gold standard" bone marrow mesencymal stem cells (Baksh *et.al* 2007). However, several reports have indicated that MSC generated from various compartments of UC vary in term of initial stem cell composition and frequency; the ability to form colony forming units (CFU) (Majore *et al*. 2010; Karahuseyinoglu *et al*. 2007; Sarugaser *et al*. 2005;).

In this present study, two distinguished anatomical parts of human umbilical cord Wharton's jelly were consumed to generate MSC. These two anatomical parts are foetal distal end (FE) of UC that connected to the new-born baby and the placental distal end (PE) of UC which is connected to the placenta. The determination of an appropriate distal end of umbilical cord to generate MSC which potentially yield greater quantity and better quality of MSC will validate a fundamental character of WJ-MSC from different anatomical parts of human umbilical cord. Moreover, the findings from this study will be potentially useful for cryo-banking and also economical in term of space and cost. Hence, the early phase of this study is focused on generation of MSC derived from FE(FE-WJMSC) and PE(PE-WJMSC), followed by a standard characterisation using panel of surface markers and the ability to differentiate into osteocytes and adipocytes. The immunosuppressive activity of MSC also was investigated. Finally, the generated

WJ-MSC from two distal ends were co-culture with T cells and Jurkat cells to further explore the immunomodulatory effect and rescuing ability of WJ-MSC from serum-deprived cell death.

General objective

To generate, characterise and compare the mesenchymal stem cells that derived from foetal and placental distal ends of human umbilical cord Wharton's jelly.

Specific objectives:

1. To generate MSC from foetal distal end (FE) and placental distal end (PE) of human umbilical cord Wharton's jelly.
2. To compare the growth kinetics of MSC that derived from foetal and placental distal ends of human umbilical cord Wharton's jelly.
3. To characterise MSC that generated from both FE and PE by a standard immunophenotyping and differentiation potential.
4. To compare the immunosuppressive activity of MSC derived from FE and PE of human umbilical cord Wharton's jelly.
5. To evaluate the protective ability of FE-WJMSC and PE-WJMSC on serum-deprived Jurkat cells.

The hypotheses of this research are:

1. Colony forming adherent cells can be generated from both distal ends of human umbilical cord Wharton's jelly.
2. Adherent cells generated from both distal ends are able to express MSC specific surface makers and differentiate into mesodermal lineage cells.
3. Mesenchymal stem cell derived from FE of human umbilical cord Wharton's jelly will serve as a better source for mesenchymal stem cell with respect to their proliferation capability, differentiation potential and immunosuppressive activity as compared to PE.

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