UNIVERSITI PUTRA MALAYSIA

SKELETAL MUSCLE TISSUE ENGINEERING USING BIOLOGICAL SCAFFOLDS FOR REPAIR OF ABDOMINAL WALL DEFECTS IN A RABBIT MODEL

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By

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

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SKELETAL MUSCLE TISSUE ENGINEERING USING BIOLOGICAL SCAFFOLDS FOR REPAIR OF ABDOMINAL WALL DEFECTS IN A RABBIT MODEL

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October 2009

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Abdominal wall defects caused by trauma, tumour ablation, muscle deficiency or postsurgical loss of muscle mass may lead to the need for restoration of damaged muscle tissues as loss of functional muscle tissue could cause severe impairments of the functionality of skeletal muscle. Hence, the present study was aimed mainly to engineer skeletal muscle tissue using myoblast seeded bovine pericardium (BP) and bovine tunica vaginalis (BTV) scaffolds in a rabbit model. Myoblast were harvested successfully from soleus muscles of 5-day-old male White New Zealand rabbit and based on the purity test using immunocytochemistry (desmin staining) and flow cytometric analysis, more than 97% of the isolated skeletal myoblast have got myogenic phenotype. Myoblast were labelled with PKH26-fluorescent dye and seeded onto the scaffolds and incubated *in vitro* for 5 days. The *in vitro* findings of myoblast-seeded BP and BTV scaffolds

suggest that myoblast harvested from primary culture are able to form myotube on both types of scaffolds and these naturally derived collagen-based scaffolds showed a tremendous potential for *in vitro* cultivation of skeletal muscle that can be used as substrate for filling of wound bed or for the delivery of cells. A total of thirty-six male New Zealand white rabbits which were divided into two groups (BP and BTV groups) of eighteen rabbits each were used in this study. The rabbits in each group were further subdivided into two groups of nine rabbits each: the treatment groups (I and II) and control groups (III and IV). Myoblast seeded-BP and myoblast seeded-BTV scaffolds were implanted on the artificially created 3 x 4 cm^2 defects at mid-ventral abdominal wall on nine rabbits of the treatment groups I and II, respectively. Whereas, control groups III and IV were repaired with non-seeded BP and BTV scaffolds, respectively. Three rabbits from each group were euthanized at 7^{th}, 14^{th} and 30^{th} days of post-implantation and their ex-implanted specimens were examined macroscopically and microscopically. Macroscopic examination of the abdominal wall post-implantation showed no evidence of herniation, signs of rejection and infection in both treatment and control groups of both type of scaffolds. However, 33.33% and 22.22% mild type of adhesion were found in the control groups III and IV, respectively. Whereas, 11.11% mild type of adhesion and absence of adhesion were found in the treatment groups I and II, respectively. At 7^{th} day of post-implantation, microscopic examinations

revealed more intense infiltration of granulocytes and macrophages in the treatment and control groups of both types of scaffolds. Whereas, on 14th and 30th days of post-implantation, the fibroblast migration, deposition of newly-formed collagen, neovascularisation and skeletal muscle cells ingrowths were detected in the treatment groups: I and II. However, not a single of skeletal-muscle cell were found in the control groups III and IV. In conclusion, this study demonstrated that myoblast seeded BP and BTV can be successfully transplanted into abdominal wall defects and resulted in the regeneration of skeletal muscle tissue.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KEJURUTERAAN TISU OTOT SKELET MENGGUNAKAN PERANCAH BIOLOGI UNTUK MEMPERBAIKI KECACATAN PADA DINDING ABDOMEN DALAM MODEL ARNAB

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Kecacatan dinding abdomen akibat kesan trauma, pembuangan tumor, kelemahan otot atau kehilangan otot pasca pembedahan boleh membawa kepada keperluan untuk pemulihan tisu otot yang tercedera kerana kehilangan fungsi tisu otot akan menyebabkan ketaksempurnaan yang teruk pada kefungsian otot skelet. Oleh yang demikian, kajian ini dijalankan bertujuan untuk menjurutera tisu otot skelet menggunakan perancah pericardia lembu (BP) dan tunika vaginalis lembu (BTV) yang disemai dengan mioblas dalam model arnab. Mioblas telah berjaya diperolehi dari otot soleus yang diambil dari arnab New Zealand White yang berusia 5 hari dan berdasarkan ujian ketulenan menggunakan imunositokimia (pewarnaan desmin) serta analisis aliran sitometri, didapati lebih dari 97% otot mioblas yang dipencilkan mempunyai fenotip miogen. Mioblas telah dilabel dengan pewarna pendarfluor PKH26 dan disemai pada perancah dan dieram secara

in vitro selama 5 hari. Penemuan dari kajian *in vitro* perancah BP dan BTV yang telah disemai dengan mioblas menunjukkan bahawa mioblas yang diperolehi dari kultur primer berkebolehan membentuk miotiub pada kedua-dua jenis perancah dan perancah yang terhasil secara semulajadi dari kolagen ini menunjukkan potensi yang besar untuk implantasi *in vitro* otot skelet yang boleh digunakan sebagai substrat untuk mengisi dasar luka atau untuk pembawaan sel. Sejumlah tiga puluh enam ekor arnab jantan New Zealand white telah dibahagikan kepada dua kumpulan yang terdiri daripada lapan belas ekor arnab setiap kumpulan telah digunakan untuk perancah BP dan BTV. Arnab dalam setiap kumpulan seterusnya dibahagikan kepada dua kumpulan (kumpulan rawatan: I dan II dan kumpulan kawalan: III dan IV) yang masing-masing terdiri daripada sembilan ekor arnab. BP dan BTV yang telah disemai dengan mioblas masing-masing telah diimplan pada kerosakan buatan berukuran 3 x 4 cm^2 pada bahagian tengah-ventral dinding abdomen sembilan ekor arnab dalam kumpulan I dan II. Manakala, kumpulan rawatan III dan IV masing-masing telah dirawat dengan perancah tanpa semaian. Tiga ekor arnab dari setiap kumpulan telah dikorbankan pada hari ke-7, 14 dan 30 selepas implantasi dan specimen eksplan tersebut telah dikaji secara makroskopi dan mikroskopi. Kajian makroskopi terhadap dinding abdomen selepas implantasi menunjukkan tiada bukti berlakunya hernia, tanda-tanda penolakan dan jangkitan pada kumpulan rawatan dan kawalan untuk

kedua-dua jenis perancah. Walau bagaimanapun, 33.33% dan 22.22% pelekatan sederhana dijumpai masing-masing dalam kumpulan III dan IV. Manakala, 11.11% pelekatan sederhana dan ketiadaan pelekatan masing-masing dijumpai dalam kumpulan rawatan I dan II. Pada hari ke-7 selepas implantasi, kajian mikroskopi menunjukkan penyusupan granulosit dan makrofaj yang lebih ketara dalam kumpulan rawatan dan kawalan bagi kedua-dua jenis perancah. Manakala, pada hari ke-14 dan 30 selepas implantasi, penghijrahan fibroblast, pengendapan kolagen yang baru terbentuk, neovaskularisasi dan pertumbuhan otot skelet telah dikesan dalam kumpulan rawatan I dan II. Namun begitu, tiada satu pun sel otot skelet yang dijumpai dalam kumpulan rawatan III dan IV. Kesimpulannya, kajian ini telah membuktikan bahawa BP dan BTV yang telah disemai dengan mioblas boleh digunakan sebagai transplan pada dinding abdomen yang mengalami kecacatan dan membawa kepada penjanaan semula tisu otot skelet.

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I certify that a Thesis Examination Committee has met on 26 October, 2009 to conduct the final examination of Ayele Taddese Tsedeke on his thesis entitled "Skeletal Muscle Tissue Engineering Using Biological Scaffolds for Repair of Abdominal Wall Defects in a Rabbit Model" in accordance with Universities and University Collages 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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The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 14 January 2010

DECLARATION

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

Ayele Taddese Tsedeke

Date**:**

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LIST OF ABBREVIATIONS

BP	Bovine Pericardium
BSA	Bovine Serum Albumin
BTV	Bovine Tunica Vaginalis
cm	Centimeter
^{o}C	Degree Celcius
DMSO	Dimethyl sulphoxide
DPX	Di-n-butylPhthalate in Xylene
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethlenediaminetetraacetic acid
FVM	Faculty of Veterinary Medicine
FITC	Fluorescein Isothiocyanate
g	Gram
hrs	Hours
H& E	Haematoxylin & Eosin
pH	Hydrogen Ion Concentration
KGy	Kilo Gray
kV	kilo Voltage
MINT	Malaysian Institute of Nuclear Technology
µg	Microgram

µl	Microliter
µm	Micrometer
mbar	Millibar
ml	Millilitre
mM	Millimolar
nm	Nanometre
OCT	Optimum Cutter Temperature
%	Percent
PBS	Phosphate Buffered Saline
rpm	Revolution Per Minute
SEM	Scanning Electron Microscopy

CHAPTER ONE

GENERAL INTRODUCTION

Tissue engineering is an interdisciplinary field which applies the principles and methods of engineering and the life sciences towards the fundamental understanding of structural and functional relationships in normal and pathological tissue and the development of biological substitutes to restore, maintain or improve function (Skalak and Fox, 1988).

The creation of skeletal muscle tissue using tissue engineering methods holds promise for the treatment of a variety of muscle diseases, including skeletal myopathies such as muscular dystrophy or spinal muscular atrophy, traumatic injury and aggressive tumor ablation (Guettier-Sigrist *et al*., 1998; Law *et al*., 1993). Tissues that are engineered using the patient's own cells, or immunologically inactive allogenic or xenogenic cells have the potential to overcome current problems of replacing lost tissue function and offer new therapeutic options for diseases where currently no options are available. Moreover, this technology can play a vital role in the future management of paediatrics patients (Saxena *et al*., 1999a).

In general "Tissue engineering" refers to the science of creating living tissue to replace, repair or augment diseased tissue. The engineered tissue may be created *in vitro* and subsequently implanted into the patient or the tissue may be created entirely *in vivo*. Regardless of the technique, tissue engineering requires at least three components: a growth-inducing stimulus (induction), responsive cells (production), and a scaffold (biomaterials) to support tissue formation (Bronzino, 2006).

Biomaterials are any material used to make devices to replace a part or a function of the body in a safe, reliable, economic and physiologically acceptable manner (Hench and Erthridge, 1982). The use of biomaterial for repair of abdominal wall defects is gaining increasing recognition and the use of biomaterials to achieve a tension–free repair has resulted in a significant reduction in post-operative pain, length of recovery period and the number of recurrence (Amid, 1997).

Currently there is an increasing demand for cheap and ideal biomaterials which can be used in reconstructive surgery for repair of traumatic wounds suffer during war, traffic accidental and natural disaster and in the restore of the functions of diseased tissues or organs. Biomaterials are either synthetic (prosthesis) such as ceramic, polymeric and composite or biologic (bioprosthesis) such as heart valve, skin and other types of tissue graft (Black,

1992). The ideal biomaterials for abdominal wall repair should possess adequate strength, no hypersensitivity reactions and biocompatibility to facilitate tissue ingrowths, which may help long term maintenance of mechanical strength (Lai *et al*., 2003).

Recently, new biodegradable biomaterials developed from biological materials mainly of collagen in nature have been tested for repair of body wall instead of the non-biodegradable synthetic materials. BP, human cadaveric fascia lata, human dura mater and collagen-based materials derived from porcine small intestine submucosa have been investigated for reconstruction of abdominal wall defects (Ueno *et al*., 2004; Saaverda *et al*., 2001; Santillan *et al.* 1995; Rodgers *et al.,* 1981). However in most research, it is indicated that these collagen based biomaterials are failed to be replaced by skeletal muscle tissue or regeneration of muscle tissue is not observed as whole therefore optimal muscle recovery or regeneration may require the use of novel technology like tissue engineering.

Skeletal muscle comprises approximately 48% of the body mass and is responsible for voluntary control and active movement of the body. Application of tissue engineering techniques and successful fabrication of skeletal muscle mass holds now a promising future for the restoration of 3-dimentional contour as well as the loss of function for the affected part of the

body. In order to generate skeletal muscle tissue, myoblasts which are skeletal muscle tissue precursors, have been employed (Saxena, 2005).

One of the strategies for muscle tissue engineering involves the harvesting of satellite cells, their expansion *in vitro*, and their subsequent autologous implantation *in vivo* into the sites requiring repair or replacement. One of the main obstacles in the formation of new muscle tissue is the lack of an adequate support for expanded satellite cells. To overcome this obstacle, many researcher groups are trying to develop adequate synthetic and biological delivery systems for implanted cells (Conconi *et al.,* 2005).

Currently myoblast transplantations have been predominantly performed by injection of myoblast cell suspensions into mature skeletal muscle. These single cells have been shown to fuse with the host myofibers (Wernig *et al*., 2000). Saxena *et al.* (1999b) were the first to implant successfully *in vitro* cultured myoblasts into a non-muscular environment. Their group used a polyglycolic acid (PGA) mesh as a scaffold for skeletal muscle cells (Saxena *et al.*, 2001; Saxena *et al.,* 1999b). Myoblasts have also been seeded onto polyglycolic acid porous polymers with successful generation of vascularized new skeletal muscle *in vivo* (Saxena and Willital, 2000).

Synthetic materials, such as Dacron and Polytetrafluorethylene, have been used to repair congenital muscles defects, e.g. Onfalocele and gastrochisis (Bauer *et al.,* 1999; Calzolari *et al*., 1995; Meddings *et al.,* 1993). However, all of these materials do not allow cell growth and do not follow host development. Evidence has been provided that biological materials can support *in vivo* and *in vitro* cell adhesion and proliferation.

Bovine pericardium has been used as source of natural biomaterials for a wide range of clinical applications (Jose *et al.,* 2001; Won *et al*., 2000; Marques *et al*., 1995). However, few clinical data are available in current literature about grafting of BTV for surgical use, although up to 10x7cm or larger collagen rich sheet of BTV can be obtained from a testis of adult cattle. Naturally derived materials, including glutaraldehyde tanned BP (James *et al.,* 1991), small intestine submucosa (Clarke *et al.*, 1996; Prevel *et al.,* 1995) and also lyophilized and glycerolized BP and BTV (Hafeez, 2005), have been tried in animal models. These biomaterials are less susceptible to infection and cause less foreign body response (Badylak *et al.,* 1998; Hiles *et al.,* 1995). Thus, the utilization of non-edible bovine offal's of collagenous nature such as BP and BTV for the development of cheap and safe surgical patches for clinical use will be of economical importance in developing countries. However, fail to recover muscle tissue and also lack of strength over time is a concern for clinical application in which adequate tensile properties are

necessary. Thus, for this reason, it is important to understand not only the biological response to degradable biomaterials, but also the expected mechanical properties of implant and replacement of tissue over time. These new collagen based biomaterials has to be improved its morphological and biomechanical properties just by seeding it with myoblast cells and must be evaluated first in animals' model before being approved for test in human.

It was hypothesized that seeding of myoblast on the scaffold facilitates better tissue regeneration and tissue ingrowths, and also helps to enhance regeneration of skeletal muscles. Therefore, the general objective of this study was to engineer skeletal muscle tissues for reconstructive surgery of abdominal wall defects.

The specific objectives of the study were to:-

i. prepare biomaterial scaffolds from bovine parietal pericardium and bovine parietal tunica vaginalis.
ii. isolate of myoblast from primary cell culture.
iii. proliferate and differentiate myoblast onto the scaffolds and to evaluate their *in vitro* morphology.
iv. evaluate myoblast-seeded and non-seeded scaffolds post-implantation in rabbit model.

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