UNIVERSITI PUTRA MALAYSIA

CLINICAL, MECHANICAL AND HISTOLOGICAL EVALUATION OF ANTIFREEZE PEPTIDE TYPE 1M CRYOPRESERVED SKIN IN TISSUE TRANSPLANTATION

SAHAR MOHAMMED IBRAHIM

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By

SAHAR MOHAMMED IBRAHIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

November 2017

DEDICATION

I would like to dedicate this study with love and gratitude

To,

The soul of my beloved Mother may Almighty “Allah” bless her who was the candle and the motivation that gave me strength to continue in spite of everything to get this certificate and make her proud of me

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Loqman Haji Mohamad Yusof, PhD
Faculty : Veterinary Medicine

Cryopreservation of tissues and cells has witnessed a magnificent attention in the scientific society since 1949, when Polge had discovered the cryoprotective properties of glycerol to preserve sperm. Later with the progress of using of autogenic and allogeneic tissues, the need for developing new compounds and techniques for the cryopreservation of living tissues is increasing but the freezing damage represent one of the biggest challenging issues facing the maintenance of functional and structural integrity of living tissues after cryopreservation. All approaches for cryopreservation aim to overcome the biological, chemical, mechanical and thermal stresses of ice crystal formation and re-crystallization associated with subzero cryopreservation. The objectives of our study is to establish a cryopreservation technique to preserve living tissues by using antifreeze peptide type 1m (Afp1m) and to evaluate its ex vivo in vivo and in vitro effects on the mechanical, clinical, and histological properties of skin grafts by using different microscopic imaging techniques. This study was able to determine the effects of freezing on skin samples, skin fibroblasts (*M.dunni* clone (1118C), and skin grafts cryopreserved with Afp1m in different concentrations (3 and 5 mg/ml; at -4 and/or -8 ^{o}C for up to 72 hrs). Histological and immunohistological examination of ex vivo skin samples showed that skin graft cryopreserved in 5mg/ml at 24, 48 and 72 hrs. have the best expression of TGF and VEGF and also have the best preserved morphological appearance as compared to 3mg/ml and control (80% glycerolized tissue) at -4 and -8 ^{o}C. Cell viability studies showed that the viability of skin fibroblasts cryopreserved in 5mg/ml at 24, 48 and 72 hrs. was better as compared to 3mg and control at -4 and -8^{o}C. Cell toxicity assessment indicates that Afp1m do not have toxic effects on skin fibroblasts growth. While the level of DNA damage in skin fibroblast

cryopreserved with AFP was higher at -8 °C especially at lower concentration as compared to -4 °C .There was significant difference (P <0.05) between DNA damage at two temperatures, the level of DNA damage in cell cryopreserved with 3 mg/ml of AFP was higher than those cryopreserved with 5 mg/ml of AFP at -8 °C. The in vivo study was performed on 24 rats with 208 ± 31 g body weight (mean ± SD), and age eight- weeks old. Rats (n=24) were divided into four groups. One-two circular full-thickness 1.5-2.5 cm diameter wounds were created on the backs of rats. Non-cryopreserved and cryopreserved auto skin grafting were placed onto the wound area and stitched. Grafts were bandages. Wounds were monitored macroscopically and evaluated clinically at days 5, 8, 11, 14, 17, 20 and 22 post-operation. All skin grafts were subjected to histological and mechanical examinations at the end of experiment, while blood samples were collected from all rats at week one and at end of experiment for toxicity analysis. The clinical results showed a significant difference (P <0.05) among 4 groups in pliability of skin grafts ,adherence to the underling bed, and in the color of skin grafts (P <0.05), (P <0.05), and (P <0.05) respectively. The results of mechanical properties showed no significant difference (P >0.05) among the 4 experimental groups in maximum load, tensile stress at maximum load, tensile strain at maximum load, and elasticity (P >0.05), (P >0.05), (P >0.05), and (P >0.05) respectively .However the results of non transplanted skin strips cryopreserved in glycerol, Afp1m showed significant difference (P <0.05) in maximum load only. The histological results showed a significant difference among 4 groups in epidermal integrity, dermal-epidermal junction (P <0.05) and (P <0.05) respectively. The results of liver enzymes ALT & AST and total bilirubin showed no significant difference between weeks 1 and 3 (P > 0.05), (P >0.05), and (P >0.05) respectively and among the 4 experimental groups as compared to normal values (P >0.05), (P >0.05), and (P >0.05) respectively. In conclusion, Afp1m was found beneficial that could serve as an efficient agent for skin grafts cryopreservation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN KLINIKAL, MEKANIKAL DAN HISTOLOGI KE ATAS PENGGUNAAN KULIT DIKRIOAWET PEPTIDA BAHAN ANTIBEKU JENIS 1M DI BIDANG PEMINDAHAN TISU

Oleh

SAHAR MOHAMMED IBRAHIM

November 2017

Pengerusi : Loqman Haji Mohamad Yusof, PhD
Fakulti : Perubatan Veterinar

Krioawetan tisu dan sel telah menyaksikan perhatian yang semakin meningkat di kalangan masyarakat saintifik sejak 1949, apabila Polge menemui sifat krioperlindungan gliserol untuk mengawet sperma. Kemudian dengan kemajuan penggunaan tisu autogenik dan allogenik, keperluan untuk membangunkan kompaun-kompaun dan teknik-teknik baru untuk krioawetan tisu hidup telah meningkat tetapi kerosakan akibat dari pembekuan merupakan salah satu isu yang paling besar dan mencabar yang dihadapi oleh pemeliharaan integriti fungsi dan struktur tisu hidup selepas krioawetan. Semua pendekatan krioawetan bertujuan untuk mengatasi tekanan biologi, kimia, mekanikal dan terma oleh pembentukan hablur ais dan penghabluran semula berkaitan dengan krioawetan sub-sifar. Objektif kajian kami adalah untuk mewujudkan satu teknik krioawetan untuk memelihara tisu hidup dengan menggunakan bahan antibeku jenis peptida 1m (Afp1m) dan untuk menilai kesannya secara ex vivo, in vivo dan in vitro ke atas sifat mekanikal, klinikal dan histologi cantuman kulit dengan menggunakan teknik pengimejan mikroskopik yang berbeza-beza. Kajian ini telah dapat menentukan kesan pembekuan ke atas sampel kulit, sel-sel fibroblast kulit (M. Dunni (Clone (1118C), dan cantuman kulit dikrioawet dengan Afp1m dengan kepekatan yang berbeza (3 dan 5 m/ml; di paras -4 dan/atau -8 ^{o} C sehingga 72 jam). Pemeriksaan histologi dan imunohistologi contoh kulit in vivo menunjukkan bahawa cantuman kulit dikrioawet di paras 5 mg/ml pada 24, 48, dan 72 jam mempunyai keamatan terbaik TGF dan VEGF dan juga mempunyai penampilan morfologi yang terbaik dipelihara berbanding dengan 3 mg/ml dan kawalan (80% tisu tergliserol) di suhu -4 dan -8 ^{o}C. Penilaian ketoksikan sel menunjukkan bahawa Afp1m tidak mendatangkan kesan toksik ke atas tumbesaran fibroblast, manakala paras kerosakan DNA di fibroblast kulit

dikrioawetkan dengan Afp1m adalah lebih tinggi di suhu -8 ^{o}C terutama dengan kepekatan yang lebih rendah berbanding dengan -4 ^{o}C. Ada perbezaan yang bermakna (P <0.05) di antara kerosakan DNA di kedua-dua suhu ini; paras kerosakan DNA pada sel-sel dikrioawetkan dengan 3 mg/ml Afp1m lebih tinggi berbanding dengan yang dikrioawetkan dengan 5 mg/ml Afp1m dengan suhu -8 ^{o}C. Kajian in vivo telah dilakukan ke atas 24 tikus dengan 208 ± 31 g berat badan (min ± SD), dan usia lapan minggu. Tikus (n = 24) telah dibahagikan kepada empat kumpulan. Satu hingga dua luka bulat berdiameter 1.5-2.5 cm berketebalan-penuh telah diwujudkan di belakang tikus-tikus tersebut. Cantuman kulit auto tidak-dikrioawet dan dikrioawet ditempatkan ke kawasan luka dan dijahit. Cantuman dibalut. Luka-luka dipantau secara makroskopik dan dinilai secara klinikal di hari ke 5, 8, 11, 14, 17, 20 dan 22 pasca operasi. Semua cantuman kulit tertakluk kepada pemeriksaan histologi dan mekanikal pada penghujung eksperimen, manakala sampel darah diambil dari semua tikus di minggu pertama dan pada akhir eksperimen untuk analisis ketoksikan. Keputusan klinikal menunjukkan perbezaan yang signifikan (P <0.05) di kalangan 4 kumpulan itu dari segi sifat kelenturan cantuman kulit itu, pelekatan kepada permukaan dasar, dan dari segi warna cantuman kulit (P < 0.05), (P <0.05), dan (P <0.05) masing-masing. Keputusan untuk sifat mekanikal tidak menunjukkan perbezaan yang signifikan (P >0.05) antara 4 kumpulan eksperimen itu dari segi beban maksimum, tegasan tegangan pada beban maksimum, terikan tegangan pada beban maksimum, dan keanjalan (P >0.05), (P >0.05), (P >0.05), dan (P >0.05) masing-masing. Walau bagaimanapun bagi keputusan jalur kulit tidak-dipindahkan yang dikrioawet dalam gliserol, Afp1m menunjukkan perbezaan yang signifikan (P <0.05) untuk beban maksimum sahaja. Keputusan histologi menunjukkan perbezaan ketara di kalangan 4 kumpulan itu dari segi integriti epidermis dan simpang dermis-epidermis (P <0.05) dan (P <0.05) masing-masing. Keputusan enzim hati ALT dan AST dan jumlah bilirubin menunjukkan tiada perbezaan yang signifikan antara minggu 1 dan 3 (P >0.05), (P >0.05), dan (P >0.05) masing-masing di kalangan 4 kumpulan eksperimen berbanding dengan nilai-nilai normal (P >0.05), (P >0.05), dan (P >0.05) masing-masing. Kesimpulannya, Afp1m didapati bermanfaat dan boleh bertindak sebagai agen yang berkesan untuk krioawetan cantuman kulit.

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Loqman Haji Mohamad Yusof, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohd Basyaruddin Bin Abdul Rahman, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Noordin B Mohamed Mustapha, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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LIST OF ABBREVIATIONS

°C	Degree centigrade
%	Percentage
>	Greater than
<	Lesser than
&	And
α	Alpha
β	Beta
µm	Micro meter
µg	Microgram
µl	Microliter
Cm_2	Square centimeter
ml	Milliliter
mm	Millimeter
mM	Millimolar
nm	nanometer
AATB	American Association of Tissue Banks
AD	Adipose tissue integrity
AFGP	Antifreeze glycopeptides
AFP	Antifreeze Proteins/peptides
AFP I	Antifreeze Protein type I
AFP II	Antifreeze Protein type II
AFP III	Antifreeze Protein type III
AFP IV	Antifreeze Protein type IV

AK	Adenylate Kinase
Ala-Ala-Thr	Alanine-Alanine-Threonine
ALT	Alanine aminotransferase
ACUC	Animal Care and Use Committee
ARP	Aldehyde Reactive Probe
AP site	Apurinic/apyrimidinic site
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate.
ANOVA	Analysis of variance
BL	Ballooning of the epithelial cells
BC	Bowman's capsules
BV	Blood vessels
Ca^{2+}	Free Calcium
CFSE	Carboxyfluorescein diacetate N-succinimidyl ester
CFB	Connective tissue fibres breakage
CM	Cardiac Myocytes fibres
CN	Central Nuclei of myocytes fibres
CO_2	Carbon dioxide
CPAs	Cryoprotectant agent/agents
DAB	3,3'-Diaminobenzidine
DGL	Dermal glands appearance
DEJ	Dermo -epidermal junction
DIS	Dynamic ice shaping
DCT	Distal Convoluted Tubules

DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DK	De-keratinization
DPX	Distyrene, a plasticizer, and xylene used as a Synthetic resin mounting media
dH_2O	Double distal water
EAFS	EG-acetamide-Ficoll-sucrose
EI	Epithelial integrity
EDTA	Ethylenediaminetetraacetic acid
EC	Euro-Collins solution
ECs	Epithelial cells
ENC	Endothelial cells
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
Gln	Glutamine
G	Glomerulus
Gp	Group
HPLC	High Performance Liquid Chromatography
hESC	Human embryonic stem cells
HSCs	Hepatic Stellate Cells
HIV	Human Immunodeficiency Virus
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and Eosin
HRP	Horseradish Peroxidase
IAPs	Ice-active proteins

IIF	Intracellular Ice Formation
IRI	Ice-recrystallization inhibition
IU	International units
kD	Kilo Daltons
LDH	Lactate Dehydrogenase
Liquid N_2	Liquid Nitrogen
Mg	Milligram
MZ	Marginal Zone
MTT Assay	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
MPa	Megapascal unit of pressure
mM	Milimoler
N	Newton
NC	Negative control
OD	Optical density
PBMCs	Peripheral Blood Mononuclear Cells
PCC	Primary Cell Culture
PBS	Phosphate-buffered saline
PC	Positive control
PH	Potential hydrogen
PCT	Proximal Convoluted Tubules
RP	Red Pulp
RI	Recrystallization inhibition
RNA	Ribonucleic acid
SD	Sprague dewily

SD	Standard deviation
SM	Smooth muscles structural changes
SF	Spleen Follicle
TGF	Transforming growth factors
TBST	Tris-Buffered Saline
THPs	Thermal hysteresis proteins
TH	Thermal hysteresis
UW	University of Wisconsin solution
UV	Ultras violate light
VEGF	Vascular endothelial growth factor
Vs	Against
WP	White Pulp
xg	Times gravity

CHAPTER 1

INTRODUCTION

Skin is one of the largest and complex organs comprising and covering the whole external surface of the body. It acts as a protective physical barrier between the environment and the body, that preventing losses of water and electrolytes, reducing penetration by chemicals, and protecting against pathogenic microorganisms. The skin is also essential in body temperature regulation and immunologic surveillance. It also has autonomic and sensory nerves as well as various sensory receptors that have the ability to detect different incoming stimuli of vibration, pain, itching, temperature, pressure, and touch (Khavkin and Ellis, 2011).

In medicine usage of autogenic and allogeneic materials, such as blood vessels, skin, and heart valves has become an ordinary therapeutic option of skin damage (Baust and Baust, 2006). Beside that, magnificent progress has been made during the past 25 years in the development of in vitro-engineered compounds that mimic human skin, either as in vitro skin models or as grafts (Groeber *et al*., 2011).

Skin grafts and flaps are the two main basic surgical therapeutic options to repair skin loss. A graft is a segment of skin, of variable size and thickness that are completely removed and detached from its original position and moved to cover the area to be repaired. While a flap is a full-thickness segment of skin that retains its own blood supply removed and isolated peripherally from the surrounding tissue, except along one side, and transfer to another site (Andreassi *et al*., 2005).

Usage of skin grafting as good reconstructive tools to repair and restore tissue structure and function is an ancient strategy many past centuries (about 2500 to 3000 years) ago in India (Ratner, 1998). As a result of their limited availability, in addition to the increasing demand for various kinds of skin grafts that are widely used for treatment of different serious injuries such as skin disorders, extensive burns and wounds, and also for reconstructive and plastic surgical procedures .

The demands for developing new compounds and techniques for the cryopreservation is increasing but effective cryopreservation is not easy to be establish (Ben-Bassat, 2005). Cryoprotectant (CPA) is a technically-derived term invented to define any chemical that can be added and used with cells, tissues before storage at freezing which produces a high survival rate post-thaw as compered with its absence. During the 19th and early 20th century, some of the earliest theories of cryoprotection were established by biologists

who studied cold hardiness, freezing, and frost resistance in the environment, especially in plants (Fuller, 2004). With the development of regenerative medicine, cryopreservation of tissues which means the preservation of various living tissues in a viable condition of a temporary cessation of their vital functions without death at subzero temperatures (Bakhach, 2014; Pegg, 2007) has witnessed a huge interests.

The viability in transplanted skin is a basic demand to guarantee the supplementation of a high quality skin for clinical repair of various wounds. It is broadly recognized that 'take' of allograft tissues is highly influenced by viability of tissue (Castagnoli *et al.*, 2003) so the maintenance of different biological material in a stable and steady condition is an essential demands in all agricultural, medical, biological, and biotechnological sciences. The storage system have protected lifesaving banks of cells, tissues, and organs which can be readily use at any time for transfusion and transplantation, and has ensured experimental work calibration over time. Also this process has supported the conservation programs of species which lead to the protection of survival endangered critical germs (Bakhach, 2014; Day and Stacey, 2007; Pegg, 2007).

The American Association of Tissue Banks (AATB) declares that practicality of skin viability is a fundamental necessity for a better quality grafts for the proper closure of wounds (Baxter, 1985). Conservation of structural integrity and tissue viability are both considered to be vital for the engraftment and neovascularization of skin grafts (Kua *et al*., 2012). It is, therefore, a basic inquiry to guarantee that the viability of the skin tissue experiencing cryopreservation is and remains good. (Franchini *et al*., 2009).

All over the world tissue banks have their own techniques for storage and preservation of skin grafts, but the most useful procedures that are commonly in use include: refrigeration at 4°C, preservation by using high concentration of glycerol, and cryopreservation at subzero temperatures (Başaran *et al*., 2006; Gaucher *et al*., 2012; Gaucher *et al*., 2010).

The issue that facing storage and preservation of various biological tissues is that, the most simple common preservation techniques such as refrigeration, have many problems including, the fast decrease in fresh skin viability during storage at 4 °C (Gaucher *et al*., 2012), restricted shelf-life, high expenses, risk of genetic drift or contamination so a more acceptable choice like cryopreservation is required. This is a strategy that based on an effective suspension of all physical, biological, and chemical processes at cryogenic temperature (Karlsson and Toner, 1996) whereas cryopreservation theoretically avoids imminent expiration and maintains some viability, in spite of the cellular trauma of freezing and thawing (Gaucher *et al*., 2012). Cryopreservation has been shown to maintain cell viability up to 50 % (Bravo

et al., 2000) but glycerol preservation results in non-viable skin grafts (Kua *et al*., 2012).

Cryopreservation of different biological tissue and cells has received great attention in the scientific society all over the world since 1949, when Polge and his colleagues discovered the cryoprotective activity of glycerol. Cryopreservation of composite organs and biological tissues is stimulating the scientific community with the aim of saving organs from a living donor pending a recipient. The object is at least slow down or even to block the cellular functions while conserving their physiochemical structures (Bakhach, 2014).

In 1949 the successful addition of cryoprotectant to the freezing media became an ordinary strategy as a result of the discovery of the CPA properties (Polge *et al*., 1949). Predicting the cells behavior during freezing through the use of mathematical models by Mazur (1963), paved way for the following successes in predicting the damage of various cells as a result of intracellular ice formation (Karlsson and Toner, 2000).

Ice crystals formation during subzero temperature constitutes a practical true obstacle to many technological procedures. Thus, there is a need to improve various strategies to decrease ice crystal recrystallization for human tissues storage and distribution in transplantation and regenerative medicine (Congdon *et al*., 2013).

Anti-freeze peptides are kind of antifreeze molecules that are usually derived from native AFPs and used in various scientific applications. They consist of small number of amino acids not less than 25. This kind of antifreeze agents can act as useful antifreeze tool to inhibit ice crystals formation, decrease recrystallization, and reduce TH value in various scientific applications (Kim *et al*., 2017; Kong *et al*., 2017; Shah *et al*., 2012).

Developing effective techniques for cryopreservation of tissues is an important step in the preservation of different types of tissues. The CPAs improve cell viability after thawing by reducing the physical and chemical cell damage that occurs during the freezing and thawing process (Cho *et al.*, 2014).

The needs for successful improved storage of cell, tissue and organ are increasing as a result of the increased industrial production of the different regenerative medical products to cover the various clinic demands. One of the most critical issues in regenerative medicine is the safety of cell and tissue cryopreservation material (Stolzing *et al*., 2012).The possibility of post transplantation complications and graft failure will usually decrease when there is a viable cell population (Karlsson and Toner, 1996; McNally and McCaa, 1988).

Various strategies and techniques for storage and preservation of human skin allografts. The most frequently used techniques are cryopreservation, glycerol-preservation with increased concentration of glycerol and, less frequently, storage at 4 °C. Therefore, it is possible to divide allografts into two criteria depending on the used preservation process: viable (or partly viable) and non-viable. Allografts cryopreserved or stored at 4 °C are viable while glycerol-preserved allografts are not viable. Viability of fresh skin decline quickly during storage at 4 °C whereas cryopreservation maintains better viability, in spite of the adverse effects of freezing and thawing that causing cellular trauma (Gaucher *et al*., 2012).

Cryopreservation has witnessed an extensive development and become an important issue in the field of biomedical science and biopreservation of different donor tissues and artificial products. Biopreservation has become a vital enabling knowledge for the development of regenerative medical technology and transplantation. There are many approaches for tissue preservation, the optimum proper choice of which is depends on the required length of storage and the nature and complexity of the tissue to be stored, but the maintenance and preservation of functional and structural integrity of living tissues is always demanding. All approaches for tissue preservation target to stabilize tissues biology by delaying the biochemical and chemical processes responsible for degradation and decreasing metabolism significantly during *ex vivo* storage (Baust and Baust, 2006; Brockbank and Taylor, 2006).

Problem statement

Tissue auto-grafts and allografts derived from donors are widely used in the treatment of serious injuries and other related conditions (Baust and Baust, 2006), but the main concern is the level of tissue viability and functionality after cryopreservation. An effective method of cryopreservation is still required in order to:

- Optimize a better technique for skin graft cryopreservation that, overcome the freezing effects and recrystallization due to subzero temperature which represent a big challenging issue facing the tissue transplantation.
- Enable them to be transported and preserved safely.
- Render these grafts safe for clinical use.

Hypothesis

The hypothesis of the current study implies that tissue grafts could be effectively cryopreserved by using antifreeze peptides as a sole CPA.

The objectives of the current study are:

I. To determine the histological and structural changes associated with use of Afp1m as a skin CPA
II. To evaluate the effects of Afp1m as a CPA on cell viability and tissue functionality
III. To evaluate the macroscopic properties of Afp1m cryopreserved skin grafts
IV. To investigate the physical properties of Afp1m cryopreserved skin grafts
IV. To determine the toxicity associated with the use of Afp1m as a skin graft CPA

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