Bovine Adipose Tissue Derived Stem Cells Bioengineering: Isolation, Characterization and Differentiation Studies

by

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A thesis submitted in fulfilment of the requirements for the degree of
Master of Science Bioprocess Engineering

School of Bioprocess Engineering
UNIVERSITI MALAYSIA PERLIS
2013

UNIVERSITI MALAYSIA PERLIS

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Academic Session : 2012/2013

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ACKNOWLEDGEMENT

Alhamdulillah….All praise to Allah…for the strength and his blessing upon completing this thesis.

First and foremost, I would like to express my gratitude and appreciation to my Supervisor, Associate Professor Dr. Mohamed Zulkali Mohamed Daud, for his supervision, guidance and constant support in this research. I appreciate his time, opinions and academic comments and suggestions throughout the journey of completing this research.

My sincere appreciation also goes to my co-supervisor, Dr. Mukhzeer Mohd Shahimin. His continuous advice and encouragement throughout the research are very much appreciated and I am grateful to have learned his insights a lot. To my co-supervisor Profesor Dr. Ruszymah Hj Idrus, I would like to thank her for her support in many possible ways, guidance throughout experiments and the warm welcome of possible access to facilities available in Tissue Engineering Centre, UKM Medical Centre.

To Associate Professor Dr. Nurina Anuar, I sincerely would like to thank for her support, guidance and comments on the research. My appreciation also to her for allowing me to finished my research experiments in her laboratory in Cell Technology Laboratory, Faculty of Engineering and Built Environment, UKM.

To my husband Adly, children Afi, Dania, Rhea and families…thank you for all the support, understanding and encouragement. Life wouldn't be the same without them. The love of a family is life's greatest gift and blessing.

To staffs, friends and laboratory mates from School of Bioprocess UniMAP, Tissue Engineering Centre UKM Medical Centre, Cell Technology Laboratory UKM and Faculty of Pharmacy UKM…I cannot list all the name here, but I always remember all of your kindness, understanding and help throughout the research. With such a hearts of gold, may Allah blessed all of you…Thank you so much.

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LIST OF ABBREVIATIONS

ASCs	Adipose tissue derived stem cells
CFD	Computational Fluid Dynamic
DOE	Design of Experiment
EDTA	Ethylenediamminetetraacetate acid
EGF	Epidermal Growth Factor
ESCs	Embryonic stem cells
FTIR	Fourier Transform Infra-Red
bFGF	Fibroblast Growth Factor
$KHCO_3$	Potassium bicarbonate
GCI	Grid Convergence Index
MSCs	Mesenchymal stem cells
NH_4Cl	Ammonium chloride
SCNT	Somatic Cell Nuclear Transfer
SVF	Stromal vascular fraction
VOF	Volume of Fluid

LIST OF SYMBOLS

Quantity	Unit	Symbol
Density	Pa.s	ρ
Rotational speed	Rev/sec	N
Diameter magnetic bead	cm	D
Viscosity	kg/m^3	μ
Total number cell		N
Grid refinement factor		r
Wall shear stress		T_w
Dynamic viscosity		μ
Flow velocity parallel to the wall		u
Distance to the wall		y

Biokejuruteraan sel stem dari tisu lemak (adipos) bovin:
Isolasi, pencirian dan pengajian pembezaan

ABSTRAK

Terdapat potensi yang besar untuk sel stem dieksploitasikan bagi perubatan regeneratif. Sel stem bertindak sebagai sistem membaiki organ badan dengan dibezakan ke sel yang khusus dan menghasilkan semula sel-sel di dalam organ-organ regeneratif seperti kulit atau tisu usus. Terapi sel yang kini secara konvensional, sel-sel yang boleh dibaharui dan dibezakan ini kebanyakannya diperolehi dari sumber tulang sum-sum namun jumlah sel-sel stem mesenchymal (MSCs) yang dapat dihasilkan adalah rendah dan melibatkan prosedur yang menyakitkan di bawah bius. Di dalam bidang kejuruteraan tisu, biopemprosesan sel stem adalah penting, oleh itu sel stem daripada sumber tisu adipos telah ditemui sebagai sumber baru MSCs yang mempunyai harapan cerah. Sumber fraksi stroma vaskular (SVF) yang banyak memerlukan memerlukan alat biopemprosesan yang baik bagi mengoptimumkan jumlahnya. . Pengasingan ASCs lembu yang baik adalah dengan menggunakan collagenase 0,075% Type 1 dengan pergolakan selama 2 jam. Inokulasi sebanyak 1.0 x 10^4 di dalam plat 6 telaga adalah yang terbaik untuk kultur statik (2D). Bagi kultur pengampaian (3D), pengkulturan terbaik adalah pada 200rpm, 5ml DMEM: F12 / MesenPRO RS™ dan inokulasi 1.0 x 104 sel/ml in tiub 50ml. Kultur pengampaian (3D) berjaya meghasilkan sfera agregat sel dengan kepadatan 1.07 x 10^6 ASCs berbanding kultur statik (2D) iaitu sebanyak 5 x 10^5 ASCs secara purata. Walau bagaimanapun, bilangan sel hidup kultur statik adalah lebih baik daripada kultur pengampaian pada pergolakan tinggi yang mencadangkan bahawa parameter persekitaran yang tidak memihak kepada kultur sel di dalam tiub 50ml. Pembezaan osteoblast yang berjaya boleh dinilai menggunakan FTIR selain cara konvensional iaitu Van Kossa. Simulasi kultur pengampaian (3D) di bawah CFD menggunakan model VOF dapat membantu dalam membangunkan protokol kultur ASCs yang dioptimumkan vis-à-vis pengkulturan konvensional static (2D) dalam kelalang.

Bovine adipose tissue derived stem cells bioengineering: Isolation, characterization and differentiation studies

ABSTRACT

There is a significant potential for stem cells to be exploited for regenerative medicine. Stem cells act as a repair system for the body by differentiating into specialised cells and replenishing cells in regenerative organs such as skin or intestinal tissues. Conventionally in the current cell therapy, the renewed, differentiated cells were mostly sourced form bone marrow mesenchymal stem cell (MSCs) yielding low number of cells and involved a painful procedure under general anaesthesia. In tissue engineering, bioprocessing stem cells are crucial thus, stem cells from adipose tissue were revealed as a new promising source of MSCs. Abundant source of stromal vascular fraction (SVF) needs a good bioprocessing tools for an optimize culture. Isolation of bovine adipose tissue derived stem cells (ASCs) are found to better perform in 0.075% collagenase Type 1 and agitated for 2 hours from the experiment carried out. The static cultures were best inoculated at 1.0 x 10^4 cells/ml in 6 well plate. The suspension cultures were cultured best in 200rpm, 5ml DMEM: F12 / MesenPRO RS™ at 1.0 x 10^4 cells/ml in 50ml tube. Suspension (3D) culture of sphere aggregates yield cells density of 1.07 x 10^6 ASCs upon culture compared to static (2D) culture of 5 x 10^5 ASCs on average. However the viability of static culture are found to be more superior to suspension culture at high volume, agitation and inoculation sizes suggesting that the parameter are an unfavourable environment to culture in 50ml tubes. Successful differentiation of osteoblast cells were evaluated via Fourier Transform Infra-Red (FTIR) and conventional Van Kossa staining. Simulation of suspension (3D) culture via Computational Fluid Dynamic (CFD) using Volume of Fluid (VOF) model assist in developing an optimised ASCs culture protocol vis-à-vis conventional static (2D) culturing in flask.

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Mammalian cell cultures have given tremendous impact to the world of biotechnology per se. Monoclonal antibodies, viral vaccines, polypeptides growth factors, enzymes, hormones and immune regulators are among commercial biological product from mammalian cells that have the world.

In a world of medicine, biotechnology and sciences, finding an alternative ways of healing miscellaneous diseases and conditions are the highest priority. In the advance of technology, interests of finding a better substitution plan for the conventional treatments are overwhelmed. Challenges in the field of regenerative medicine are to direct cells, tissue and organ into replacement, replenished and restructure process. Regenerative medicine thus offers new cells, tissue and organ to prospective patients.

Stem cells have lead researches exploring many researches in the field of regenerative medicine. It is the most exciting topics due to their potential of regenerating new cells. General classification of stem cells is actually by its potency or ability to differentiate. Embryonic stem cells (ESCs) differentiation ability is pluripotent characterized by its ability to differentiate into all the three germ layers (ectoderm, mesoderm and endoderm) except extra embryonic tissue. While the multipotent adult stem cells have a restricted differentiation ability only to its cell sub lineage.

In the early discovery of cell culture, ESCs was the earliest stem cell discovery given most of research advantages. The protocols of isolation were widely available

however reports on clinical evidence in most papers have not been very strong as the papers described only numerous successful cases (Thomas & Moon, 2011; Assady et al., 2001; Wollert & Drexler, 2005; Cervantes, Stringer, Shao, Tischfield & Stambrook, 2002). Reasons discovered were the potential of the cells to change its DNA altering the cells into a potential tumour. Finding revealed that all the ESCs formed teratoma in all three germ layer origination from the inner cell mass (Zhu et al., 2009). It was also reported that the mutation event are lower in somatic cells or MSCs (Bosnakovski et al., 2005). The most successful research in ESCs was the Somatic Cell Nuclear Transfer (SCNT) procedure which is famous by the lamb named Dolly with initial purposed to make transgenic animal or for breeding (Derubeis, Pennesi & Cancedda, 2006). It was however have risen ethical issues in a few countries. With regards of future potential of stem cells, a few guidelines for scientist to conduct research on stem cells were implied.

Economically, the potential of stem cells was tremendously large. Besides regenerative medicine (tissue engineering and cell/gene therapy), other applications were for functional genomics study, biological process study, drug discovery development and cell based therapy.

In Malaysia, National Institute of Health and Ministry of Health has taken an initiative to publicly endorsed guideline for stem cell research and therapy to address emerging issues regarding research and clinical therapy using stem cells. Researchers were held to this guideline to protect from missed use, purpose and non-ethical handling of researches in stem cells.

The history of mesenchymal stem cell (MSCs) starts at year 1960's, where Mc Culloch and Till have first discovered the cells. It was revealed by the potential of mice hemopoietic tissue they cultured given rose surprisingly to millions of cells

(Siminovitch, McCulloch & Till, 1963). MSCs can be also from non hemopoietic source such as bone marrow stroma, periosteum, fat, umbilical cord, brain, skeletal muscle, skin etc (Neupane, Chang, Kiupel & Gurkan, 2008; Miller & Jee, 1987). Adipose tissue was the preferable depots for isolation of stem cells as the advantages of abundant of sources from patient, minimum pain resulting from anaesthesia and availability of isolation protocol. These factors had given much credit for adult stem cells for research. MSCs could be differentiated into various multiple cell lineages, such as adipocytes, myocytes, osteocytes, hepatocytes, chondrocytes, neurons, or astrocytes via appropriate cell culture stimulation and conditions by certain exogenous or endogenous (gene transfer) bioactive factors (Picou, 2007).

Realizing the potential of these cells, a few problems revealed upon the efforts to isolate, culture, expand the cells for further used. The current practice to harvest stem cells was from bone marrow traditional procurement procedure from iliac crest. The procedure had a few disadvantages as it was a painful procedure, involving general anaesthesia and yielding a low number of cells. Mononucleated hematopoietic cells must be expanded at least 2×10^9 in 7–10 days for adults during high-dose chemotherapy (Knocke & Vogt, 2008). Prolonged passage of MSCs more than 4 months might transform the cells in cancer cells (Mizuno, 2009). MSCs yield from bone marrow aspirate the highest at about 10^5 cells (Zuk et al., 2001). In another paper reported that a mean of 1.4-0.7 x 10^5 were yield from first passage from 1 x 10^6 of bone marrow mononucleated cells (Bakash, Song & Tuan, 2004). Pertinent report in a different study, suggested that MSCs yield from 1 gram adipose tissue was about 5 x 10^3 stem cells (Mizuno, 2009). There were varies in the number of MSCs yield which may be due to the amount of samples, quality of samples and methods of culturing.

These numbers of cells would be sufficient for personal treatment but however were insufficient for the purpose of tissue engineering.

The standard culturing methods of stem cells were using common mammalian cells anchorage dependent culture methods. Cells were grown in small scales using T-flask and 6 wells plate created variability in cultures due to consumption of many flask and plates to achieve desirable number of cells. Culturing in bigger static flask volume (e.g 235cm^2 T-flask) is still a tedious process and produce insufficient numbers of cells and longer culture time. Although MSCs survived in long term culture with retained pluripotency and high yield (more than 10 times in a week) in 2D culture (Wagner et al., 2005), another problems risen where there was only one culturing mode (batch mode). This created difficulty in monitoring and controlling factors affecting the cultures. Thus, possibilities for scaling up were narrow as many parameters were not measured and unknown.

Suspension culture was later introduced to encounter previous culture problem. Suspension culture is an anchorage independent culture. Cells were grown without support or form single spheres or aggregates of cells. This creates a possibility of scaling up in bioreactor where it facilitates cells to grow in large number for tissue engineering purpose or termed bioprocessing. Bioprocessing of stem cells is a cultivation of cells in a bioreactor with controlled environment in order to harvest consistent, reproducible and maximize amount of cells. Unknown culture parameter in bioreactor can be determined later by an optimization process.

Bioreactor volumes for individual patient treatment may ranges from 1 – 3 litres. There was limited knowledge on the parameters for up scaling stem cells in bioreactor. Screenings parameter for purpose of scaling up using big bioreactor is expensive and

requires more time. Culturing in stirrer flask from minimum 125ml -500ml for autologous stem cell harvesting from one patient for purpose of cell therapy or tissue engineering is another option for up scaling. However for screening process in this study, it was suggested using 50ml centrifuge tubes with magnetic stirrer as it was more economical and easy alternative of culturing bovine ASCs in suspension.

To suspend cells in bioreactor is by using micro carrier. The disadvantages however, was the cost of culturing will be more expensive and adding another step in the procedure. This would complicate and delayed the culturing process. Thus, an inspiration from neurosphere assay (for culturing neuron stem cells anchorage independently) would facilitate all the possibility of having consistent, reproducible and high quality stem cell (maintaining the phenotype) at the end of 1 week culture. The neurosphere assay would help cells to form spheres and floats in the medium independently.

1.2 SCOPE & OBJECTIVES

In this research, the objectives were 1) to identify isolation process of mesenchymal stem cells (MSCs) from bovine adipose tissue. 2) to characterize the isolated bovine ASCs and differentiated osteoblast cells. 3) to study the *in vitro* differentiation of bovine ASCs into osteogenic lineage 4) to compare cell growth in static culture (2D) and suspension 50ml culture (3D) of bovine ASCs with minor objectives of i) to determine the cell density and viability in static culture and suspension culture at day 7 ii) to identify flow pattern and hydrodynamic shear via simulation in CFD. The hypothesis of the research was 1) Bovine ASCs grow in suspension yield higher number of cells and high viability than in static.

The working strategy was by culturing from fresh bovine adipose (primary culture) into six well plate later transfers into 25cm^3 T-flask and 75cm^3 T-flask for static culture and into 50ml centrifuge tube with magnetic stirrer for suspension culture. The cells were passage when confluent and media was changed when needed. After 7 days of culture, cells were harvested for counting the density and viability determination. The 7 day old cells were then differentiated into osteoblast cells by changing the media. After 14 days, cells were harvested for staining with Van Kossa and for determining the carbonate and phosphate peak using FTIR. Computational Fluid Dynamic (CFD) software FLUENT and assisted with GAMBIT was used for fluid dynamic simulation of the culture media in the 50ml centrifuge tubes.

1.3 THESIS STRUCTURE

This thesis is organized in 5 chapters. Chapter 1 introduced the main topic or subject in this research which was stem cells. It addressed the problem statement, objective and scope of research. Once the goals had been set, working strategies guidelines were lay out.

Chapter 2 revealed knowledge backgrounds for further understanding on the topic discuss. Starting with background on definition and properties of stem cells, a comprise information on advantages of culturing ASCs were revised. The applications of ASCs in regenerative medicine specifically in bone engineering were also discussed following the comparison on between static and suspension culture. Hydrodynamic of cells in suspension culture were included and some revision on types of small bioreactor available in market were compared. Due to this is a first time effort to culture bovine ASCs, a concise review on process studies includes isolation, characterization and