CRYOPRESERVATION OF MESENCHYMAL STEM CELL AND TISSUE ENGINEERED CONSTRUCTS USING NON-TOXIC CRYOPROTECTIVE AGENTS

A THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

in

Biotechnology and Medical Engineering

By

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2013

Dedicated To

My Parents

ACKNOWLEDGEMENT

I avail this opportunity to express my indebtedness, deep gratitude and sincere thanks to my supervisor Prof. (Mrs.) K. Pramanik ,Professor and Head of the Department of Biotechnology and Medical Engineering, National institute of technology, Rourkela for her in depth supervision and guidance, constant encouragement and co-operative attitude for bringing out this thesis work successfully. I enjoyed the freedom she gave me throughout this work that was instrumental in discovering a scientific "self" within me. I feel proud that I am one of her doctoral students and I consider myself extremely lucky to get opportunity to work under the guidance of such a dynamic personality.

My special thanks are due to Prof. S. K. Sarangi, Director, National Institute of Technology, Rourkela for his encouragement, valuable suggestion and providing all the facilities to successfully complete this dissertation work.

I would like to extend a special thanks to Prof. S. K. Mahapatra for guidance to successfully complete this work. I am also very thankful to all the members of my doctoral scrutiny committee – Prof. M. K. Gupta and Prof A. Biswas of the Department of Biotechnology and Medical Engineering, Prof. S. Mishra of the Department of Chemical Engineering and Prof. S. Das of the Department of Life science for their thoughtful advice, inspiration and encouragement throughout the research work. I take this opportunity to thank the other faculty members and the supporting staff members of the Department of Biotechnology and Medical Engineering for their timely co-operation and support at various phases of experimental work. I would like to extend a special thanks to my dear friends Divyanshu Mahajan, Nadeem Siddique, Biswadeep Chaudhuri, Niladri Nath panda, Varshini Vishwanath and Bhisma Narayan singh for their valuable suggestions and encouragement.

And it goes without saying, that I am indebted to my parents Mr. Sarat Chandra Bissoyi and Mrs. Shanti Bissoyi and brother Sabyasachi Bissoyi, whose patience, support and endurance made completion of my thesis. Above all, I would like to thank the Almighty for his enormous blessings.

I greatly indebted to my wife, Sumana Bissoyi who had always been very supportive, caring and everlasting support she has given me.

ABSTRACT

The thesis work deals with the development of cryopreservation strategy for long term storage of MSCs and MSCs seeded tissue engineered constructs using non toxic cryoprotective agents as freezing medium. In the first phase, different freezing medium consisting of a combination of natural extracellular cryoprotectants namely trehalose, hydroxyl ethyl starch, polyvinyl pyrolidine and intracellular CPAs like erythritol, taurine and ectoin were used for cryopreservation of MNCs following the Taguchi Orthogonal Array method. Among the various combinations, freezing medium consisting of trehalose (0.05mM), ectoin (0.10mM) and catalase (100µg/ml) has shown maximum MNCs viability. These CPAs were further investigated individually as well as in combination to see their effectiveness towards long term preservation of MSCs. Among the freezing solutions, solution prepared using trehalose (0.3mM), ectoin (0.3mM), and catalase (100µg/ml) was found to be the most effective in preserving MSCs in long term basis. The viability of MSCs (73%) is found to be higher than the viability achieved with 10% (v/v) Me_2SO (61%) used as control. The apoptotic study has indicated that the addition of general caspase and calpain inhibitors can reduce the apoptosis rate upto 10-15% thereby achieving increased cell viability of 80%. The optimum condition for the controlled rate freezing of MSCs was established as prenucleation cooling rate -1^oC/min, nucleation temperature -7.5^oC, cold spike -80^oC/min, post nucleation holding time 5min, post nucleation cooling rate -1^oC/min, cell density (3×10^6/ml/cell) and storage temperature (-150°C) using the most effective freezing medium achieving cell viability of 85%. The developed freezing medium has also shown its ability to preserve MSCs seeded tissue engineered construct. The maximum viability of 80% achieved at optimum controlled rate freezing of TECs was established as cooling rate -1^oC/min, nucleation temperature -7.5^oC and freezing medium consisting of trehalose (0.3mM), ectoin (0.3mM), catalase (100µg/ml) in presence of caspase (50µg) and calpain (50µg) inhibitors. Overall, it is demonstrated that the developed freezing medium may pave the way for long term preservation of MSCs and also MSCs seeded scaffold.

Keywords: Cryopreservation, scaffold, caspase inhibitors, trehalose, ectoin, tissue engineered constructs, control rate freezer

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ADP	Adenosine diphosphate
ASC	AT derived Stromal Cells
AIF	Apoptosis inducing factor
Apo3L	Apo3 ligand
Apo2L	Apo2 ligand
Apaf-1	Apoptotic protease activating factor 1
BAG	BCL2 associated athanogene
BA	Bongkrekic acid IAPs
BAD	BCL2 antagonist of cell death
BAK	BCL2 antagonist killer 1
BAX	Bcl-2–associated X protein
Bcl-w	BCL2 like 2 protein
Bcl-x	BCL2 like 1
βME	β-mercaptoethanol
BM-MSCs	Bone Marrow derived MSCs
BIK	BCL2 interacting killer
Bcl-XL	BCL2 related protein, long isoform
Bcl-XS	BCL2 related protein, short isoform
Bcl-2	B-cell lymphoma 2
BIM	BCL2 interacting protein BIM
BNIP3	BCL2/adenovirus
BSA	Bovine Serum Albumin

CAD	Caspase-activated deoxyribonuclease
Caspase c	Cysteinyl aspartic acid-protease
CD	Cluster of Differentiation
c-FLIP	Cellular form of FLICE-inhibitory protein
CFU-F	Fibroblast Colony Forming Unit
CIDOCD	Cryopreservation-induced, delayed-onset cell death
C100	Catalase 100µg
CPDA	Citrate Phosphate Dextrose Adenine
CB	Cord Blood
dATP	Deoxyadenosine triphosphate
DD	Death domain
D10	10% v/v Dimethyl sulfoxide
DR3	Death receptor 3
DR4	Death receptor 4
DR5	Death receptor 5
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DMEM-LG	Dulbecco's modified Eagle's media-low glucose
EndoG	Endonuclease G
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
E30	Ectoin (30mM)
ELISA	Enzyme Linked Immuno-sorbent Assay
ERK	Extracellular signal-regulated kinase
FACS	Flow Activated Cell Sorter
FADD	Fas-associated death domain
FasL	Fatty acid synthetase ligand

FBS	Fetal Bovine Serum
FSC	Forward Scatter
FasR	Fatty acid synthetase receptor
IAPs	Inhibitors of apoptosis proteins
GAGs	Glycosaminoglycans
IGH	Ispat General Hospital
JNK	c-Jun N-terminal kinases
hAdMSCs	human omentum Adipose derived MSCs
HLA	Human leukocyte antigen
HTS	Hypothermosol
HtrA2/Omi	High-temperature requirement
KV	Kilo Volts
kDa	Kilo Dalton
MAP kinase	Mitogen activated protein kinase
Me_2SO	Dimethyl sulfoxide
MPT	Mitochondrial permeability transition pores
MNCs	Mononuclear Cells
Me_2SO	Dimethyl sulfoxide or DMSO
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
N_2	Nitrogen
p53	Tumor protein 53
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
ROS	Reactive oxygen species
RIP	Receptor interacting protein
RT-PCR	Reverse Transcription Polymerase Chain
RPMI	Rosewell Park Memorial Institute

SEM	Scanning Electron Microscope
SSC	Side Scatter
SVF	Stromal Vascular Fraction
SF	Silk fibroin
SMAC/DIABLO	Second mitochondria-derived activator of Caspases/direct IAP binding protein with low PI
SD-282	Indole-5-carboxamide (ATP-competitive inhibitor of p38 kinase)
S/N ratio	Signal to noise ratio
tbid	truncated Bid
TNF	Tumor necrosis factor
TNF-a	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TRADD	Tumor necrosis factor receptor type 1- associated DEATH domain protein
TRAIL	TNF-related apoptosis-inducing ligand
$T_{30}/E_{30}C_{100}/I$	Trehalose (30mM), Ectoin (30mM), Catalase (30mM) & apoptosis inhibitor
T_{30}	Trehalose (30mM)
UC	Umbilical Cord
UCB	Umbilical Cord Blood
ZVAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

GENERAL INTRODUCTION

1.1 Background

Millions of people worldwide have been suffering from various tissue and blood related diseases such as diabetes, parkinson diseases, osteoarthritis, bone, cartilage, tendon, muscle, neuronal etc. due to the consequence of trauma, degeneration, birth defects, infection and others. The current clinical treatment methods involving autograft, allograft and xenograft suffer from several limitations including immune-rejection, donor site morbidity and disease transmission [1]. As a result, none of these methods is able to fully cure the patients from such diseases and ultimately suffering continues. In this context, cell and tissue based therapies have emerged as promising techniques for the treatment of such diseases [2]. However, for success of this technique, a wide variety of cells and cell-scaffold constructs (cell seeded on artificial extra cellular matrix) are needed to be supplied to the health sector.

Mesenchymal stromal cells or mesenchymal stem cells (MSCs) are multipotent cells present in all tissues and have the ability to differentiate into several cell types including osteoblast, adipocytes, chondrocytes, skeletal muscle cell, myoblast and the like. Recently, MSCs and MSCs derived tissue engineered constructs (TECs) have been considered as the most promising clinical products that can be used for a variety of therapeutic and tissue engineering applications. This has lead to many researchers worldwide to produce MSCs and MSCs seeded tissue engineered constructs (TECs) for their therapeutic applications in repairing and replacing defect and/or diseased tissues and organs. However, the long-term storage of these cells and constructs is the most critical aspect to ensure the off-the-shelf availability of such products for clinical applications at the time of injury or diseases. In this context, cryopreservation using freezing medium consisting of a suitable cryoprotective agent has been considered as an effective preservation method. Freezing solution containing Me_2SO as cryoprotective agents is normally used for preservation of various cells and other biological species. However, freezing protocol using Me_2SO as cryoprotective agent in freezing solution is reported to have several detrimental effects on the cryopreserved cells that are harmful to patients. Therefore, an urgent research program calls for the development of an appropriate

cryopreservation strategy using freezing medium using a cryoprotective agent alternative to toxic Me_2SO to meet the growing demand of MSCs and TECs for clinical applications.

1.3 Cryopreservation of cells and tissues

1.3.1 Principle of cryopreservation

Cryobiology is a new modern science studies effect of low temperature on biological system. Cryobiology is the branch of science, which deals with the effects of reduced temperatures on living organisms, their constituent parts, and their products. [3] Cryobiology is the study of the effects of extremely low temperatures on biological systems, such as cells or organisms diversity of cells and other biological materials. The main aim of cryobiology is to protect and preserve viable cells and tissues at very low temperature for a long duration of time without alteration of their characteristics.

1.3.2 History of cryopreservation

In 1949, Polge and colleagues discovered the effectiveness of glycerol as a cryoprotective agent for fowl spermatozoa [8]. These early studies resulted in the development of the discipline that is now known as cryobiology. Within a few short years, human red blood cells were successfully cryopreserved. In the 1960s, Peter Mazur conducted extensive experiments to model the responses of microorganisms when subjected to low temperatures and freezing [6]. Through the 1960's other call lines were successfully cryopreserved along with unstable, complex bio-organic molecules especially labile proteins, In 1972 the methodologies necessary for the maintenance of "frozen" embryos were developed [7] and new concepts in-vitro fertilization were adopted. To date, list of cell lines and tissue types that are routinely cryopreserved is extensive. Both profit and non-profit banking system exist worldwide.

1.4 Important factors involved in cryopreservation

1.4.1 Ice formation in a cell suspension

An aqueous solution usually supercools at few degrees below its freezing point before freezing is initiated by heterogeneous nucleation that is by a foreign particle (nucleating agent) that mimics the organization of water molecules in ice and catalyzes ice crystallization.

Once nucleation occurred ice crystal growth starts, the amount of water that separates from the solution to form ice increases progressively with decreasing temperature [8].Evidence to suggest that although intracellular freezing is initiated by heterogeneous nucleation, intracellular nucleators are very inefficient [9]. Consequently, when a cell suspension is cooled, the cells super cool to a greater extent than the bathing medium, and freezing occurs almost exclusively in the extracellular solution. The resulting increase in extracellular solute concentration lowers the chemical potential of water outside the cells, which are, therefore, no longer in osmotic equilibrium be with the external solution. Under these conditions, the chemical potential of water inside the cells could be lowered and osmotic equilibrium is restored in three ways: by an efflux of water, by an influx of solute, or by intracellular freezing. As a consequence of the semi permeable properties of the plasma membrane, many types of cell respond as osmometers to changes in the extracellular concentration of nonpermeating solutes. Osmotic equilibrium is restored, therefore, by the movement of water, rather than solute, across the plasma membrane; water moves from the region of higher to the region of lower chemical potential until the chemical potential of water is once more the same on both sides of the plasma membrane. Consequently, cell volume is a linear function of the reciprocal of external osmolality [10].

1.4.2 Rates of cooling and warming

Cooling rate is one of the major factors affecting cell survival after freezing and thawing. Each cell type has its own characteristic optimum cooling rate, which is, to a large extent, determined by the water permeability of the cell but also influenced by the presence of cryoprotectants. The terms *rapid* and *slow* cooling for a particular cell type are defined in relation to the optimum cooling rate. The decline in survival at rates higher than the optimum is due to the likelihood that cells contain ice [11]. Although the formation of intracellular ice during cooling is not necessarily harmful, the consequences of the presence of intracellular ice during warming are usually lethal [12-18].

Several mechanisms have been proposed for slow-cooling injury: namely, direct damage to the membrane caused by high electrolyte concentrations [19-20]; excessive cellular shrinkage, which results in damaging stresses in the plasma membrane [21-25]; thermal shock and dilution shock [26]; and changes in the volume of the liquid phase in the ice lattice [27].

Some types of cell, however, survive rapid cooling when they are cooled by the so-called two-step method [28-35]. Cells are initially cooled to a relatively high subzero temperature (e.g., -25°C), held at that temperature for a few minutes, and then cooled rapidly by immersion in liquid nitrogen. The period spent at the intermediate temperature allows the cells time to dehydrate sufficiently to avoid intracellular ice formation during subsequent rapid cooling in liquid nitrogen.

The survival of cryopreserved cells is also dependent on the rate of warming [36-38]. When cells are cooled at rates higher than the optimum, they are likely to contain intracellular ice; the survival of these cells is higher when warming is rapid than when it is slow. Furthermore, cells cooled at suboptimal rates survive better when; for example, the first successful recovery of mouse embryos was achieved only when they were both cooled and warmed slowly [39].

1.5 Cryoprotectants

Cryoprotectants are chemical compounds that are able to protect cells against the stresses of freezing and thawing. Glycerol and Me_2S0 are the most widely used cryoprotectants, but there is a large range of chemically diverse additives that possess cryoprotective properties [40, 41]. The only common features that these compounds seem to possess are high solubility in water and low toxicity to cells. Cryoprotectants are divided broadly on the basis of whether they permeate cells; for example, cells are permeable to glycerol and Me_2S0 but are impermeable to sucrose and dextran. Although direct interactions between cryoprotectants and cells cannot be excluded, the main effect of additives, such as glycerol, is to reduce both the amount of ice formed and the rise in electrolyte concentration at any given temperature [11]. The high molecular weight of polymers, such as polyvinylpyrrolidone, precludes a similar colligative effect. However, their extremely non ideal behavior at high concentrations can reduce the amount of water that crystallizes [42, 43]. Cryoprotectants do not mitigate the effects of intracellular freezing; instead, the presence of a cryoprotectant can reduce the cooling rate at which intracellular nucleation first occurs [44]. Cryoprotectants are therefore only effective at protecting cells against slow cooling injury, and as the initial concentration of cryoprotectant is increased, the optimum cooling rate is reduced and survival improves [45]. In two-step cooling procedures the main action of a cryoprotectant is thought to permit survival of the cells at the intermediate

holding temperature, rather than during subsequent rapid cooling. The optimum holding temperature is itself dependent on cryoprotectant concentration [46, 47]. Cryoprotectants are divided into two types: membrane-permeating (e.g., glycerol, ethylene glycol (EG), dimethyl sulfoxide (Me_2S0), propanediol) and membrane non-permeating (e.g., sucrose, glucose, Ficol, proteins, lipoproteins).

1.5.3 Cryoprotectant and toxicity

Cryoprotectants are reported to have adverse effects on cells due to chemical toxicity, or they could be a result of osmotic stress during the addition of the cryoprotectant before freezing and during dilution of the cryoprotectant after thawing. If a cell is more permeable to water than to a solute, then a sharp increase in the concentration of that solute in the bathing medium results in cellular shrinkage as water moves out of the cell to restore osmotic equilibrium.. It is accompanied by water, and the cell returns asymptotically to its normal volume. Conversely, if the external concentration of the solute is reduced abruptly, the cell will swell as water enters to restore osmotic equilibrium. The cell then returns to its normal volume as solute and water leave the cell simultaneously. Reducing the rate of change of solute concentration reduces the associated osmotic stress. Stepwise addition and dilution protocols to minimize osmotic stress can be devised in a rational manner, instead of empirically, providing that values for certain parameters are available for the cell: namely, the limits to which the cell can shrink and swell without damage and the membrane permeability coefficients.

1.6 Stem cells

Stem cells are exceptional cells with unique ability to differentiate into diverse cell types in the body, right from the time of nativity to death of an organism [3]. They are found in internal repair systems where they divide without limit, to either repair or replace damaged and diseased cells throughout a person's life. A stem cell has the capability of forming a new cell that either has the potential of a stem cell or becomes a more dedicated type of cell with definite function. Stem cells can be defined as undifferentiated cells with high proliferative capacity, self renewal ability and are found in all multi-cellular organisms. Stem cells are distinguished from other cell types by two important characteristics: i) they are undifferentiated cells, capable of self-renewing for continued periods of time. ii) They can be

differentiated into a more dedicated lineage with specialized functions under certain specific physiological environmental conditions.

1.6.1 Types of stem cells

Stem cells are of three types based on their proliferation potential namely totipotent stem cells, pluripotent and multipotent stem cells. totipotent stem cells are obtained after initial cell division from a fertilized ovum and have the capacity to become all cell types including placenta. After blastocyst stage, cells become pluripotent, which develop into cells from the 3 primary germ layers. Multipotent cells can develop into cells that are closely related to where they reside and self renew for a lifetime. Some sources of stem cells have a potential to differentiate to lineages of endoderm, ectoderm and mesoderm which form the entire human body whereas others are more specialized to differentiate into a few lineages only. There are two types of stem cells namely embryonic stem cells and somatic or adult stem cells. Embryonic stem cells (ESCs) are derived from the inner cell mass of 3 to 5 day old embryo. ESCs can give rise to the entire body of the organism, including all of the many specialized cell types and organs. An adult stem cell is an undifferentiated cell, found among differentiated cells in a tissue or organ [5]. The primary role of adult stem cells in a living organism is to maintain and repair the tissue in which it is found [3-7]. However, in recent past experiments have shown that they can differentiate to cells that are not closely related to them by the phenomenon called transdifferentiation [5, 8]. Adult stem cells are immunocompatible, immunomodulators and immunosuppressant, and there are no ethical concerns with their use.

1.6.3 Mesenchymal stem cells

The most promising attractive stem cell sources for the regeneration or repair of damaged tissues. MSCs have the ability to proliferate in vitro and differentiate into a series of mesoderm-type lineages, including osteoblasts, chondrocytes, adipocytes, myocytes, vascular cells and the like [9-14]. The advantages of MSCs as potential source for tissue engineering are: ease of availability and large quantity of starting samples for isolating cells [12], high proliferation ability, no sign of senescence for >16 passages [10], immuno-compatible and immunosuppressant, useful in autologous and allologous stem cell transplantation, no formation of teratomas or tumor as in case of ESCs [15-16], immuno-modulator property,

useful in GHVD and inflammation [17-18], no ethical concerns. MSCs were first identified by Friedenstein and Petrakova (1968) [19] from rat marrow as plastic adherent bone-forming progenitor cells. MSCs in culture have a fibroblastic morphology and adhere to the tissue culture substrate [20]. MSCs isolated from bone marrow are obtained by invasive procedure and it has been found that the passaging capacity, maximum culture lifetime as well as differention potential [21-24]. The potential sources are bone marrow, umbilical cord (UC), umbilical cord blood (CB), placental tissue (PT), adipose tissue (AT) , amniotic fluid (AF) and the like. The MSCs s derived from all these sources may but differ in proliferation and differentiation capability [25-26].

However the advantage of using birth associated tissues like UC, CB, AF and PT are ease of their availability in large quantity, non invasive procedure to harvest, usually discarded as waste and ability to proliferate and maintain self renewal capacity for longer period of time in culture, unlike BM-MSCs [6]. AT is also less invasive source available in large quantity and AT derived stromal cells (ASC) has shown similar properties as bone marrow derived mesenchymal stem cells (BM-MSCs) [12, 27]. Phenotypically, the minimal criteria to consider human MSCs are adherence to plastic surface, >95% expression of CD105, CD73, CD90; < 2% of CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR and differentiation to osteoblasts, adipocytes, chondrocytes [28].

1.7 Cryopreservation of Tissue Engineering Construct

Tissue defects are one of the most frequent, devastating, and costly problems in human health care. The need for substitutes to repair restores, or replaces tissues due to disease, trauma, or congenital problems is over whelming. In recent years, tissue-engineering constructs (TECs) that can be developed by seeding of cells on the scaffold (artificial ECM) through tissue engineering technique. TECs have the potentiality to revolutionize current methods of health care treatment and significantly improve the quality of life of patients when compared with the three major types of clinical treatment methods are currently utilized: autograft, allograft and xenograft. However, living TECs are not easily available due to time and space constraints. One of the major obstacles in the industrialization and large- scale clinical applications of tissue engineering is the preservation of TECs. TECs stored at ambient temperature require expensive human involvement to satisfy metabolic demands and can be

potentially infected and biologically altered. Safe and long-term preservation methods for these engineered tissues are needed [29-31].

Organisation of thesis

The work embodied in this thesis has been presented in the following six chapters

Chapter I: Presents a general introduction emphasizing on the background of the study, preservation techniques for cells and tissue, principle of cryopreservation, history of cryopreservation and important factors involved in cryopreservation. The problem associated with cryopreservation of stem cells and cryopreserved TECs have been highlighted.

Chapter II: An extensive literature survey on the preservation of MSCs, cryoprotectants alternative to DMSO for preserving MSCs, cryopreservation of TECs and cryopreserved induced cell death has been presented.

Chapter III: Includes the aims and scope of the present work.

Chapter IV: Describes the materials and detailed experimental procedure to carry out various stages of present research work including i) collection and isolation of MNCs ii) sorting and culturing of MSCs iii) Characterizations before and after cryopreservation of MSCs iv) Cryopreservation study on MNCs and MSCs iv) Cryopreservation study of MSCs seeded TECs vi) Characterizations of TECs before and after cryopreservation.

Chapter V: Deals with the results and discussion on the experimental work. The results and discussions section is divided into three phases. The first phase includes the cryopreservation of MNCs for the selection of most efficient natural CPAs included in this phase. The second phase involves the Cryopreservation of MSCs using most efficient CPA with the aim to improve long term cryopreservation efficacy MSCs. In the last phase of this chapter Cryopreservation of TECs using most efficient freezing solution has been presented.

Chapter VI: Includes a brief summary and conclusion of the whole investigation.

CHAPTER II

Literature Review

LITERATURE REVIEW

2.1 Preservation of MSCs

Cryopreservation plays a vital role in obtaining off the-shelf availability for a variety of tissues and cells[4]. Recently MSCs are considered to be the main cell source for tissue engineering and stem cell therapy. The availability of stem cell and amount makes cryopreservation important [32]. If stem cells can be cryopreserved for long duration with retention of high level of viability and potentiality to differentiate into tissue-specific cells, their clinical applications are greatly simplified. However, an important prerequisite for prospering applications of MSCs is to cryopreserved these cells under well defined conditions to satisfy needs in clinical applications. Currently, there are available approaches for cryopreservation of cells such as (i) typical slow cooling and (ii) vitrification using Me_2SO as CPA.

Slow freezing has the advantage of using low concentrations of CPAs, which are reported to be associated with chemical toxicity and osmotic shock [33-34]. More significantly slow freezing can handle large quantity of cells, which makes it more clinically relevant. After the invention of glycerol as cryopreservation of sperm in 1949 [5], it has become a common practice to add one or several CPAs to the freezing media [6]. Afterwards, the discovery of Me_2SO as cryoprotectant increases the growth of cryopreservation applications. Recent studies have confirmed that 10% Me_2SO and slow cooling/rapid warming does not affect the viability or differentiation potential of adipose-derived MSCs [7]. Adult MSCs derived from human dental pulp also showed high post-thaw recovery and trilineage differentiation potential after slow cooling in 1–1.5mol/l Me_2SO [8]. Me_2SO is known to have an effect on the epigenetic profile of, and induce differentiation in, murine stem cells [9]. Cryopreserved cells treated with Me_2SO may cause other adverse effect

including gene mutation. Therefore, it is important to reduce the levels of Me_2SO in freezing solutions even if complete elimination is not possible.

2.1.1 Alternative cryoprotective agents for preserving MSCs

The use of polyvinylpyrrolidone (PVP), an extracellular cryoprotectant, has been investigated as an alternative to Me_2SO and fetal calf serum (FCS) [14-13] for cryopreservation of MSCs. Recovery of MSCs cryopreserved in 10% PVP with human serum was slightly lower than cells cryopreserved in Me_2SO with animal serum as reported[10]. A similar study utilizing methylcellulose either individually or in conjunction with reduced levels of Me_2SO indicated that human serum could replace in standard Me_2SO mixtures without affecting the recovery of cells [11] and that 1% methylcellulose produced comparable results with Me_2SO concentrations as low as 2% when an annexin V apoptosis assay was used to analyze cells after 24 h post-thaw [12]. In an another study, MSCs exposed to trehalose loading medium for 24 h prior to addition of cryoprotectant mixture (10% Me_2SO and serum)[13] showed significant increase in post-thaw viability of more than 50% . They showed a beneficial effect of trehalose in the elution solution, Polyampholytes (polyelectrolytes bearing both cationic and anionic repeat group) such as poly-L-lysine have been used successfully to cryopreserve MSCs isolated from rat [14] and may offer an alternative to Me_2SO. Sericin (a protein derived from the silkworm) has been shown to improve the attachment of cryopreserved hepatocytes and mammalian cells [15-17].

2.1.2 Use of antioxidants and inhibitors as additive in freezing medium

Antioxidants in the form of β-mercaptoethanol are routinely present in culture medium of human embryonic stem cells (hESCs). But the overproduction of reactive oxygen species (ROS) during cryopreservation has led to the addition of antioxidants to the freezing solution in an attempt to reduce damage [35- 36]. Addition of glutathione to the cryoprotectant and post-thaw recovery solution has shown an improvement in the survival of embryonic stem cells following cryopreservation [18-20]. Rho-associated protein kinase (ROCK) inhibitors have been reported to improve post-thaw survival in a number of studies [38-42]. It has been demonstrated that Y-27632 added to the post-thaw culture medium increased hES survival rate 10-fold, with colony growth rates similar to unfrozen controls [21]. Furthermore, the effect of ROCK inhibitor Y-27632 has shown to have similar effects on the recovery from cryopreservation of both adult stem

cells and bone marrow-derived MSCs[22] as well as human iPS cells in both feeder-associated and feeder-free conditions [43].

It is reported that Rho GTPase enzyme target rho-associated kinase I (ROCK-I) is cleaved by caspase inhibitor during apoptosis and activated ROCK-I brings about bleb formation in apoptotic cells [27] which is one of the essential characteristics of apoptosis. A potent ROCK matter Y-27632 inhibits apoptosis and is proved to be effective in increasing post thaw recovery and survival of many cryopreserved cells like human embryonic cells , induced pluripotent stem cells (iPS cells), human mesenchymal cells, cord blood-derived CD34+ hematopoietic the cells and frozen human embryos. Targeting and inhibiting Furthermore, various molecules including proteases, signaling molecules and other proteins bring about apoptosis and necrosis down the cell death cascades is the prime goal of the researchers in this field.

Various caspase inhibitors are reported to provide protection against apoptosis [44]. The most widely studied is the synthetic broad-spectrum irreversible inhibitor of caspase enzymes and N-Benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone (zVAD-fmk) [45]. However, incorporation of only zVAD-fmk in the freezing medium, does not much improve post thaw cell viability. But an increase in cell viability is observed when it is added to post thaw culture medium and incorporated in both freezing and post thaw culture medium [38]. In another study, zVAD-fmk not only inhibited caspase activity in cryopreserved cells but also reduced mitochondrial injury by caspase inhibition by increasing mitochondrial membrane potential in cryopreserved porcine hepatocytes [39]. As mentioned earlier, cryopreservation induced stress activates mitochondrial death pathway resulting in an increase in mitochondrial membrane permeability and reduction in mitochondrial membrane potential. Besides zVAD-fmk, there are many other potential caspase inhibitors has been reported. Recently Xia Xu et. Al.1999 studied caspase-9 inhibitor and zVAD-fmk [46]. They reported that though these inhibitors significantly inhibited caspase-8 and caspase-9 activity, but they did not enhance post thaw cell viability [23]. This implies that inhibition of caspase activity does not protect the cells [24]. Hence it is stated that caspase 8 and caspase 9 acts as downstream effectors in cryopreservation induced cell death and their inhibition does not improve cell recovery.

Ferrusola et. al. (2010), reported that the addition of bongkrekic acid (BA), a specific inhibitor of mitochondrial permeability transition pore (PT–pore), during cryopreservation of sperm stallion,

significantly reduced caspase activity after thawing, reduced the increase in membrane permeability, and increased the mitochondrial membrane potential of thawed sperm cells [47]. The addition of broad spectrum protease inhibitors to the cryopreservation medium significantly reduces the cleavage of apoptosis proteins like caspase 3 and 8 as reported [48]. The p38 MAPK is one of the mitogen-activated protein kinase (MAPK) signal transduction pathways which can be activated in response to various environmental stimuli and induce apoptosis. It is shown to be activated during freezing-thawing and cryopreservation processes [49-51]. Inclusion of p38 MAPK inhibitor (SD-282) inhibits the phosphorylation of p38 MAPK substrate, heat shock protein (HSP27), which in turn reduces cell damage/apoptosis, thereby results in the improvement in viability of cryopreserved cells [52].

The F-actin cytoskeleton plays a vital role in various cellular functions like cell motility, shape and regulating complex signaling systems [53]. It has been observed that alteration of the equilibrium between G-actin and F-actin in response to environmental stimuli can induce apoptosis in mammalian cells [54]. Cytoskeleton may be affected by hypertonic conditions and may bring about remodeling of actin [55]. It is well known fact that during cryopreservation, cells are exposed to anisotonic conditions, which result in elevation of F-actin levels inside cellular cytoplasm [56]. This elevation in F-actin levels itself may be the reason for low recovery after cryopreservation as alteration in cellular F-actin levels causes apoptosis [57]. Rho GTPases is reported to regulate cytoskeleton dynamics. In particular, ROCK triggers a signalling pathway that leads to actin-myosin coupling to the plasma membrane leading to the contraction of actin-myosin and blebbing of plasma membrane [58-62]. It has been reported that ROCK pathway can be activated by a variety of external stimuli including osmotic stress [63]. Pizzolla D. et al. has reported that ROCK leads to phosphorylation of ezrin, resulting in clustering of FAS and ultimately apoptosis via extrinsic pathway [64]. A potent ROCK inhibitor Y-27632 inhibits apoptosis and is proved to be effective in improving the post thaw recovery and survival of many cryopreserved cells like human embryonic cells hESCs), induced pluripotent stem cells (iPS cells), human mesenchymal cells and frozen human embryos[65-75] except for cord blood-derived CD34+ hematopoietic progenitor cells [76]. It has been reported that the inhibition of ROCK by Y-27632 following cryopreservation reduces caspase 8 activity [64]. Thus ROCK is involved in the regulation of activation of caspase 8 during cryopreservation which may be through ezrin phosphorylation and induces apoptosis via extrinsic pathway. However, it is still not

clear whether caspase 8 induces apoptosis directly or via mitochondrial pathway. Thus the mechanism of cryopreservation induced ROCK pathway and relation between F-actin and ROCK needs to be further studied. Recently, Barbaric et. al. found an alternative to the potent ROCK inhibitor Y-27632. They found an FDA approved drug, pinacidil through a high-throughput screening assay with similar properties to that of Y-27632. Like Y-27632, the compound is reported to promote attachment of post thawed hESCs, reduce apoptosis and enhance their survival when added to the post thaw culture medium [76]. The advantage of pinacidil over Y-27632 is that it has been used over many years as a vasodilator drug for treating hypertension, its availability and much cheaper than Y-27632, thus, it is expected to develop cryopreservation and post thaw culture medium economical.

As mentioned earlier, in response to various stimuli, p53 plays an important role in regulation of apoptosis [52]. At low levels of reactive oxygen species (ROS), p53 acts as a survival factor but at higher concentrations, p53 induces the expression of pro-oxidant genes [76]. Xu et al. have reported that Bax-peptide inhibitor promotes cell recovery to some extent and also demonstrated and correlated the high level of intracellular ROS and p53 activation caused by the procedure of freezing in cryopreservation. They have also reported that in presence of p53 inhibitor, pifithrin μ, there was a reduction in caspase 9 activity but not caspase 8 activity [77]. This suggests that apoptosis following cryopreservation may be induced by activation and accumulation of p53 in the cytosol through the intrinsic pathway. Furthermore, study has shown that there is an increased level of LC3-II (phagophore protein to lysosomal degradation) during storage of stallion spermatozoa [78]. Autophagy is reported to be detected after cold storage of fatty livers [79]. However, further study is essential to understand the exact relationship between autophagy and cell survival due to cryopreservation.

2.2 Cryopreservation of TECs

Biopreservation techniques that maintain ex-vivo TECs viability and function represents the foundation of modern tissue engineering. The ability to preserve the integrity of TECs outside the native environment for extended periods has not only separated donors and recipient's time, but also has made it possible for bio-banks to provide safe, high quality TECs products in an efficient and effective manner. It is fact that successful cryopreservation of ready to use TECs would support the implementation of routine cryopreservation practices during preparation of engineered

cells and tissues for clinical applications and benefit high throughput cell based assays by reducing batch to batch variation and eliminating the time consuming, labor intensive process of inoculation and expansion from a frozen vial of cells, thus reducing time between cell storage and use in experimental settings. For this reason, cryopreservation of not only cells, but also tissue engineered constructs (TECs) is an important part.

Various natural and synthetic materials have been used to construct extracellular matrices (ECMs) for in vitro cell culture and in vivo tissue regeneration. The biomaterials used as scaffolds are natural or synthetic polymers such as polysaccharides, hydrogels or thermoplastic elastomers. However, there is currently no scaffolding material that simultaneously offers superior biocompatibility, biofunctionality, effective mechanical properties and tractability.

There are several challenges of cryopreservation of TECs such as preservation of integrity of TECs (Table 1) adapted from Sarangi et al [25]. Maintenance of cell-cell interaction, cell substrate contact, cell viability, cell functions, cell proliferation and differentiation ability, It was hence the objective of this review to discuss how the surface properties of biomaterial affect cryopreservation outcome of adherent hMSCs.

Table1: Factors influencing cryopreservation of TEC

Irregular physical dimensions of TECs	Uneven distributions of cryoprotectant occur, some regions are not sufficiently protected due to low concentration of cryoprotectant and some are damaged by over exposure to the cryoprotectant.
Non uniform rates of temperature change	Optimal cooling and warming rates are difficult to achieve in large sections of the tissue constructs.
Thermal gradients difference	Uneven expansion or contraction in the TECs
Vascularization of tissue	Vascularisation results in extracellular ice formation inside the intravascular space, ultimately resulting in over distention and rupture of blood vessels and sinusoids

Stem cell transplantation has been in clinical use for decades to treat diseases. The principal sources for those therapeutic strategies are stem cells derived from umbilical cord blood, non hematopoietic stem cells, such as embryonic and induced pluripotent stem cells. The therapeutic strategies utilizing such cellular products depend heavily on the effective preservation of those

cell products for clinical use. The need for ready availability of such products calls for appropriate storage procedures with favorable graft survival rates and a tolerable toxicity profile. Broad research on stem cells open an area of promising therapeutic ability of stem cells has shed light on shortage of tissue grafts. Stem cells derived from bone marrow, cord blood, cord, adipose tissue, dental pulp are considered as an important source for tissue regeneration. These stem cells have shown good proliferation and differentiation ability into various functional cell types of mesenchymal tissues, including bone, cartilage, tendon, fat and have been considered appropriate candidates for various tissue regeneration. MSCs derived from various source can be successfully cryopreserved in suspension, such as embryonic stem cells, with high recovery of viability and functions, but this technology has not been successfully extended to cells on substrates/scaffolds. Only limited success has been achieved in cryopreservation of encapsulated hepatocytes. The majority of cell scaffold construct have been shown not to accommodate freezing protocols due to various factors such as various location in the tissue changes the concentration of cryoprotectant and temperature which leads to non uniform mass and heat transfer [26-27].

2.2.1 Cryopreservation of TECs by slow freezing

Polge et al. in 1949 was first reported the successful slow cooling protocol using glycerol as cryoprotectant[28]. Slow cooling consists of steps of various steps like CPA addition, seeding, cooling, thawing and CPA removal. The traditional methods used for cryopreservation of TECs are based on the slow cooling approach. For this, TECs are usually cooled in presence of one or more cryoprotectant, at a selected cooling rate in a programmable freezing device. The slow cooling rate allows TECs to partial dehydration by maintaining equilibrium with the frozen extra-cellular environment. For successful cryopreservation, cooling rate is most important part as it determines the rate of water efflux from cell to extracellular component. However, slow freezing method is not cost-effective as it requires a controlled rate freezer. More significantly, it is extremely tricky to preserve the intactness of biomaterials and the integrity of neo-tissue during the liquid-ice phase transition[4, 29]. Uncontrolled freezing i.e. at -20ºc and -80ºc is reported to more injurious for fragile TECs seeded with stem cells. Table 2 shows the some of the important cryopreservation study on tissue engineering constructs.

2.2.2 Cryopreservation of TECs by vitrification

Vitrification refers to the physical phenomenon relating the solidification of any solutions into a glass state. Vitrification occurs due to rise in viscosity during sudden cooling which eliminate ice formation. Vitrification of cells depends on various factors such as (a) cooling and warming rates (b) concentration of cryoprotectant (c) substitution of an amino group for the hydroxyl (OH^-) group of an alcohol (d) hydrostatic pressure of the solution (e) reduction of C_v (f) sample size and carrier systems [80]. Table 2 shows some of the important cryopreservation study on tissue engineering constructs.

Table 2: Cryopreservation of tissue engineering constructs by vitrification as reported

Year	Author	Cryoprotectant	Application	Morphology/viability
2004	Pegg, D	Me_2S0	Cartilage	Inadequate and variable
2006	Han, D	10%Me_2SO & 20% FBS	Osteoblasts	50%
2006	Wang	10% Me_2SO	PGA seeded dermal cells	Regain its natural shape & No fracture was observed
2013	Chen	10% Me_2SO	Alginate encapsulation stem cells	Gave 74% (mESC) and 80%(hMSC) survival rates
2010	Malpique	CryoStor	Neurospheres	60%
2009	Malpique	CryoStor & 10% Me_2SO	N2a, Caco-2	91 ± 15% and 68 ± 15%
2011	Khalil	12% Me_2SO/Celsior+/-	Liver Spheroids	Improve multi-cellular constructs
2009	Miyoshi	10% Me_2SO	Bioartificial livers	65% cell attached to scaffold
2006	Lehle	10% Me_2SO	Vascular tissue	Between 60% and 80%
2009	Sui	10% Me_2SO	3Dgelatin constructs	78.7 ± 3.94%
2011	Sambu	20 % (v/v) Me_2SO	ESCs alginate beads	60 %
2013	Massie	15% Me_2SO	HepG2 monolayer cell & Alginate encapsulation	65% to 80%
2011	Umemura	10% ethylene glycol 1.0M sucrose & 0.00075 M (PVP)	3D gelatin	49% to 96%
2012	Costa	Me_2SO	Starch & PCL blend	Off-The-Shelf Engineered Tissue Substitutes
2012	Xu	10% Me_2SO	glass,gelatin, matrigel and a matrigel sandwich	cell viability of adherent hMSCs is significantly lower (up to 35%)

2011	Chen	Me_2SO & trehalose	Epithelial sheets	Mechanical property decreased

2.3 Cryopreservation induced cell death

Cryopreservation is defined as the promising technique of storing biological living cells and tissues at sub freezing temperatures below -80°C and particularly at a cryogenic temperature below -140°C. In long term storage of cells, tissues and other biological materials, during cryopreservation, cells face various physical and chemical stresses such as cold stress, formation of intracellular and extracellular ice crystals, osmotic stress during cryoprotectant addition and removal, solution effects and chemical toxicity of solutes added as potential cryoprotectants [30]. Despite all the marked developments in cryopreservation protocols, post thaw cell death is still significant, especially within the first 24 h of post thaw culturing. However, the majority of cryoinjuries occur during the freezing and thawing processes [81].

During the development of preservation protocol and preservation medium, scientists have always focused on overcoming cryopreservation induced cell death that mainly occurs due to intracellular ice formation and chemo-osmotic stress leading to morphological and functional damage of cells and subsequent necrosis. To overcome this difficulty, many investigators have used cryoprotective agents like Me_2SO and glycerol to preserve biologics in an extracellular-like carrier solution i.e. standard cell culture medium [1-5]. However, low survival rate of cryopreserved cells is still observed in spite of using most effective cryoprotectants like 10% Me_2SO [6]. The involvement of molecular-based mode of cell death following cell preservation is first reported by Nagle et. al. (1990) under non freezing condition. This was further studied by Baust et. al. (2009) under freezing condition [82-85]. Later on many scientists have reported cryopreservation induced cell death and given efforts to modify cryopreservation solution accordingly. Baust et al. (2000) used Hypothermosol® (HTS) as an intracellular-like hypothermic maintenance solution and modified it by adding 5% Me_2SO and caspase I inhibitor V to HTS to control cryopreservation induced cell death and traumatic necrosis [85]. The modified HTS is known as CryoStor® CS 5N. In their study, the addition of a caspase inhibitor showed a remarkable improvement in cryopreservation outcome as compared to the parent solution without adding inhibitor.

2.3.1 Modes of cell death

Multicellular organisms eliminate unwanted, damaged or dying cells by two main mechanisms: necrosis and apoptosis. Necrosis or pathological cell death is an energy independent process that is characterized by cellular and organelle swelling, formation of blebs and vacuoles, disaggregation and detachment of ribosomes, loss of organelle and cell membrane integrity and rupture, random DNA degradation by endonucleases resulting in cell lysis and activation of active immune and inflammatory response due to release of cytokines [86-92]. Necrosis is triggered by multiple stresses such as osmotic shock, hypothermia and cryopreservation, ischemia, ionic disregulation and exposure to toxic agents. On the other hand, apoptosis or programmed cell death is an energy dependent process involved in homeostatic maintenance of cell number and tissue size in complex organisms. It is characterized by the activation of intracellular proteases from their inactive zymogens (procaspases to caspases), cellular shrinking, chromatin condensation, non random cleavage of DNA into 180 KDa fragments by exonucleases, maintenance of intact cell membrane, externalization of phosphotidylserine, and formation of membrane blebs (apoptotic bodies). There is no recruitment of immune and inflammatory response. Apoptosis may be initiated by various stresses such as anoxia, nutrient depletion, withdrawal of essential growth factor, radiation, cytotoxic agents and exposure to extreme temperature [92-100].

It is believed in many cases that when a cell commits to cell death, first apoptosis is activated and then continues via the classical apoptotic pathway till stresses experienced by the cell exceed normal limits or the energy reserves become very low for apoptosis to continue. It is at this point the cells switch from apoptotic to necrotic pathway of cell death called secondary necrosis [101, 102].

2.3.2 Apoptosis

Apoptosis may be defined as a physiological cell death that occur in response to environmental and developmental signals during which dying cells silently vanish out, without any traces being left behind. In mammals, apoptosis can be triggered by mainly two different pathways (Figure 1) extrinsic pathway (Death receptor pathway) and intrinsic pathway (Mitochondrial pathway). There is an additional pathway termed as the granzyme pathway that involves T cell mediated cytotoxicity and perforin granzyme dependent killing of cell via either granzyme A or granzyme

B. The extrinsic, intrinsic and granzyme B pathways finally converge to a common execution path of apoptosis that involves proteolytic activation of caspases -3 and /or -7 from their inactive procaspases [103-109]. The granzyme-A pathway is a caspase independent cell death pathway that is characterized by single stranded DNA damage [110].

Caspases play a major role in the final stage of "execution" in apoptosis. These are highly conserved family of cysteine proteases with specificity for aspartic acid in their substrates. The cleaveage of these substrates induces the cell death. Caspases are constitutively present in the cytoplasm of most of the cells in the form of single chain proenzyme (Procaspase). These proteins undergo two proteolytic cleavages, wherein first divides the chain into large and small domains and second one cleaves the N-terminal domain (prodomain) to give its fully activated and functional caspase. Caspases have been broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) [29, 30]. Caspase cascade activation and DNA fragmentation are reported to the main features of apoptosis [30]. Apoptotic cells also exhibit phosphatidyl serine externalisation [110].

2.3.3 Extrinsic and intrinsic pathway

The receptors for extrinsic pathways located in the plasma membrane are activated by ligands present in extracellular membrane. Some of the well characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 [111]. These receptors contain a death domain on their cytosolic side. The binding of FasL to FasR results in the recruitment of adaptor protein FADD (Fas-Associated protein with Death Domain). The attachment of TNF ligand to TNF receptor results in the binding of TRADD (tumor necrosis factor receptor type 1-associated DEATH domain protein) and recruitment of FADD and RIP (receptor interacting protein)[39, 40]. FADD then binds to procaspae-8 via dimerization of their death domain (DD) to the DD on the cytoplasmic side of the receptors [111] resulting in the formation of death inducing signalling complex (DISC) [112-117]. Formation of DISC results in the autocatalytic activation of initiator procaspases [31] which activates downstream effector caspases (caspases-3 or -7) that further carry out the degradation of cellular targets. On the other hand, activated caspase 8 can also cleave BH3 only protein Bid resulting in the formation of truncated Bid (tBid). tBid then translocates to the mitochondria, changes the permeability of inner mitochondrial membrane and induces release of cytochrome C, leading to the activation of

caspase 9 and caspase 3 [118]. Extrinsic pathway can be blocked by the expression of c-FLIP (cellular form of FLICE-inhibitory protein), which is physiologically present as a negative caspase-8 that leads to the formation of a signalling non-functional DISC [119-124].

The intrinsic or mitochondrial pathway is triggered by a variety of extracellular and intracellular stresses such as high temperature, oxidative stress (excess of reactive oxygen species), irradiation etc. All these stimuli result in opening of the mitochondrial permeability transition pore (MPT). This results in loss of mitochondrial membrane potential that leads to the release of two specialised types of proteins from the intermembrane space into the cytosol [124]. The first group of proteins consists of cytochrome c, serine protease HtrA2/Omi and Smac/DIABLO [125-128]. These proteins further transduce the apoptotic signal via the caspase-dependent pathway. Following release into cytosol, cytochrome c binds to Apaf-1 in conjunction with dATP, leading to the recruitment of pro-caspase-9 to this complex, termed as apoptosome [129-132] which contains several units of above molecules. Active caspase-9 further activates caspase-3 and initiates proteolytic cascade causing cell death [56]. Smac/DIABLO and Omi/HtrA2 activate caspases by neutralising the inhibitory effects of inhibitors of apoptosis proteins (IAPs) [133,134]. The second group of proteins released from the intermembrane space of mitochondria include AIF (apoptosis inducing factor) [135], endonuclease G [136] and CAD (caspase-activated deoxyribonuclease) [137]. These proteins are released generally at later stages of apoptosis. Apoptosis-inducing factor (AIF) and endonuclease G bring about DNA damage and condensation [61-63] in a caspase independent manner. CAD also results in DNA fragmentation and brings about a more advanced level of condensation [139]. Apoptosis also may occur due to p53 protein by its signalling pathway. It has been reported that signals from external stress can cause DNA damage, which can activate p53 protein. High level of p53 switches on the apoptosis genes and switches off the survival genes. The intrinsic apoptotic pathway is regulated by many p53 regulated genes. These genes contribute to the activation of caspase 9 and ultimately cause apoptosis [117]. p53 protein is normally present in the cytosol at low concentration that prevents it from entering into the nucleus. Various cellular stress activate p53, leading to apoptosis in either transcription dependent manner or transcription independent manner [140]. In transcription independent manner, p53 directly acts on mitochondria and induces apoptosis [141-144]. p53 also enters the nuclei and activates various pro-apoptotic genes in response to generation of Reactive oxygen species (ROS).

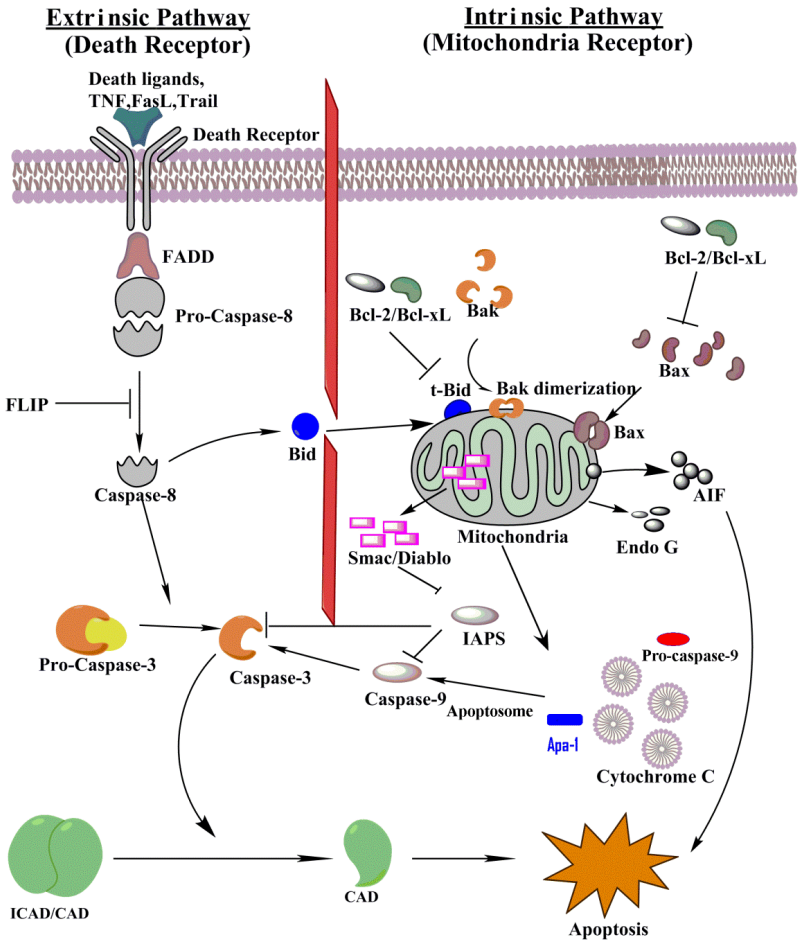


Figure 1: **Graphical representation of two distinctive apoptosis pathways namely death receptor pathway (extrinsic pathway) and mitochondrial pathway (intrinsic pathway). In the extrinsic pathway death receptors present on cell membrane ligate due to the binding of their ligands, resulting in the recruitment of adaptor protein Fas-associated protein with death domain (FADD) and procaspase 8. The complex is an "apoptosome" in which the aggregated procaspase transactivates. The active caspase 8 then acts to cleave and activate the downstream caspases. Caspase 8 also activates Bid which further represents the main link between extrinsic and intrinsic pathways. Truncated Bid (tBid) favours aggregation and permeabilization of Bax and Bak to the outer membrane of mitochondria. In the intrinsic pathway, various forms of cellular stress cause mitochondrial membrane permeabilisation which triggers mitochondrial release of cytochrome c, which binds to Apaf1, which in turn self-associates and binds procaspase-9, resulting in an apoptosome. Transactivation of the complex procaspase-9 to active caspase-9 cleaves and activates downstream caspases. Mitochondria also initiates apoptosis in a caspase independent manner by releasing pro-apoptotic proteins like Smac/Diablo, Omi/HtrA2 (caspase dependent), AIF and Endo G.**

Mitochondrial pathway is basically regulated by two types of proteins namely pro-apoptotic and anti-apoptotic proteins belonging to the Bcl (B-cell lymphoma 2) family of proteins [144] which are controlled and regulated by the tumor suppressor protein *p53* [145]. The pro-apoptotic proteins like Bcl-10, Bax, Bak, Bid, Bad, Bim and Bik insert into the mitochondrial membrane, resulting in release of cytochrome c from the intermembranal space of mitochondria into the cytosol [146]. In contrast, anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG, prevent cytochrome release by binding and inhibiting pro-apoptotic proteins.

2.3.4 Necrosis and autophagy

Necrosis also known as pathological cell death is a term used to define "cellular murder" [65]. Morphologically, apoptosis and necrosis are very different. However, with the development of the techniques which differentiate apoptosis and necrosis, it is revealed that there are many examples when biochemical and morphological characteristics of both apoptosis and necrosis are found in the same cell. This indicates that apoptosis and necrosis are just two extremes of a series of cell death program that occur in a dying cell. Furthermore, apoptosis is considered as a process of cell elimination without disruption of the plasma membrane, while necrosis is the result of rapid efflux of cell constituents into the extracellular space. However, it is reported that in vitro apoptosis finally leads to the disruption of plasma membrane due to absence of phagocytosis.

Members of the TNF (tumor necrosis factor) receptor family Fas, and TNF-related apoptosis-inducing ligand (TRAIL) not only initiate apoptosis but also necrosis [147,148]. RIP 1 is a death-domain-containing kinase associated with the death receptors that is required for activating necrosis by the death receptor ligands (FasL or TNFα) [147]. Another important sensor is massive DNA damage brought about due to excessive ROS production. Excess of DNA damage activates Poly (ADP-ribose) polymerase (PARP), thus depleting ATP and activating necrosis due to energy depletion [149,150]. PARP is a nuclear enzyme activated in response to excessive DNA breaks. Upon activation it binds to oligomers of ADP-ribose, thus utilizing ATP for synthesis of PARP substrate NAD^+, Ca^{2+} ions plays an important role as an inducer and secondary messenger involved in receptor mediated necrosis [151]. Mitochondria play a crucial role in determining cell fate under stress conditions. It is a source of death promoting factors as it can initiate and amplify caspase dependent death pathway by effluxing cytochrome C, or initiate cell death in a caspase

independent manner by secreting AIF or EndoG. It also produces ROS that may trigger necrosis [152-153]. Being the factory for ATP production, mitochondria chooses between ATP dependent and independent processes depending upon its availability for bringing about cell death under stress conditions. The anti-apoptotic members of Bcl-2 family (Bcl-2, Bcl-XL) can inhibit both apoptosis and necrosis [154-158]. A protein belonging to Bcl-2 family named BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) has been discovered possessing pronecrotic activity [158]. Stress kinases like JNK (c-Jun N-terminal kinases) and p38 activated by ligation of death receptors, oxidative stress and DNA damage have the ability to initiate both apoptotic and necrotic programs. Inhibition and suppression of these stress kinases decreased necrotic death in many cells [159, 160]. On the other hand, stress induced apoptosis protected by AKT kinase and MAP kinase ERK can also provide protection against necrotic cell death [161-164]. This indicates that proapoptotic (JNK and p38) and antiapoptotic (AKT and ERK) function in similar manner in necrosis (Figure 2).

Finally, the terminal step of necrosis is the activation of proteases. Caspases may or may not be involved in the execution stage of necrosis as it has been observed in many cases that inhibition of caspases during stress conditions may trigger necrosis. Calpains, cathepsins and serine proteases are the main proteases involved in necrosis [165].

Besides apoptosis and necrosis, autophagy has also an important role in cell death machinery. Autophagy is a degradative process where cyoplasmic organelles are hydrolysed to provide metabolic precursor to fulfill the energy demand of the cells which are under stress condition such as starvation. Though, initially it is believed that autophagy plays an important role in cell survival by elimination of damaged organelles, but recently it has been reported that Autophagy also kills the cells [166]. This autophagy cell death mechanism is also called as type II cell death mechanism. It works as secondary death mechanism when apoptosis death pathways are not available. Though apoptosis and autophagy have distinct mechanism of action, but both are regulated by each other [167].

2.3.5 New Generation Cryopreservation and Post thaw culture medium

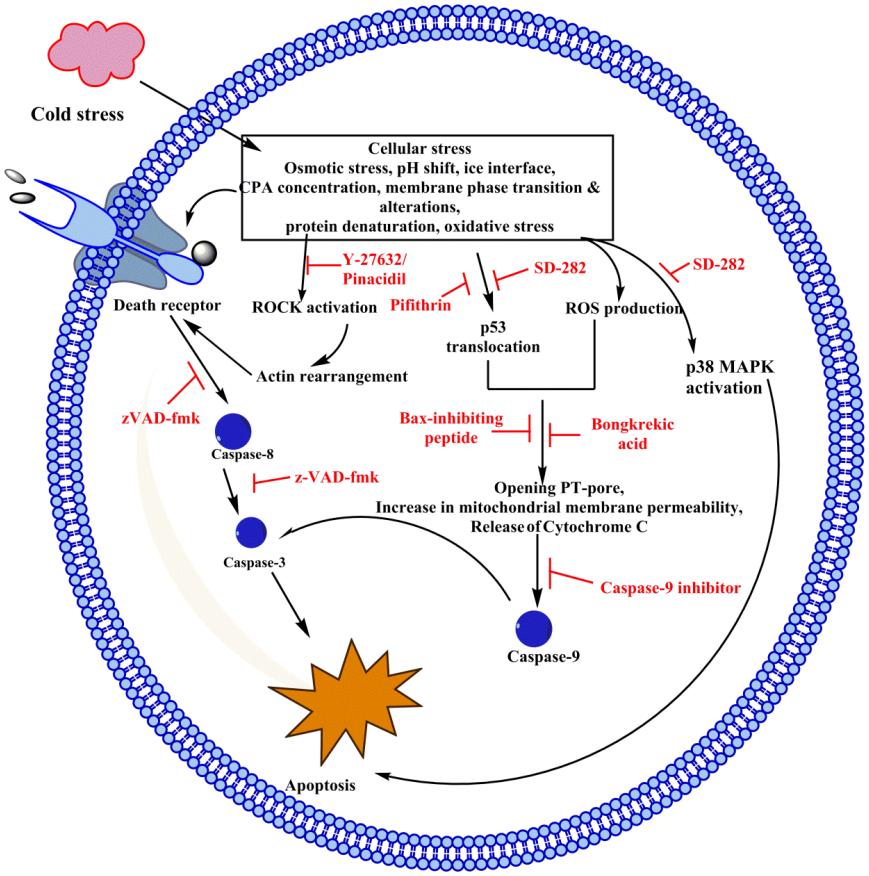


Figure 2: Mediators of apoptosis during cryopreservation and effective apoptosis inhibitors [254]

LC3B-II protein is the indicator of Autophagy [168]. The unprocessed form of LC3 (pro LC3) is proteolytically cleaved by Atg4 protease, resulting in formation of LC3-I (carboxy terminal with exposed glycine).Upon activation of autophagy the LC3-I is conjugated with phosphatidylethanolamine (PE) moiety in presences of Atg7, Atg3, Atg12, Atg5 and Atg16L multimers to generate LC3-II. LC3-II is the only well characterized protein that is phagophore to lysosomal degradation [168].

2.3.6 Motivation for cryopreservation of MSCs

Mesenchymal stem cells (MSCs) are becoming increasingly important source of stem cells that have the potentiality to be used for the regenerative and or repair of damaged tissue and thus restore the tissue functionality. MSCs have the ability to proliferate and differentiate into a series of mesoderm type lineages includes osteoblast/osteocytes [32], chondrocytes [33], skeletal

muscles[34], adipocyte and myocytes, vascular cells and the like [35]. With the advancement of MSCs isolation and expansion, the development of technology and or strategy for storing and preserving of MSCs for long time without change in its properties is of paramount importance so that these MSCs can be used when patient needs. The current technique using Me_2SO as CPA has potentiality for preservation of various cells and tissue. However, the use of Me_2SO has detrimental effects causing cell death. Me_2SO is considered to possess an impact on the genetic profile and induce differentiation in murine stem cells[36]. Use of nontraditional cryoprotectant in preservation of stem cells a crucial facet because the entire long term storages are done with customary Me_2SO and glycerol. Several studies have already been done on the cryopreservation of mesenchymal stem cell with trehalose and few with ectoin also [13, 37-47]. However, very little work has been done on long term cryopreservation of hMSC using natural osmolytes and impact of these cryoprotectants on long time storage of MSCs. The aim of our present study is, therefore, to investigate the effect of trehalose and ectoin as major cryoprotectant using a reduced amount of Me_2SO for long term cryopreservation of MSCs.

Furthermore, many factors affect the effectiveness of MSCs cryopreservation, including incubation temperature, the freezing medium, cooling rate, storage temperature, and cell concentration during freezing and duration of storage, thawing temperature, and thawing medium. And also the cooling profile which is important for the cell viability is not taken into consideration [125]. Long time biopreservation technique that maintains ex-vivo TECs viability and functioning is one of the most important research aspects of modern tissue engineering and regenerative medicine. The ability to preserve the integrity of TECs outside the native environment for an extended period shall not only save time of the donors and the recipients, but it may also be possible to establish cell bio-banks worldwide for the safe preservation of high quality TECs products for use when need arises. Such a ready to use TECs would support the implementation of routine cryopreservation practices during preparation of even engineered cells and tissues for the clinical applications when needed.

Chapter III

Scope and Objective

Mesenchymal stem cell is an important and promising cell source for various therapeutic applications and development of various tissue constructs/tissue grafts through tissue engineering approach. Thus there is a worldwide demand of mesenchymal stem cells (MSCs) and MSCs seeded scaffold (TECs) in clinical sector for the treatment of variety of blood and tissue related diseases. With the advancement of isolation and expansion of MSCs and development of tissue

engineering products utilizing MSCs as an important cell source, the development of technology and/or strategy for storing and preserving of such clinical products for long time without change in their properties is of paramount importance keeping in view of their actual use when patient needs. Cryopreservation has been found to be the most effective method of preserving cells using Me_2SO as CPA. However, the use of Me_2SO in cryopreservation medium cause cell death and also harmful to the patient.

In this context, one of the key challenges is the development of a cryopreservation strategy using an alternative freezing medium that can preserve MSCs and MSCs seeded scaffold for a longer period of time with high recovery of cells, differentiation ability and structural integrity of the scaffold used in TECs formation.

Therefore, the main objective of the present research is the development of cryopreservation strategy using natural osmolytes for long term cryopreservation of MSCs and MSCs seeded scaffold.

The specific **objectives** of the present research work are as follows:

1. To select potential non-toxic (natural) cryoprotective agents
2. To evaluate the potentiality of natural osmolytes in preserving MSCs
3. To study the role of apoptosis pathways in cryopreserved induced cell death of MSCs
4. To optimize the controlled rate freezing condition for cryopreservation of MSCs using natural CPAs
5. To evaluate the potentiality of natural CPAs in preserving MSCs seeded constructs
6. To optimize the controlled rate freezing MSCs seeded constructs using natural CPAs

Scope of work

Cryopreservation is proven to be an efficient method for preserving varies of cells and other biological species. This method uses Me_2SO is as a potential CPA which is reported to have several detrimental effect causing cell death. To overcome the limitation and adverse effect of

Me_2SO, the recent research is directed towards the search of natural CPAs which seem to be promising without having harmful effect like Me_2SO. However there is very limited research has been done on the study of these natural osmolytes for its actual use in long term preservation of MSCs and other clinical product using MSCs.

Therefore the present investigation aimed to formulate efficient cryopreservation medium from potential natural CPAs for preserving MSCs and develop a cryopreservation system that will be helpful to preserve MSCs and other tissue products on long term basis.

The specific scope of work under this research includes the following

1. **Selection of potential CPAs alternative to Me_2SO**

In this part, efforts would be given to select a single or a combination of potential natural cryoprotective agents by a preliminary study on cryopreservation of umbilical cord blood (UCB) derived MNCs in a controlled rate freezer. Different batches of cryopreservation solutions prepared using extracellular cryoprotectants like trehalose, hydroxyl ethyl starch, polyvinyl pyrrolidine and intracellular CPAs like erythritol, taurine and ectoin will be investigated and the most effective CPAs will be selected for further study on cryopreservation of MSCs.

The formation of reactive oxygen is another important factor which causes cellular damage during low temperature storage. In this context, the addition of selective antioxidants in cryopreservation medium is reported to be beneficial to overcome this oxidative effect and thus improve post-thaw cell viability. Keeping this in view, cryopreservation experiments will be performed to investigate the performance of the best obtained freezing solution in presence of potential antioxidants such as catalase, co-enzyme Q10 and N-acetyl cystine and thus an optimal freezing solution is expected to be obtained giving maximum cell viability after cryopreservation.

2. **Development of an effective freezing solution for cryopreservation MSCs**

This phase of research will focus on the development of a freezing solution from the selected CPAs and antioxidant for long term preservation of MSCs. Different batches of freezing solutions will be prepared using individual as well as combination of selected CPAs. The solutions will be evaluated by cryopreservation experiment to see their ability towards preserving MSCs and the most effective freezing solution will be selected.

3. Study on apoptosis signaling pathways

Apoptosis that involves program cell death is an important phenomenon that occurs in cryopreserved cells. The inhibition of cryopreserved induced apoptosis is important to improve the post thaw viability of cryopreserved cells. Therefore, an effort will be given to study the influence of important caspase and calpain inhibitors on the cryopreservation of MSCs and the potential inhibitors will be selected to improve further the effectiveness of the developed freezing solutions.

4. Optimization of cryopreservation condition for MSCs

The controlled rate freezing condition will be optimized further by studying the effect of key parameters such prenucleation cooling rate, nucleation temperature, cold spike temperature, post nucleation holding temperature, post nucleation cooling rate, cell concentration and storage temperature using the best obtained freezing solution to maximize the MSCs recovery

5. Evaluation of developed freezing solution for preserving MSCs seeded scaffold

In this phase, research work will further be extended to evaluate the potentiality of the freezing solution developed for MSCs cryopreservation towards the preservation of TECs formed from MSCs seeded on scaffold.

6. Optimization of cryopreservation condition for TECs

The controlled rate freezing condition will be further optimized by studying the effect of key parameters such as prenucleation cooling rate and nucleation temperature using the best obtained freezing solution to maximize the MSCs recovery in TECs

Chapter IV

Materials & Methods

Materials and Methods

4.1 Chemicals and culture media

4.1.1 Cryoprotective agents

The various cryoprotective agents such as trehalose, Me_2SO, hydroxy ethyl starch (HES), ectoin, erythritol and poly vinyl chloride (PVC) used in this thesis work were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.1.2 Antioxidants and inhibitors

The chemicals such as co-enzyme Q-10, ascorbic acid, catalase and N-acetyl cystine were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as antioxidants. Caspase inhibitors (z-LEHD-FMK, z-IETD-FMK, z-DEVD-FMK, z-VAD-FMK) and calpain inhibitors (3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid) were purchased from BD pharminogen (Becton Dickenson, San Jose, CA, USA).

4.1.3 Cell culture media and antibodies

Dulbecco's modified eagle medium (DMEM), Penicillin-Streptomycin solution, fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), chondrogenic basal medium and supplement, Collagenase type I and 0.25% Trypsin /ethylene diamine tetra acetic acid (EDTA) solution were procured from Gibco (BRL, USA). Phalloidin-Alexa Fluor 488 conjugate, calcein green AM and JC1 were used of Invitrogen, USA. Ficol Hypaque solution (Hi sep LSM 1073), adipogenic differentiation media, Giemsa 10x stain, Triton X-100, formaldehyde, reagent for MTT assay and Commassie blue G-250 are of HiMedia labs, India. Growth factors EGF and bFGF, BD FACS lysis buffer, propidium iodide (PI), CD34-PE, CD44-FITC, CD45-PerCP/cy5.5, CD73-PE/APC, CD90-APC, CD105-PE and HLA-DR-PerCP/cy5.5, Annexin-V conjugated with FITC were from BD pharminogen (Becton Dickenson, San Jose, CA). Dexamethasone, staurosporine, α-MEM, L- glutamine, L-ascorbate, β-glycerophosphate, bovine serum albumin (BSA), Oil red O, Alizarin red S, Safranin O were from Sigma-Aldrich (St Louis, MO, USA). All the tissue culture plastic wares and RNase were from BD falcon and MP Biomedicals, USA, respectively.

4.2 Collection and processing of umbilical cord blood

Umbilical cord blood (UCB) samples were collected from Ispat General Hospital, Rourkela, India with prior consent of the full-term delivery patients. A blood collection bag containing 17 ml of

citrate phosphate dextrose adenine (CPDA) anticoagulant was used for aseptic transfer of the cord blood sample each time and brought to our culture laboratory for processing. The Institutional ethical board approved the collection procedure.

4.3 Isolation and culture of MNCs

The MNCs were isolated from umbilical cord blood by standard Ficol hypaque density gradient method [33]**.** 30 ml of cord blood sample was diluted with RPMI 1640 in the ratio of 4:1 (v/v) and added to 20 ml of ficol hypaque solution. The solution is then centrifuged (swing bucket rotor) at 430×g for 30 min at 4°C. From the three layers formed, the middle layer (buffy coat) containing MNCs was separated, followed by the addition of 1x Lysis buffer (1:9 dilution of 10x stock buffer in distilled water) and incubated for 10min at room temperature. The cell suspension was centrifuged at 200×g for 10 min at 4°C and supernatant was discarded. The pellet was washed to remove lysis buffer and other impurities by centrifugation twice with D-PBS at 200×g for 10 min each time. Finally the pellet obtained was resuspended in expansion medium consisting of α-MEM, 10% FBS, 2mM glutamine and 1% antibiotic-antimycotic solution and plated at density of 1×10^6 cells/cm^3. The non adherent cells were discarded after incubating culture plates at 37°C with 5% humidified CO_2 for 24 h. The adherent cells were washed thoroughly with D-PBS/EDTA and then supplemented with freshly prepared expansion medium. The cells were cultured with media change weekly twice.

4.4 Sorting and culturing of MSCs

Mesenchymal stem cells (MSCs) were isolated from the cultured human mononuclear cells (hMNCs) by florescence activated cell sorting (FACS ARIA III; BD Biosciences) using antibodies like CD90-APC, CD73-APC, CD105-APC, CD44-FITC, CD45 PerCP Cy5-5, HLA-DR PerCP Cy5-5, CD34 PE. UCB derived MSCs was isolated from MNC populations by positive selection using CD44, CD90, CD73vand CD105 positive antibodies (BD Biosciences, USA). After sorting, the cells were counted by haemocytometer with Trypan Blue dye used for staining dead cells. MSCs were suspended in an expansion medium consisting of Dulbecco's modified eagle medium (DMEM), 10% FBS and 1% antibiotic-antimycotic solution and plated initially at cell density of $1x10^6$cells/cm^3 in T_{50} culture flasks (Beckon Dickinson, San José, CA, USA). Non adherent cells were discarded after incubating culture plates at 37°C with 5% humidified CO_2 for 24 h. The adherent cells were washed thoroughly with D-PBS/EDTA two or three times and then

supplemented with freshly prepared culture media. Thereafter the media was changed twice weekly.

4.5 Characterization of cells

4.5.1 Morphological characterisation

MSCs were harvested and morphology was observed from the culture flask under phase-contrast inverted microscope. Images of MSCs were captured using a Carl Zeiss Axiovert 40 microscope. No specific staining was carried out.

4.5.2 Immunophenotypic characterisation

The specific surface antigens expressed by MSCs were characterized by flow cytometry analysis. The trypsinized post-thaw cells (5×10^5 cells per sample) were stained with human monoclonal antibodies against CD44, CD45, CD73, CD90, CD105, CD34 and HLA-DR. The CD expressions were analyzed using a flow cytometer (Becton Dickinson and Co, San Jose, CA, USA).

4.6. Cryopreservation of cells

4.6.1 Preparation of cryopreservation solution

1×10^6 cells/ml MNCs were transferred into standard 1-ml cryovials (Nalge Nunc, IL, USA) containing each batch of freezing solutions prepared from 10mM extracellular cryoprotectant, 5mM intracellular cryoprotectant and 100µg of antioxidant in FBS at 6ºC. The concentrations of CPAs were selected based on the published literature [169-173]. Each time, MNCs were treated with intracellular cryoprotective agents for 20mins followed by the treatment with extracellular cryoprotectants for 10 mins. Similarly, MNCs were resuspended in 10% Me_2SO culture medium solution without FBS at 4°C as a positive control. For the culture of MSCs, medium was replaced with DMEM containing ectoin and trehalose at 0.3 M concentration each and cultured at 37 °C in CO_2 incubator for overnight [174]. All freezing vials contained 1ml of final freezing medium after the addition of cells. Different molar concentrations of osmolytes were added to the cryovials. In all cases, 100µg/ml catalase was added to the cryovials. Cryopreservation solution used for this study was prepared by adding different concentration of molar ration of trehalose, ectoin and 100 µg/ml catalase.

4.6.2 Cryopreservation experiments

The sealed cryovials containing each cryoprotectant solution and cells were placed in a controlled rate freezer (Kryo360-17, Planer, Inc.., UK) as shown in Figure 3. The cryopreservation experiments were carried out following the programmable cooling protocol [175] as- ramp 1, for 4ºC for 10min; ramp 2, -3ºC/min to -10ºC/min; ramp 3, -15ºC/min to -30ºC/min; ramp 4, -3ºC/min to -60ºC; Ramp 5, -10ºC/min to -180ºC. After achieving -180°C, the cell suspension was taken out of the freezer and stored in vapor phase storage tank (-180 ºC) for further use. Each batch of experiments was repeated three times.

Figure 3: Controlled rate freezer for cryopreservation of MSCs

4.6.3 Thawing and post-thaw culturing

The cryopreserved cells were thawed by immersing cryovials rapidly in water bath at 37°C and then subjected to gradual dilution of cryoprotectant solution with prewarmed thawing medium (DMEM + 10% FBS). The cells were then centrifuged at 200×g for 5 mins and resuspended in DMEM supplemented with 10% FBS, 100U/ml penicillin G, 100 mg/ml streptomycin and 10ng/ml epidermal growth factor (EGF). The cells were then cultured in 6-well tissue-culture plates at 37ºC in a humidified atmosphere of 5% CO_2 [177, 178].

4.6.4 Cell viability assay

After 24h of incubation, medium containing the dead cells were separated and the attached cells were trypsinized. All the cells were stained with propidium iodide (PI) and analysed by flow cytometry for cell viability. Flow cytometry analysis was done within 10min trypsinization [176].

4.6.5 Cell proliferation assay

1×10^4/ well of cell were seeded into 96-well plates with 100µl of growth medium that consists of DMEM supplemented with 10% FBS, and 10ng/ml EGF and incubated at 37ºC and 5% CO_2. These cells were detached with trypsin/EDTA mixture and the cell number counted from day 1 to 7 day. The number of viable cells was counted using automated cell counter with cells stained with 0.2% trypan blue [177].

4.6.6 MTT assay

The cell viability of cryopreserved cells was further verified by MTT (5-dimethylthiazol-2-y1]-2, 5-diphenyl tetrazolium bromide) assay using standard micro plate absorbance reader [178]. Post thaw cryopreserved MNCs was treated with 10% Me_2SO (control) to determine baseline cell viability. For viability tests of different batches of CPA solutions, the post thaw cells at concentration of 1×10^5 cells/ml were plated (in triplicate) by adding 10µl MTT solution in each well and incubated at 37ºC for 4h in 5% CO_2. The optical density of the solution is then measured using ELISA plate Reader (Perkin Elmer) at 570 nm.

4.6.7 Apoptotic assay

Post-thawed MNCs were labeled with AV-FITC (Annexin V conjugated with FITC) as per manufacturer's instruction (BD Pharmingen, San Diego, CA) [179] and 1×10^6 cells/ml were suspended in PBS (pH 7.4) supplemented with 2mM $CaCl_2$ solution. AV-FITC binding was analyzed with respect to fresh cells as positive control. The cells were incubated with AV-FITC for 15 min in dark at room temperature and then subjected to flow cytometry analysis using FACS Aria III flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 10,000 events were acquired from the region defined for MNCs and analyzed by BD FACSDiva Software 6.0 (*Becton Dickinson, San* Jose, CA). Compensation was required for the annexin V assay. Firstly, this was done by analyzing an autofluorescent control to determine the level of autofluorescence. Secondly, annexin V-FITC labeled cell population was analysed to establish optimal compensation for annexin V-FITC. Finally, only PI-labeled cells were analyzed on these settings

and the cell populations was recompensated by subtracting the overlapping of PI and FITC. Cells cryopreserved with 10% Me_2SO (v/v) were used as negative control.

4.6.8 Mitochondrial membrane potential assay

Mitochondrial membrane potential of cryopreserved MSCs were evaluated by flowcytometry using JC-1 as per method previously described [180]. Post thaw cryopreserved MSCs for 12 month were exposed to JC-1 for 15min at 10µg/ml concentration in culture medium and incubated in 5% Co_2 at 37°C. For the assessment of JC-1 staining, a total of 10,000 gated events were analyzed per sample. In addition, the entire samples were viewed by fluorescence microscopy to confirm JC-1 labeling.

4.6.9 Differentiation potential

After 12 months of storage in vapor phase liquid nitrogen, the cryopreserved MSCs were thawed by usual procedure. Both cryopreserved and fresh (non-cryopreserved) MSCs were then seeded at a concentration of 3×10^3 cells/cm^2 into a 6 well-plates coated with type I collagen and cultured in DMEM medium supplemented with 10% FBS for 24h.

Osteogenic differentiation

The osteogenic differentiation ability of cryopreserved MSCs was assessed by following the method described in literature [181]. The differentiation of hMSCs were induced by culturing with osteogenic differentiation medium consisting of DMEM supplemented with 100nM dexamethasone, 0.05µM ascorbate-2-phosphate, 10mM β-glycerophosphate, 1% antibiotic/antimycotic and 10% FBS for 14 and 21days. The culture medium was replaced twice a week. After a specific period of culture, cells were washed twice with PBS and fixed with 2% methanol for 10 min at room temperature. The cells were then stained with 1% alizarin Red S for 15min and used for assessment of mineral deposition.

Adipogenic differentiation

For the assessment of adipogenic differentiation potential, cryopreserved and unfrozen hMSCs were cultured in adipogenic differentiation medium. The medium contains DMEM supplemented with 10% FBS, 0.5 Mm isobutyl-methyl xanthine, 1µM dexamethasone, 10 µg/ml insulin, 200 µM indomethacin, and 1% antibiotic/antimycotic. The culturing was done for 7 and 14 days with

the change of media twice a week. The cells were washed with PBS and then fixed with 2% methanol. The cells were stained with oil red O to visualize the intracellular lipid droplets.

Chondrogenic differentiation

The chondrogenic differentiation of post thaw cultured cells was performed using pellet culture method as previously described [181]. Briefly, 2.5×10^5 cryopreserved and non-cryopreserved hMSCs were centrifuged at 800rpm for 15min. The pellets were resuspended in chondrogenic differention medium, the composition of which is DMEM supplemented with 10% FBS, 1% penicillin, 1% insulin-transferrin-selenium, 10^{-7}M dexamethasone, 150µM ascorbic-2-phosphate, 20 µM linoic acid and 0.1ng/ml TGF-β. The cells were incubated at 37ºC and 5%CO_2 environment for 21days. The chondrogenic culture medium was replaced twice a week. The cells were washed in PBS and fixed with methanol before staining with Saffranin O.

4.6.10 CFU-F assay

Colony forming assays were done following the procedure described in publish literature [182]. Post thawed as well as non cryopreserved MSCs were seeded in 75 cm^2 tissue culture flasks at a concentration of 8 cells/cm^2 in cell culture medium. The medium was changed every 2 to 3 days. After 12 days, cells were washed with PBS and fixed with methanol for 15 min. CFU-F colonies were stained with 0.4%w/v Giemsa (diluted with deionized water for 30 min) and the culture plate was observed under optical microscope (Leica DM750 equipped with digital camera). Colonies were considered as clusters of more than 50 cells.

4.6.11 Cell cycle analysis

Post thaw MSCs were fixed with the addition of 70% ethanol and stored at 4°C. The ethanol-suspended cells were centrifuged at 300 *g* for 5 min and the pellet was suspended in 2 ml of D-PBS. This pellet was centrifuged again to remove any ethanol and finally the pallet was suspended in 1ml D-PBS/EDTA containing 20 µg/ml PI and 100 µg/ml RNase A for 30 min. Cells were then analyzed by flow cytometry by gating on PE-area versus PE-width discriminating all the doublet population [183].

4.6.12 Western blotting

Cryopreserved MSCs were suspended in buffer containing 20 mM TRIS, 2 mM EDTA, 150 mM NaCl, 0.5%Triton X-100 and protease inhibitors (Sigma). The samples were sonicated,

centrifuged and the pellets were discarded. The cryopreserved MSCs were assayed for protein concentration following standard Bradford assay. Protein extracts of 50µg/sample, control or post-thaw cryopreserved MSCs were separated on 10% SDS PAGE and transferred to PVDF membrane. The membranes were incubated with primary antibodies such as caspase-3 (1:1000, BD Biosciences), caspase-8 (1:250, BD Pharmingen), caspase-9 (1:250, BD Pharmingen), cytochrome C (1:250, BD Pharmingen), PARP (1:500, BD Pharmingen), Bid (1:250, BD Pharmingen) and calpain (Cell Signaling Technology, Inc., Beverly, USA) for overnight [185]. The membranes were then incubated with HRP Goat anti-mouse Ig, following the manufacturers recommended protocol and visualized on film with the ECL substrate (Abcam Inc., Cambridge, MA, USA).

4.6.13 Optimization of cryopreservation process

The cryopreservation of MSCs was optimized by investigating the effects of five aspects of cryopreservation cooling profile, storage temperature and cell concentration on cell viability. The cryopreservation experiments were carried out using (Kryo Cooler 1.6 Planner Biomed, U.K). The various controlled rate freezing parameters investigated are: prenucleation cooling rate (1ºC/min, 2.5ºC/min, 5ºC/min, and 10ºC/min), holding temperature (0ºC, -2.5ºC, -5ºC, -7.5ºC and -10ºC), nucleation temperature (-2.5ºC,-5ºC, -7.5ºC and -10ºC), cold spike temperature (-20 ºC, -40ºC, -60ºC, -80ºC) and post nucleation cooling temperature (1ºC/min, 2.5ºC/min, 5ºC/min, 10ºC/min). Various MSCs concentrations were compared at 0.5, 1, 3, 6×10^6 /ml. To select optimal storage temperature, final storage temperature was maintained at -80°C, -150°C, or -196°C. For all experiments, in addition to comparing their own conditions, the medium composition used as 0.3M trehalose, 0.3M ectoin, 100µg/ml catalase and 50µm/ml of calpain inhibitor (PD 150606) and general caspase inhibitor (z-VAD-fmk) each. The cooling rate of 1°C/min, storage temperature of −180°C and cell concentration of 2×10^6 /ml. were maintained.

4.7 Cryopreservation of TECs

4.7.1 Preparation of scaffolds

3-D nanofiberous mats developed using semi automated electrospinning machine (ESPINNANO M/s. Physics Instruments Company, India) was used as scaffold for TECs formation. The detail description of the method is reported in our patent filed (Patent number: 713/KOL/2012). In brief, Silk fibroin (SF) was extracted from eri and tasar silk cocoon by degumming method using aq.

Na_2CO_3. SF blend were prepared and made electrospinnable by dissolving silk fibroin of eri and tasar silk in achloroform and formic acid mixed in a ratio of 60:40v/v. The blend was electrospinned at 15kV to generate nanofibrous scaffold of thickness 300µm and the average fiber diameter of nanofiber is measured as 350nm (range 200-500nm). The contact angle and porosity were determined as 54.7º and 80-82%.

4.7.2 Formation of TECs

The nanofibrous sheets of dimension 6mm diameter and 400µm thickness were used in this study. The scaffolds were autoclaved and then sterilized with 70% EtOH. The sample was further exposed to ultraviolet irradiation for 40min to avoid any morphological change. The sterilized scaffolds were washed thoroughly with PBS and neutralized with growth medium at 37°C and 5% CO_2. 5.0×10^5 [48-49] cells/ml (MSCs) were seeded on scaffold by static method and cultured in DMEM containing 10% MSC qualified fetal bovine serum and 1% antibiotics for a period of three weeks.

4.7.3 Preparation of cryopreservation solutions

Cryopreservation solutions were prepared with varying compositions as follows: (1)10% (v/v) Me_2SO with 50% (v/v) fetal bovine serum (D_{10}) and $T_{30}/E_{30}/C_{100}$. All solutions were prepared with DMEM media.

4.7.4 Cryopreservation experiment

The precultured TECs were exposed to cryoprotectant solutions for 10 min at 4°C and then transferred to a high-density polypropylene sterile pouch. The cryopreservation study was performed in the computer-controlled programmable freezer following two steps cooling (shown in Figure 4). TECs samples were cooled at the rate of 1°C/min down to -150°C and then immersed into liquid nitrogen bath [186,187]. After 7 days of cryopreservation, the samples were removed from liquid nitrogen and were thawed by shaking at 37°C. The TECs were transferred from pouch into a 35-mm culture dish containing DMEM solution and washed thoroughly to remove cryoprotectants from TECs. Precultured TECs without cryopreservation was used as control.

Figure 4: Controlled rate freezer for cryopreservation of TECs

4.7.5 Characterization of TECs

4.7.5.1 Cell morphology by SEM

Scanning electron microscopy was performed before and after cryopreservation at a specific interval of time. Cryopreserved TECs were fixed in 2.5% glutaraldehyde at 4ºC overnight. TECs were washed three times with deionized water and serially dehydrated with increasing gradient of ethanol. Then samples were subjected to SEM study at 15kV accelerating voltage[180-181]. Elements, especially calcium (Ca), phosphorous (P) and oxygen (O) on the surface of post-thawed TECs after culture for 28days in osteogenic media were analyzed by energy-dispersive X-ray spectroscopy (EDX, Philip Model XP 30 CP).

4.7.5.2 Cell viability

After cryopreservation study, TECs were removed from DMEM medium and washed twice with PBS buffer. MSCs were removed from TECs by treating with 0.25% trypsin and 1mM EDTA. The cells were then centrifuged at 200×g for 5 mins and resuspended in PBS. The percentage viability of cells was assessed by flow cytometric analysis using propidium iodide [190] staining.

4.7.5.3 Cell metabolic activity

The metabolic activity of MSCs in scaffolds were quantitatively determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide formazan) assay at an interval of 3, 5 and 7 days of culture period. Culture medium was aspirated and washed with 600µl prewarmed DPBS. 400µl prewarmed culture medium supplemented with 40µl MTT solution [2.5mg/ml MTT dissolved in Dulbecco's Phosphate Buffered Saline (DPBS)] was added to each sample. After 3hr incubation at 37°C, the medium was removed from each well and scaffolds were transferred to 24-well petri dish. 250 µl Me_2SO was added to each well to dissolve the formazan crystals[191-192]. The resulting solution was removed and centrifuged at 500g. The optical density of the supernatant was measured spectrophotometrically at 595 nm, using an ELISA plate Reader (Perkin Elmer).

4.7.5.4 Cytoskeleton analysis by confocal microscopy

Post-thaw TECs were fixed with 2% paraformaldehyde in PBS at room temperature for 5min. This was followed by 40-min treatment with 2% Bovine serum albumin (BSA) and 1% glycine in PBS to block any non-specific staining. Samples were then permeabilized with 0.5% Triton X-100 in PBS for 15 min. For actin staining, samples were incubated with Alexa-Fluor 488 conjugated phalloidin at 37^0 C for 40min. in dark condition. Samples were mounted on glass slides and confocal images of the samples were acquired using Leica TCS SP5 X Super continuum Confocal Microscope [193-194].

4.7.5.5 Osteo-induction assessment

The osteogenic differentiation potential of cryopreserved TECs was assessed and compared with control. Cryopreserved and non cryopreserved TECs were cultured in osteogenic medium consisting of high glucose–DMEM (Gibco, Invitrogen) supplemented with L-ascorbic acid-2-phosphate (50mg/ml), β-glycerophosphate (10 mM), and dexamethasone (100nM) (Sigma) 24 hr after cryopreservation [196-197]. Culture media was allowed to change every 3 days.

4.7.5.6 Alkaline phosphatase activity

The alkaline phosphatase (ALP) activity of post thawed TECs was determined quantitatively by 1, 7, 14, and 21days [195] of TECs culture. The cell lysate from cryopreserved TECs was prepared by treating TECs with 1% Triton X-100 for 50 min and sonication for 10 min. The cell lysate is then added to ALP substrate (para-Nitrophenylphosphate) with a ratio of 1:1. After 40 min of incubation, 1 M sodium hydroxide (Sigma) was added to block the enzymatic reaction. The OD of the solution

mixture was measured at 405nm in a microplate spectrophotometer. Each experiment was performed in triplicate.

4.7.5.7 Histological study

The mineralization of hMSCs on the scaffolds was analyzed and quantified by alizarin red-assay. The cells grown on the scaffolds were washed with PBS and fixed with paraformaldehyde for 15min. The cell-seeded constructs (TECs) were thoroughly washed with distilled water prior to the addition of 500ml of 40mM alizarin red stain (pH 4.1). The plates were incubated at room temperature for 20 min with gentle shaking. After aspiration of un-incorporated dye, the wells were washed thoroughly with distilled water with stirring for 5 min. The plates were left for 2 min to facilitate removal of excess water.

4.7.5.8 Osteogenic-specific gene expression

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the instructions of manufacturer. After the treatment of cell extract with DNase (Invitrogen), 1 μg of total RNA was used for cDNA synthesis (Superscript First-strand synthesis system, GibcoBRL, Life Technologies) following the protocol of manufacturer. RT-PCR was performed to determine the expression of genes using primers (Table-3) for the osteogenic differentiation of osteopontin [OPN], ALP and core-binding factor alpha 1[Cbfa 1]. Glyceraldehydes phosphate dehydrogenase [GAPDH] was used as a house keeping gene for internal control. The polymerase chain reaction (PCR) was carried out using BIO-RAD Mycylcer and analyzed by software Bio-Rad. Detail description of the procedure described in published literature [195].

Table 3: The specific primers used for RT-PCR

Gene	Abbr.	Forward primer	Reverse primer
Collagenase 2	COL2	GTGGGCTTCCTGGTGA	CAGCACCTGTCTCACCA
Osteocalcin	OCN	CCCAGGCGCTACCTG TATCAA	GGTCAGCCAACTCGTCACAGTC
Osteopontin	OPN	TGTGAGCTCAATCCGGACTGT	CCGATAGGCCTCCTGAAGC
Glyceraldehydes-3-phosphate dehydrogenase	GAPDH	GCACCGTCAAGGCTG AGAAC	ATGGTGGTGAAGACGCCAGT

4.7.5.9 Mechanical strength

Scaffolds were subjected to tensile tests before and after cryopreservation for determining their elastic modulus, using a Universal tensile testing machine (Instron 4505 Universal Machine) following the procedure described in the published literature [197]. The tests were performed under compression loading of 1KN with a crosshead speed of 2 mm/min till 60% strain was reached.

4.7.6 Optimization of MSCs cryopreservation parameters

The precultured TECs were exposed to cryoprotectant solutions for 10min at 4°C and then transferred to a high-density polypropylene sterile pouch. The cryopreservation study was performed in a computer-controlled programmable freezer (Kryo cooler, UK) following two steps cooling. TECs samples were cooled at different cooling rate such as 0.5°C /min, 1°C/min and 2°C/min from 5°C to -150°C. Finally the scaffold was immersed into liquid LN_2 bath. After 7 days of cryopreservation in vapors phase storages tank, the samples were removed from liquid nitrogen and were thawed by shaking at 37°C. The TECs were transferred into a 35-mm culture dish containing DMEM solution and washed thoroughly to remove cryoprotectants from TECs. Precultured TECs without cryopreservation was used as control. Seeding was performed manually at temperature -5° C,-7° C and -10° C to analyze the effect of seeding temperature.

4.7.7 Statistical analysis

Statistical differences were evaluated by the Tukey-Kramer test after one-way analysis of variance. $p < 0.05$ was considered significant using online graphpad quickcalc software.

CHAPTER IV

Results and Discussion

PART I

A preliminary study on cryopreservation of MNCs: Selection of natural cryoprotective agents & antioxidant

In recent years stem cell therapy using mesenchymal stem cells has been considered as a promising treatment option for patients suffering from various therapeutic and tissue related diseases [1-13]. Therefore, systematic efforts have been given by the scientists worldwide for the isolation and expansion of MSCs keeping in view of their commercial applications. Umbilical cord blood (UCB) is considered as a potential source of MSCs because of its easy availability as a waste product, no risk to donor, juvenile source and better proliferation potential compared to other sources. However, development of a long term preservation strategy is a prerequisite for successful application of MSCs when need arises. Cryopreservation using Me_2SO as CPA is a proven method for long term storage of human cells with high cell viability and maintenance of desired stem cell characteristics [197-200]. However, cellular damage occurs due to extracellular and intracellular ice formation and toxicity effect.of using Me_2SO. The search for novel cryoprotective agent (CPA) as alternative to Me_2SO is under active research consideration as CPA has a critical role in reducing many of these factors encountered under extreme low temperature treatment of cells.

In this context, treahalose, polyvinyl pyrrolidine (PVP) and hydroxy ethyl starch (HES) seem to be attractive extracellular [200-212] and ectoin, taurine and erythritol as intracellular CPAs for the preservation of cells [213-219]. These CPAs has important role in protecting cell membrane integrity during low temperature preservation by preventing extracellular and intracellular ice formation that lead to the damage of cell membrane and denaturation of cell. Furthermore, the use of these CPAs in combination seems to be more efficient in preserving cells at low temperature freezing conditions [22, 54]. However, not much investigation has been done so far to explore the potentiality of these natural CPAs particularly their use in combination for preserving mesenchymal stem cells. Another important aspect is that the choices of many CPAs are cell-specific and their potentiality as CPA cannot be adjudged as a priori. So, in the present study three extracellular (trehalose, PVP, HES) and three intracellular (ectoin, taurine and erythritol) CPAs were chosen to assess their ability for cryopreservation of MNCs and select the most effective CPA which can be further used for the study on MSCs preservation. The MNCs were chosen for preliminary trial with intention to avoid the use of MSCs which takes long period of time for their harvesting and the process is tedious, whereas MNCs that contains MSCs is easy to isolate and handle [220].

The formation of reactive oxygen is another important factor which is reported to cause cellular damage during low temperature storage. The addition of selective antioxidant in cryopreservation medium is reported to improve post thaw cell viability by overcoming this oxidative effect [222]. The widely used antioxidants reported are catalase, co-enzyme Q10 and N-acetyl cystine [221-223]. So the effect of these antioxidants was also investigated to enhance the cryopreservation efficiency of the freezing solution.

5.1.1 Isolation and culture MNCs

A blood bag containing CPDA solution was used for the CB collections. The blood was collected by gravity as shown in Figure 5. The cells isolated by Ficol- hypaqe technique were first cultured on plates in DMEM media with supplement of 10%FBS and 1% antibiotic. After 14 days of culture, MNCs were harvested using trypsin/EDTA solution (Figure 6).

5.1.2 Cell morphology study

The morphological variation in the cultured cells was studied by phase contrast microscopic images as shown in Figure 7(A-F). After initial culture, the cells are observed to be round in shape (Figure 7 A) and MNCs are shown to be adherent to culture plate after 48h (Figure 7B). Cells became elongated as spindle fibroblastic shape after 4 days of culture (Figure 7C) and confluence is shown to be reached after 14 days of culture (Figure 7F).

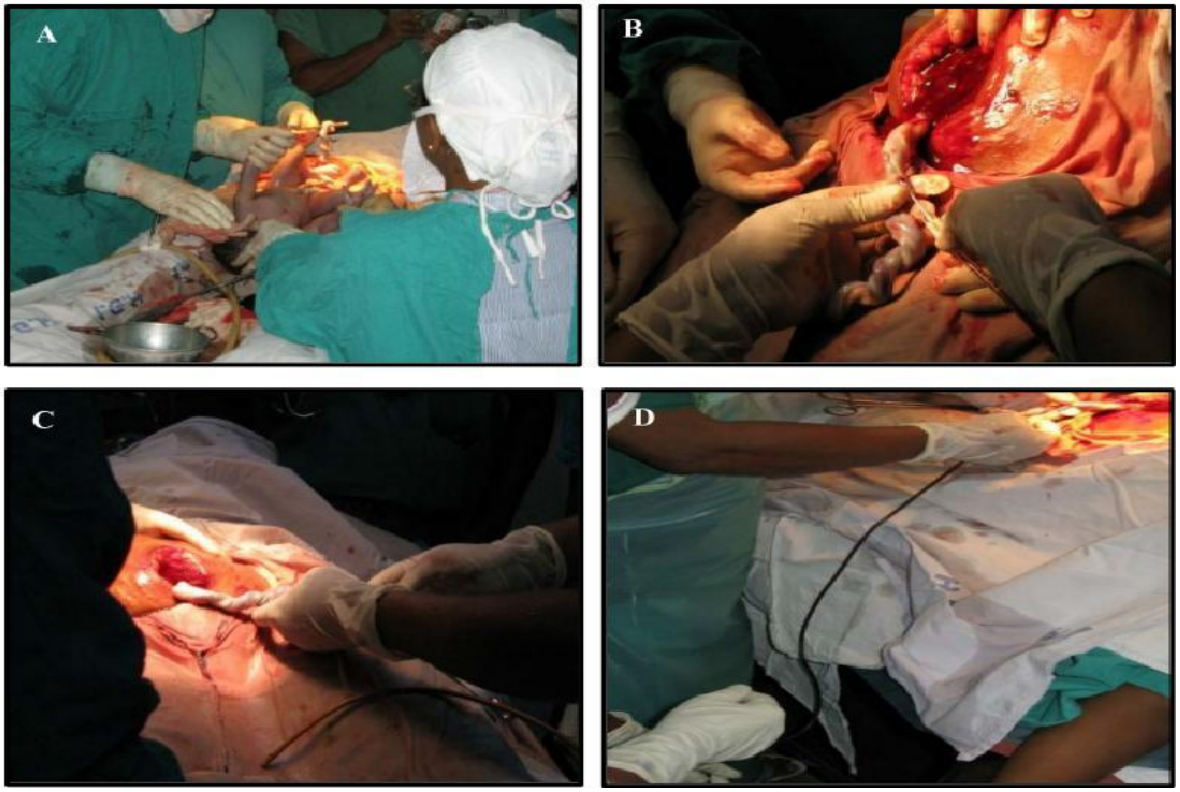


Figure 5: Collection of Cord blood: (A) Clamped and cut the cord just after the birth of baby, (B) and (C) needle is inserted in umbilical cord with collection bag, (D) cord blood allowed to fill the collection bag by gravity

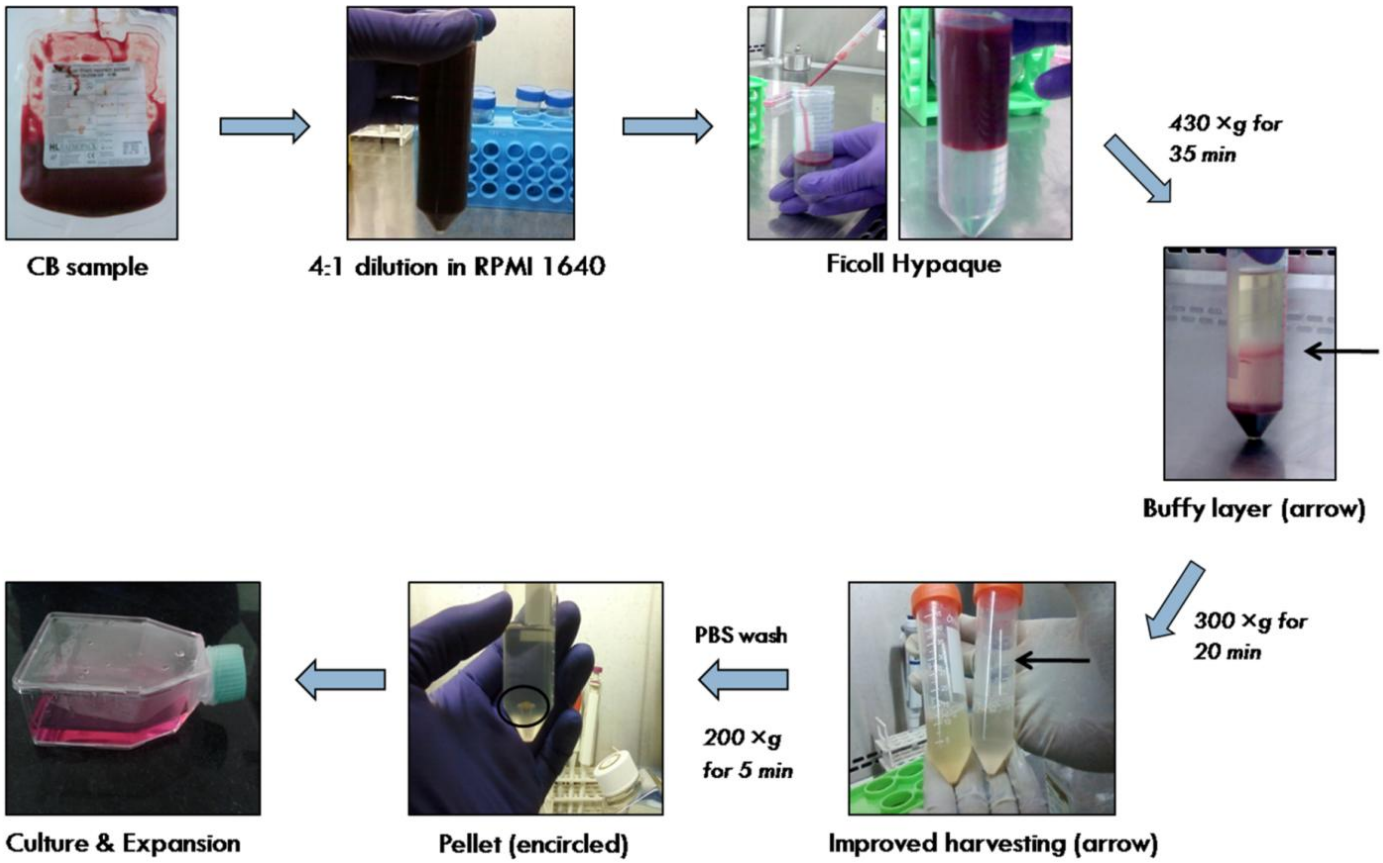


Figure 6: Schematic representation of isolation procedure of MNC from umbilical derived cord blood

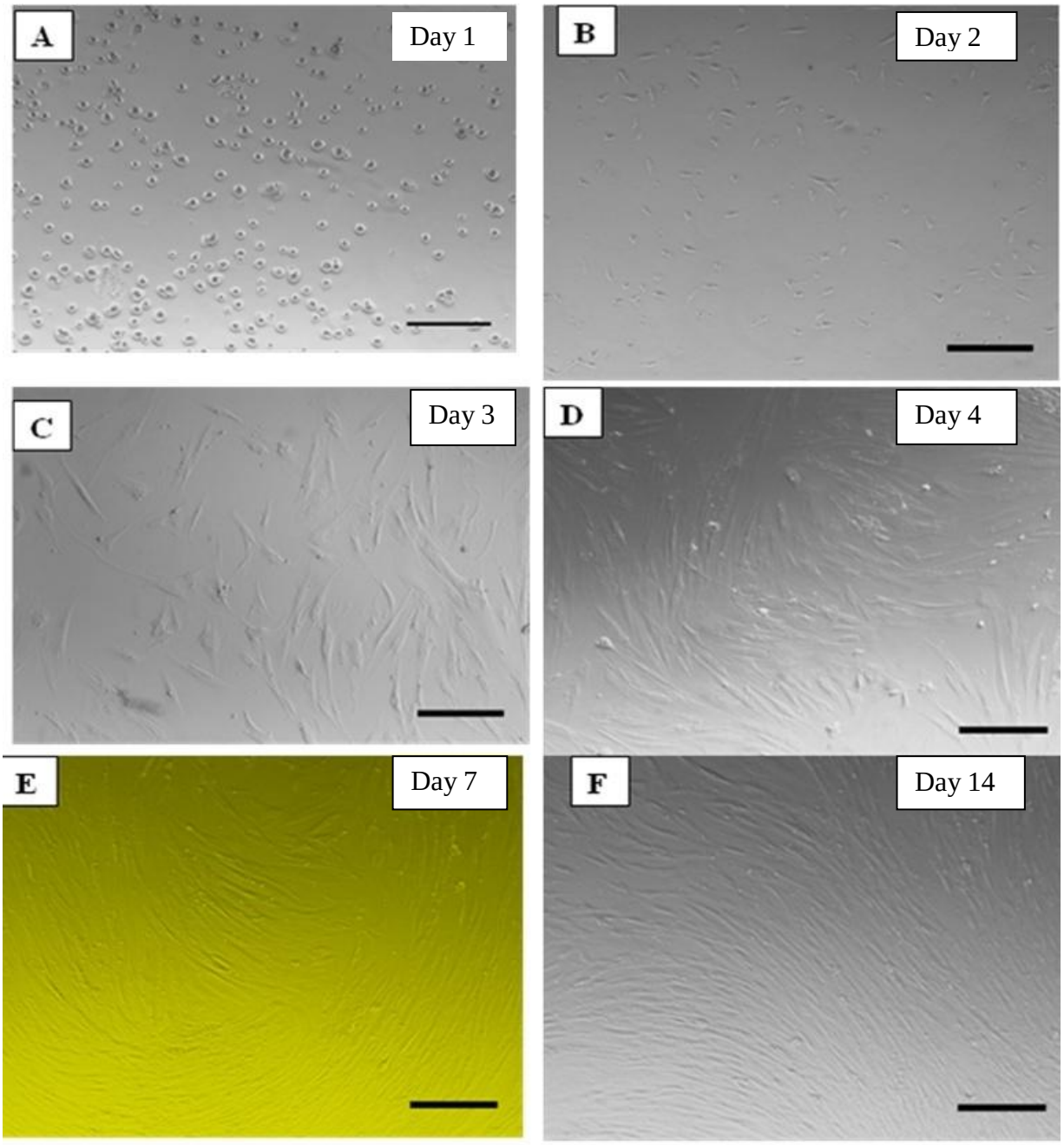


Figure 7: Phase contrast microscopic images depicting the morphological changes during culture period. After initial culture, the cells are observed to be round in shape (A) and MNCs are shown to be adherent to culture plate after 48h (B). Cells became elongated as spindle fibroblastic shape after 4 days of culture (C) and confluence is shown to be reached after 14 days of culture (F)

5.1.3 Cryopreservation solution

Different freezing solutions for cryopreservation of MNCs prepared by taguchi orthogonal L9 array are presented in Table 4.

Table- 4: **Taguchi L9 array for nine cryoprotectant solutions with different compositions**

Cryopreservation solution	**1**	**2**	**3**	**4**
	A	**B**	**C**	**D**
Solution-1	HES	Erythritol	Co-enzyme Q 10	10% FBS
Solution-2	HES	Taurine	Catalase	5% FBS
Solution-3	HES	Ectoin	N-acetyl cystine	0% FBS
Solution-4	PVP	Erythritol	Catalase	0% FBS
Solution-5	PVP	Taurine	N-acetyl cystine	10% FBS
Solution-6	PVP	Ectoin	Co-enzyme Q 10	5% FBS
Solution-7	Trehalose	Erythritol	N-acetyl cystine	5% FBS
Solution-8	Trehalose	Taurine	Co-enzyme Q 10	0% FBS
Solution-9	Trehalose	Ectoin	Catalase	10% FBS

5.1.4 Cell viability and apoptosis study

An important phenomenon that occurs in cryopreserved cells is the programmed cell death, called apoptosis. The measurement of this apoptosis is important because it permits the detection of cells that are undergoing a programmed cell death without disrupting their membrane integrity and thus it helps in selection of cryoprotective agents [50-51]. In this study, the cell viability and apoptosis of post thaw cultured MNCs were studied by flowcytometry analysis using Annexin–V FITC/PI staining. Four quadrants of dot plots consisting of Q1 -dead and necrotic cells, Q2-late apoptotic, Q3-live and Q4-late apoptotic are defined as

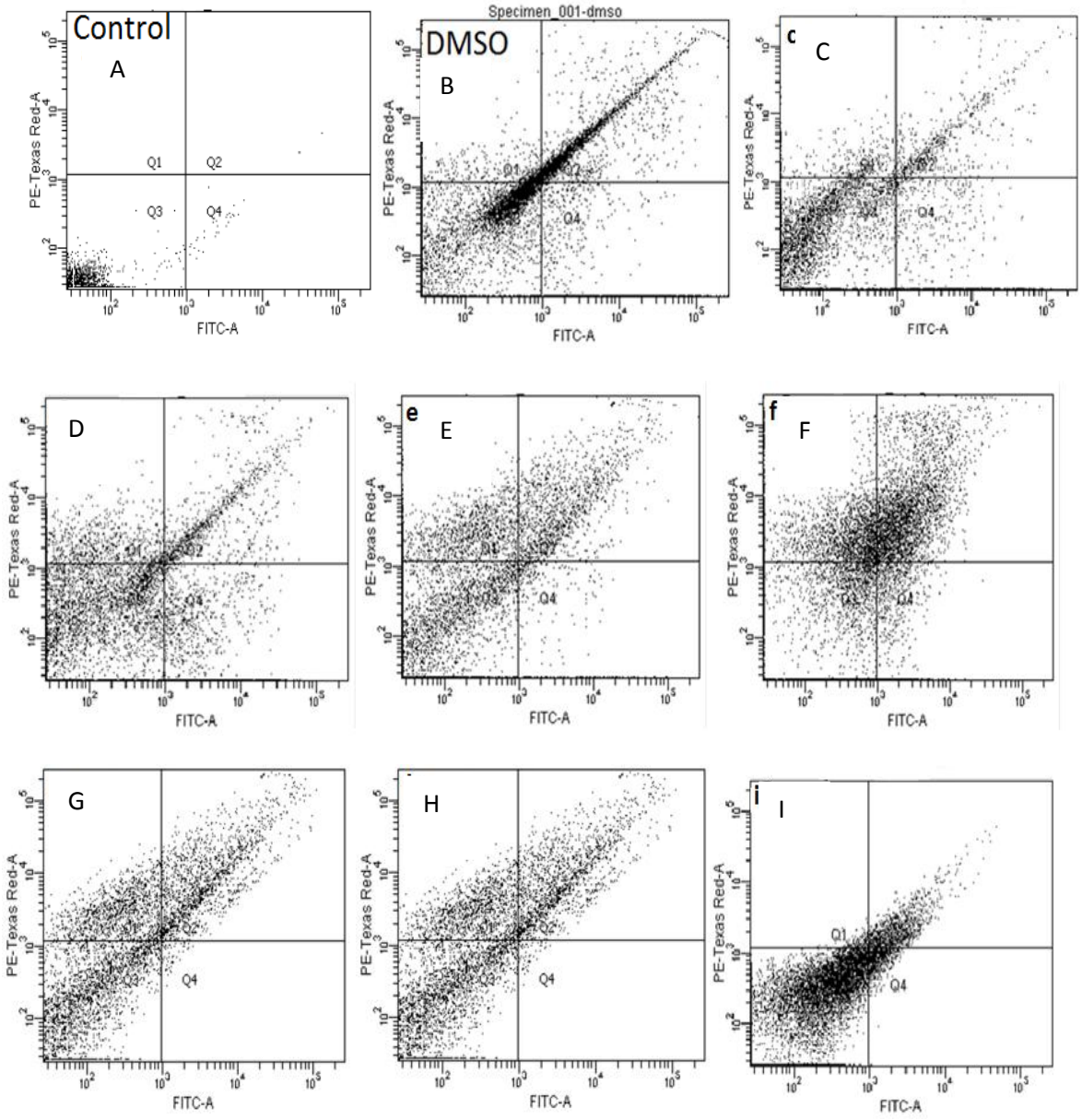
Q1-Annexin V-FITC negative & PI positive –dead and necrotic cells,
Q2-Annexin V-FITC positive & PI positive –late apoptotic cells,
Q3-Annexin V-FITC negative & PI negative -live cells,

Q4- Annexin V-FITC positive & PI negative-early apoptotic.

The dot plots of flowcytometry analysis found with post-thaw cultured samples are shown in Figure 8 and the cell viability data generated from dot plots is tabulated in Table 5. The experimental results indicate that the combination of hydroxy ethyl starch, ectoin, catalase and 10% FBS (solution-9) has shown maximum cell viability of 93.7%, 5.5% early apoptotic cells, 0.7% late apoptotic cells and 0.1% necrotic cells. But the combinations of polyvinlypyrrolidine, erythritol and co-enzyme Q10 in absence of FBS have shown the least cell viability of 36.0%. The cell viability is also compared with the cell viability obtained with the cells cryopreserved in 10% (v/v) Me_2SO as control. The cell viability was observed to be significantly reduced when cell cryopreserved in Me_2SO. The cell viability achieved in later case is 60%. Percentage of cells in Q3 quadrant was further used for Taguchi statistical analysis. Signal-to-Noise ratio (S/N), a logarithmic functions of desired output, that serves as objective functions for optimization, helps in data analysis and thus predicts the optimum results. There are three way of calculating Signal-to-Noise ratios (1) smaller-the-better (2) larger-the-better and (3) nominal-the-best. In the present study S/N ratio was calculated based on the principle of "smaller the better

Table 5: S/N ratio and percentage of cells in Q3 quadrant (viable cell)

Sample	Repetition -1	Repetition-2	Repetition-3	Average viability	S/N Ratio
Solution 1	83.70	84.4	84.2	84.1	47.5817
Solution 2	66.90	66.3	66.5	66.6	46.9271
Solution 3	69.90	70.8	67.8	69.6	41.0037
Solution 4	36.09	35.9	36.3	36.0	51.5668
Solution 5	68.80	72.0	70.0	70.5	32.8675
Solution 6	83.50	80.3	81.5	81.6	34.1221
Solution 7	80.50	79.6	78.0	79.4	42.6865
Solution 8	63.00	67.0	64.0	65.0	30.2377
Solution 9	91.60	93.8	95.5	93.7	37.4882


Control
A
Specimen_001-dmso
DMSO
B
C
D
E
F
G
H
I
PE-Texas Red-A
FITC-A
Q1
Q2
Q3
Q4

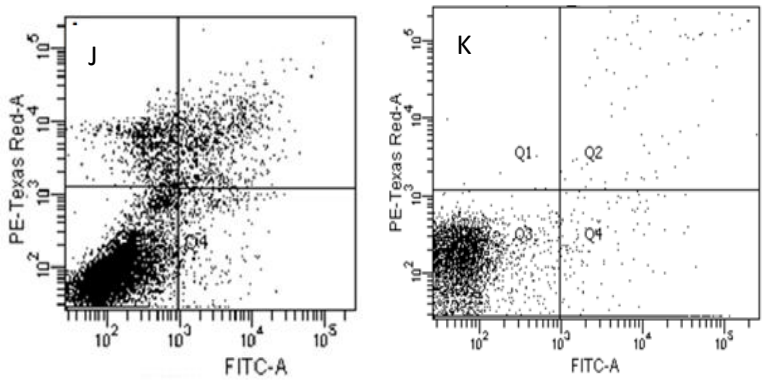


Figure 8:Dot plots of cryopreserved and non-cryopreserved MNCs: control(A), cryopreserved MSCs in Me_2SO (B), HES, erythritol, Co-enzyme Q10 and 10%FBS(C), HES, taurine, catalase and 5%FBS(D), HES, ectoin, n-acetyl cystine and 0%FBS(E), PVP, erythritol, catalase and 0% FBS(F), PVP, taurine, n-acetyl cystine and 10%FBS(G), PVP, ectoin, co-enzyme Q10 and 5%FBS(H), trehalose, erythritol, n-acetyl cystine and 5%FBS(I), trehalose, taurine, co-enzyme Q10 and 0% FBS (J), trehalose, ectoin, catalase and 10%FBS(K)

5.1.5 Statistical analysis

The S/N ratio data as shown in Figure 9, was calculated based on Taguchi's smaller the better approach (224-225). A large variation in S/N ratio is observed among the extracellular cryoprotectants, intracellular cryoprotectants and antioxidants as evident from Figure 2(C_1), (C_2) and (C_3). Furthermore, there was no significant variation in S/N ratio observed with the sample containing FBS which indicates that variation in concentration of FBS does not have any remarkable effect on cell viability. However, though a slightly increase in cell viability is achieved with freezing medium containing 5%FBS. Among various cryoprotective agents studied, trehalose and ectoin are found to be the most effective extracellular and intracellular cryoprotective agents showing maximum cell viability in terms of S/N ratio. Similarly catalase was found to be the most favorable antioxidant.

5.1.6 Analysis of Variance (ANOVA)

ANOVA helps in testing the significance of all main factors and their interactions by comparing the mean square against an estimate of the experimental errors at specific confidence levels [225]. The results are also compared with statistical data obtained by ANOVA analysis based on analysis of variance as shown in Table 6. Data confirms that the extracellular (p value=0.0003)

and intracellular CPAs (p value=0.009) have greater influence on cell viability compared to other factors such as FBS concentration (p value= 0.05) and types of antioxidants (p value= 0.037).

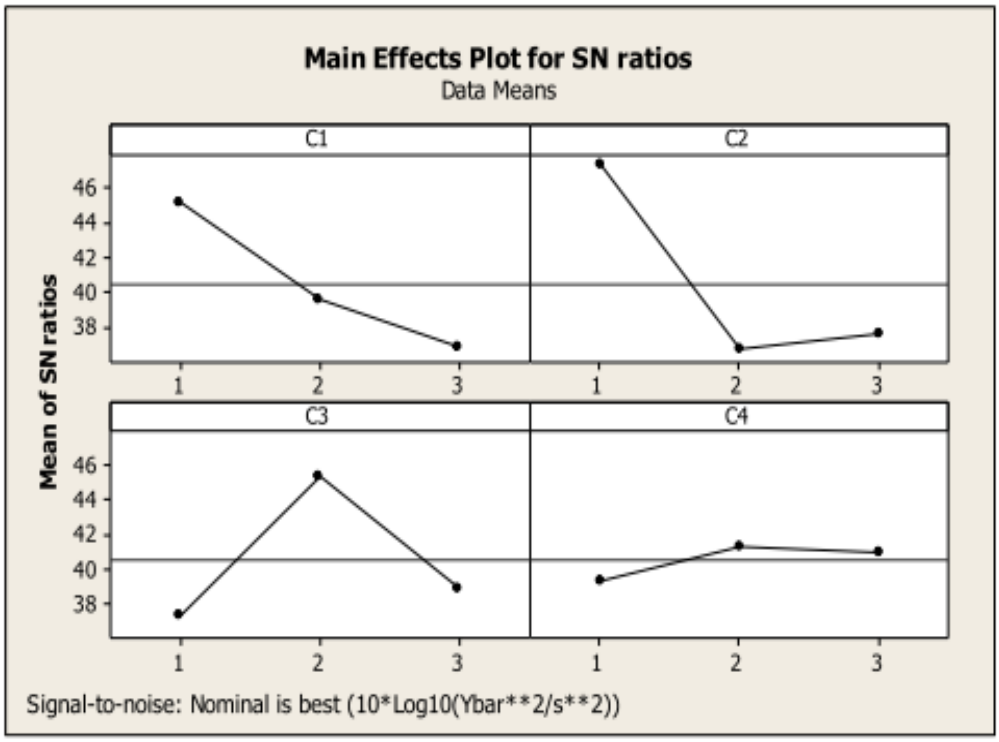


Figure9: Illustrates S/N ratio averages for each parameter at three levels. As it is observed extracellular cryoprotectants (C1) intracellular cryoprotectants (C2) and antioxidants concentrations (C3) exhibit larger variations. However, the largest variation is found in case of antioxidants

Since the Taguchi Orthogonal array adopted in this study is a factorial experiment, it is necessary to conduct verification experiment to ascertain that the entire factor included in this analysis are significant [224].Therefore, various experiments for verification under best obtained level with the respective factor were performed following the methodology published earlier [225]. The average viability is calculated based on flowcytometry as 73% which is shown to be within the confidence level, where quadrant analysis shows the distribution of viable MNCs (7AAD -ve, Annexin V-ve), apoptotic MNCs (7AAD -ve, Annexin V +ve), and necrotic MNCs (7AAD +ve, Annexin V +ve).

Table 6: ANOVA analysis of experiments consider in Taguchi orthogonal array

Factor	Degree of freedom	Sum of squares	Mean square	P-value
Extracellular cryoprotectants	2	129.4	64.7	0.0003
Natural osmolytes	2	133.5	66.7	0.009
Antioxidants	2	94.8	47.4	0.037
Concentration of FBS	2	13.1	6.5	0.050
Total	8	370.8		

5.1.7 Cell morphology analysis of cryopreserved cells

The cellular morphology of cryopreserved MNCs was studied by SEM and fluorescence microscopic images (Figure 10). The cryopreserved cells are observed to retain their normal cell morphology.

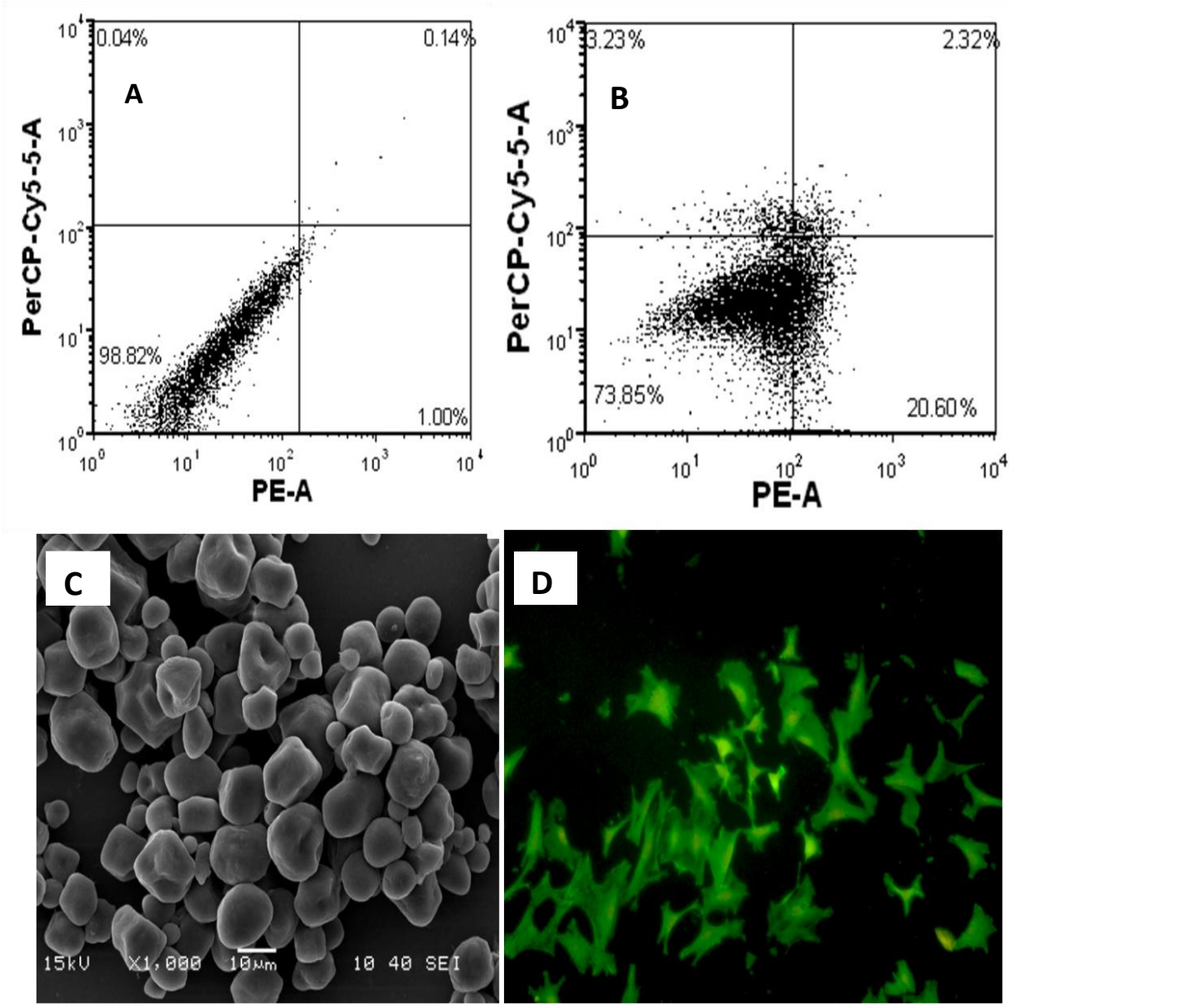


Figure 10: Conformity test for best obtained level with the respective factor. (A) Dot plot of non-cryopreserved sample (negative control), (B) Post-thaw MNCs viability, (C) SEM image of after 24h post-thaw MNCs ,(D) fluorescence image after 24h post thaw of MNCs at 5x magnification. (B), (C) and (D) show the result observed with combination of trehalose, ectoin, catalase and 5% FBS

5.1.8 MTT assay

The cell viability of cryopreserved MNCs was also measured quantitatively by MTT assay and the experimental results are shown in Figure 11. The assay result indicates that the cells preserved in solution 1, 2, 3, 7 and 9 are more viable after 24 h of post thaw compared to the cell viability observed with other CPA solutions. Furthermore, the combination consisting of

trehalose, ectoin and catalases have shown better cell viability as compared to the other antioxidant present in cryopreserved solution. However, a statistically significant increase in cell viability is observed using cryoprotectant solution 9 (***p<0.0005) followed by 3 (**p<0.05) as compared to the post thaw MNCs cryopreserved in 10% Me_2SO. The result has shown similar trend as the results obtained by using flow cytometer i.e apoptosis study. However, cell viability with traditional Me_2SO treated samples are found to be less as compared to any combination except solution 4 (**p<0.05). Furthermore, no significant variation in cell viability is observed with solution 5, 6 and 8 from the cell viability achieved with control.

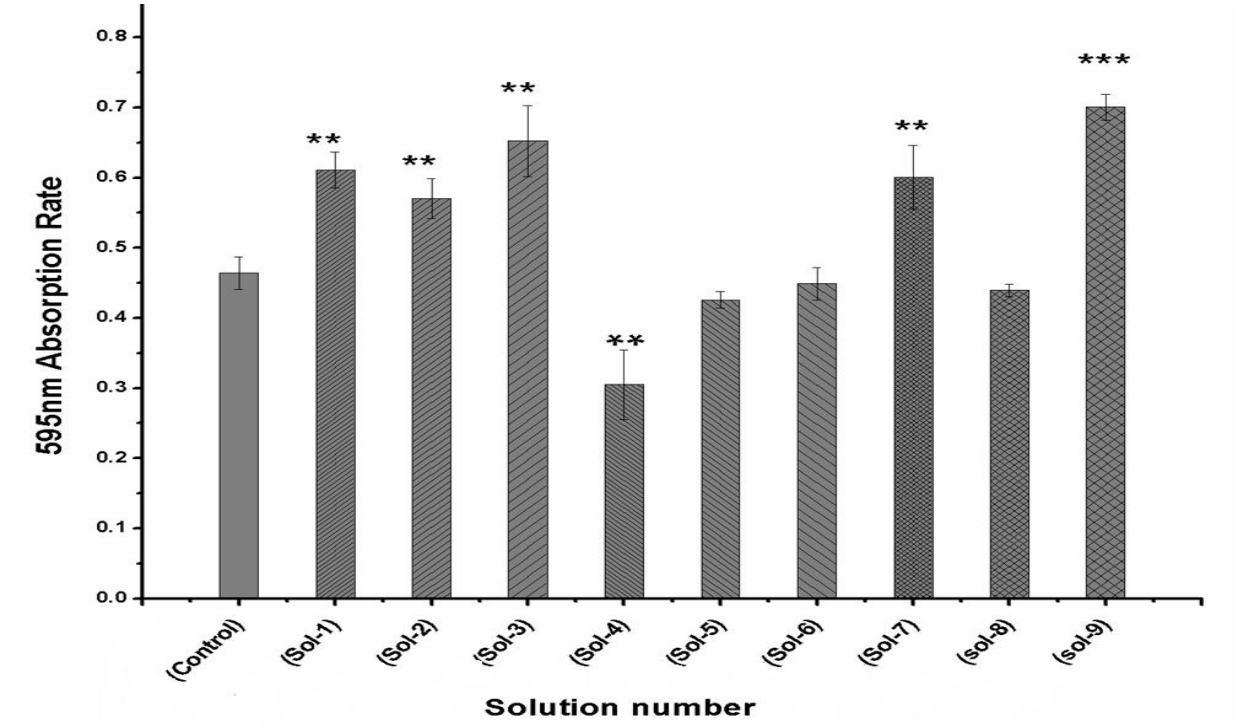


Figure 11: MTT assay of post-thawed MNCs cryopreserved in different compositions of extracellular cryoprotectant, intracellular, antioxidant and FBS. MNCs cryopreserved in 10% Me_2SO taken as control. All the data are compared with control using student t-test, Bars represent data from three separate experiments with standard deviation (*p< 0.05, **p< 0.005, *<0.0005)**

5.1.9 Post-thaw cytoskeleton assessment

F-actin, a main constituent of cytoskeleton has an important role in maintaining cellular function [226]. It is further reported that at extremely low temperature, the ultra structure of F-actin becomes distorted that results in cell damage [227]. Therefore, the effect of cryopreservation was investigated using non-toxic CPA solution on the F-actin which would be beneficial to design

cryopreservation strategy. The change in F-actin of 48h post thaw cultured samples was observed under the florescence microscope (Figure10). Non-cryopreserved cell shows prominent cytoplasmaic extension with maximum cell attached to the flask as depicted in Figure 10A and MNCs cryopreserved with solution-9 shows good cell attachment. However, the cell attachment is found to be lesser than the normal cultured cell (Figure 12B) and higher than the sample cryopreserved with 10% Me_2SO (Figure 12 C)

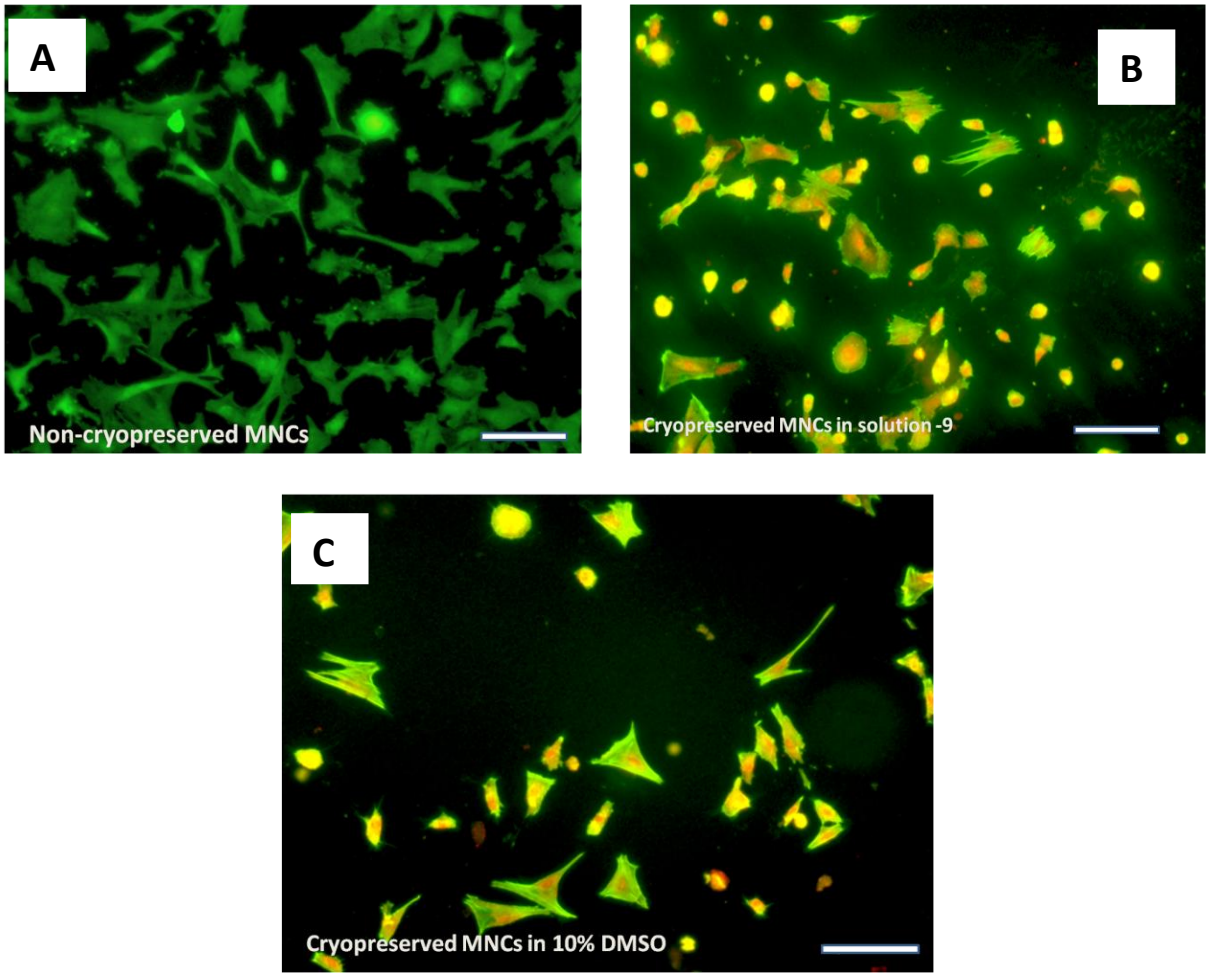


Figure 12: Growth and attachment of cryopreserved MNCs after 48 h of incubation. (A) non-cryopreserved MNCs, (B) MNCs cryopreserved in solution-9 and (C) MNCs cryopreserved in 10% Me_2SO (Scale bar 100 µm).

5.1.10 Morphological analysis of cryopreserved MNCs

The morphology and membrane integrity of cryopreserved MNCs were studied by SEM and phase contrast microscopy. Though analysis was done with all samples, but images of non-cryopreserved MNCs, cryopreserved MNCs with solution-9 and cryopreserved MNCs with 10% Me_2SO are shown in Figure 13. The MNCs cryopreserved in solution-9 are observed to retain their original shape without any bulb formation after 24h of post thaw culturing. This indicates that the combination of cryoprotective agents can be used without any adverse effect on the cell membrane. Whereas, prominent bulb formation observed in MNCs cryopreserved with 10% Me_2SO.

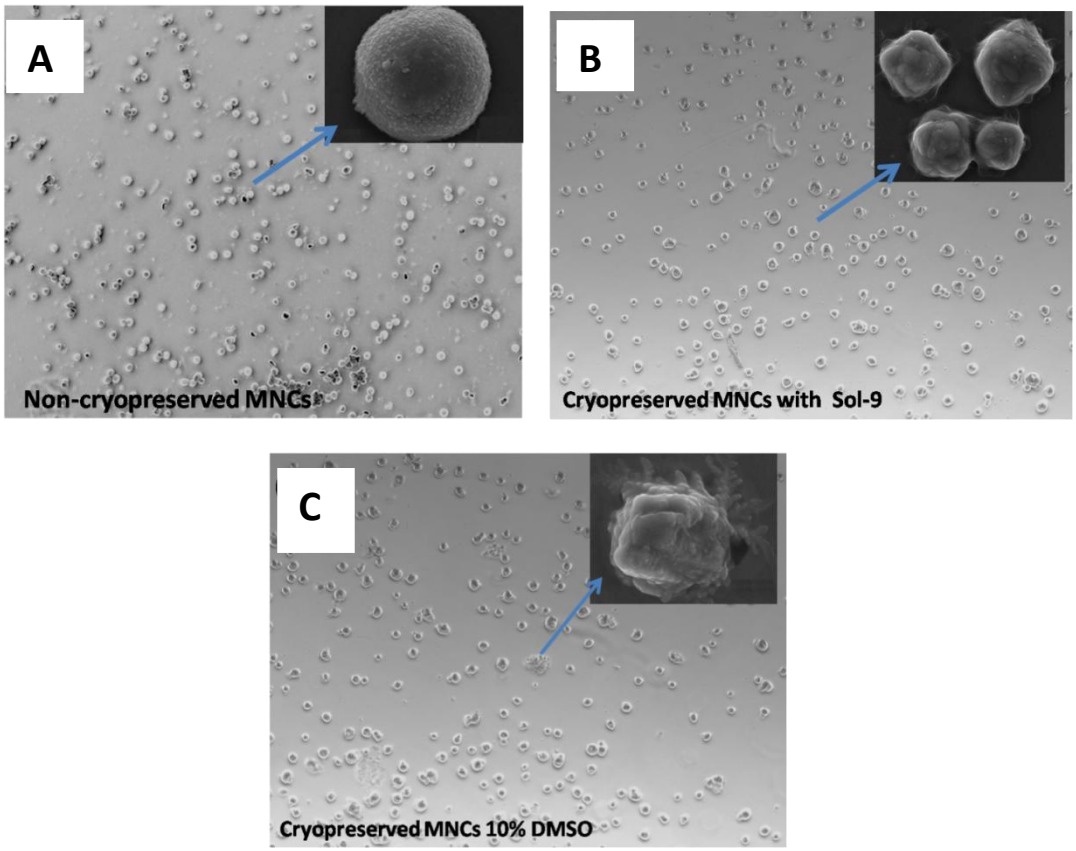


Figure 13: SEM images of UCB derived MNCs (A) non-cryopreserved MNCs, (B) MNCs cryopreserved in solution-9 and (C) MNCs cryopreserved in 10% Me_2SO. All the images were taken at a magnification of 50X (phase contrast microscope) and 1000X for SEM

5.1.11 Conclusion

In this study, a number of potential natural osmolytes and antioxidants were investigated to see their effects on post-thaw cell viability and early apoptosis of the cryopreserved MNCs isolated from UCB. Among the CPAs and antioxidants used under study, trehalose and ectoin as extracellular and intracellular CPAs and catalase as antioxidant are found to be the most effective. Furthermore, the combination of these CPAs and antioxidant has shown encouraging results towards the maintenance and survival of cell during and after freezing achieving maximum post thaw viability of 76%, which is more than 10% higher compared to the cell viability obtained using conventional Me_2SO. Thus it has been demonstrated that trehalose, ectoin and catalase can be the major constituents in developing a freezing solution for cryopreservation of MNCs and may be useful to develop a cryopreservation strategy for MSCs.

PART II

Cryopreservation of UCB derived MSCs using natural cryoprotective agents

As it has been already mentioned, transplantation of MSCs derived from umbilical cord blood is a promising treatment option for patients suffering from various blood and tissue related diseases because of their immunosuppressive properties and ability to differentiate into mesoderm type lineages including osteocytes [32], chondrocytes [33], skeletal muscles [34], adipocyte and myocytes, vascular cells and the like [35]. This has led to the advancement of isolation and expansion of MSCs, for which the development of a successful preservation strategy is of prime requirement to advance their clinical applications [59].

In previous chapter, trehalose and ectoin were found to be the most effective extracellular and intracellular natural CPAs and catalase as antioxidant for the preservation of MNCs. In this phase of thesis work, the research work has been extended further to evaluate the ability of these CPAs and catalase towards preservation of MSCs and establish an optimized controlled rate freezing protocol for its long term preservation.

5.2.1 Sorting and culturing of MSCs

Fluorescence activated cell sorting allows physical separation of sub-populations of cells of interest from a heterogeneous population and thereby a high degree of purity (95-100%) of the sorted population is obtained [60]. The cultured MNCs were sorted by this technique using CD44, CD73, CD90, CD105, CD34, CD45 and HLA-DR markers. MSCs that are positive to CD90/CD105/CD73/CD44 were sorted by cell sorter. As shown in Figure 14(A) primary gate applied on FSC-A vs SSC-A to discriminate Mononuclear cells from other population, followed by a gate to discriminate singlet from doublet with SSC-H vs SSC-W and FSC-H vs FSC-W Figure 14 (B) a gate in FSC-H vs. FSC-W was applied to divide singlet and doublets from higher level aggregates, and was refined by similar gate in SSC-H vs. SSC-W (Figure 14C). Singlet population was further analyzed for the pattern of expression of CD45/HLA DR vs. CD 34 (Figure 14D). Negative expressed cells are again gated for CD44 positive cells (Figure 12E). Stringent gating was done to remove any false CD44 positive cells based on CD34 vs CD45/HLA-DR (Figure 14E). Cells were again gated for true positive CD90/CD73/CD105 cells (Figure 14F).

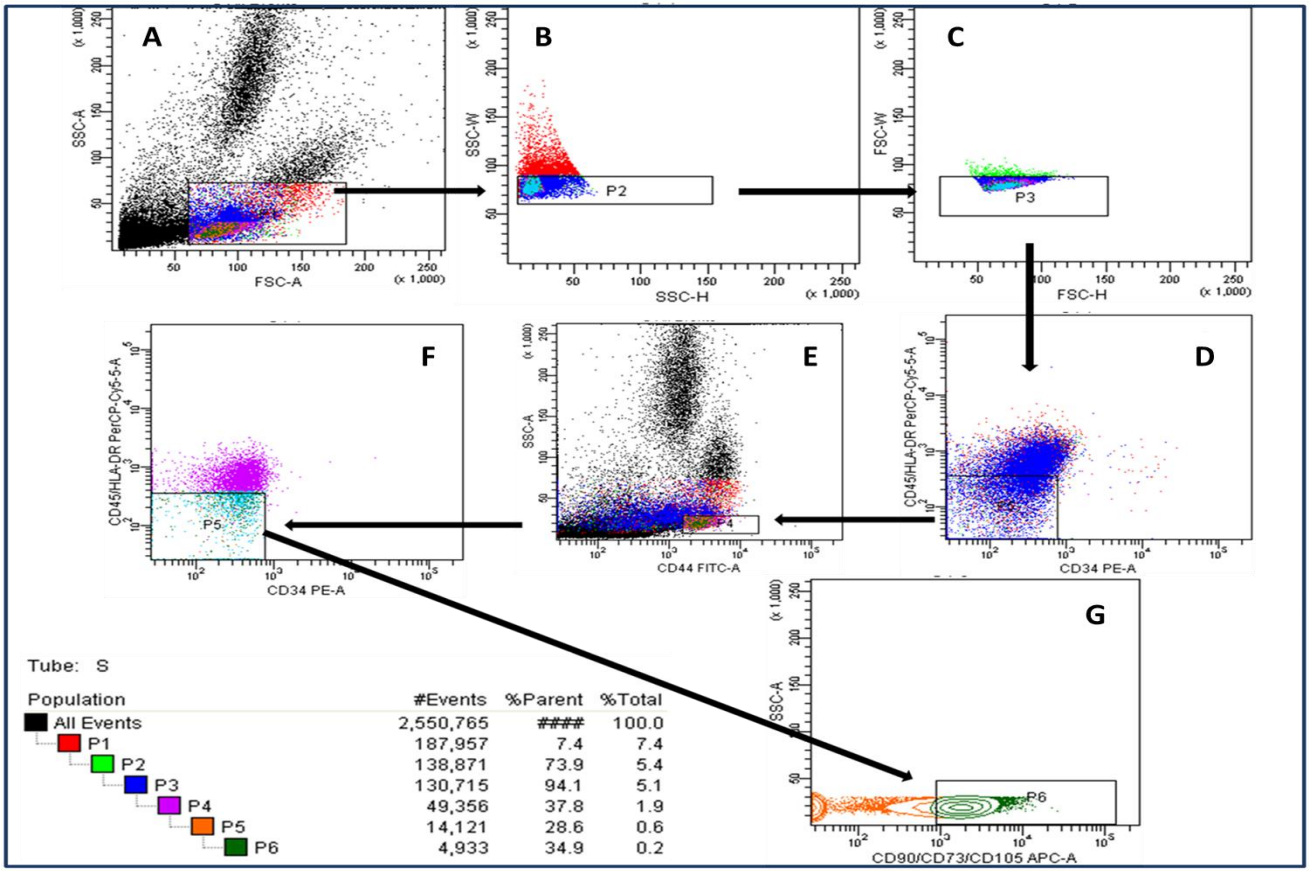


Figure24: Image shows the sorting strategy used for isolation of MSCs. As shown in Figure 14A primary gate applied on FSC-A vs SSC-A to discriminate Mononuclear cells from other population, followed by a gate to discriminate singlet from doublet with SSC-H vs SSC-W and FSC-H vs FSC-W Figure 14 B, a gate in FSC-H vs. FSC-W was applied to divide singlet and doublets from higher level aggregates, and was refined by similar gate in SSC-H vs. SSC-W (Figure 14C). Singlet population was further analyzed for the pattern of expression of CD45/HLA DR vs. CD 34(Figure 14D). Negative expressed cells are again gated for CD44 positive cells (Figure 12E). Stringent gating was done to remove any false CD44 positive cells based on CD34 vs CD45/HLA-DR (Figure 14E). Cells were again gated for true positive CD90/CD73/CD105 cells (Figure 14F)

5.2.2 Cell morphology study

After initial culture, cells were observed to be round in shape as depicted in Figure 15 A. After 48 h of culture cells were shown to be adherent to culture plate (Figure 15B).

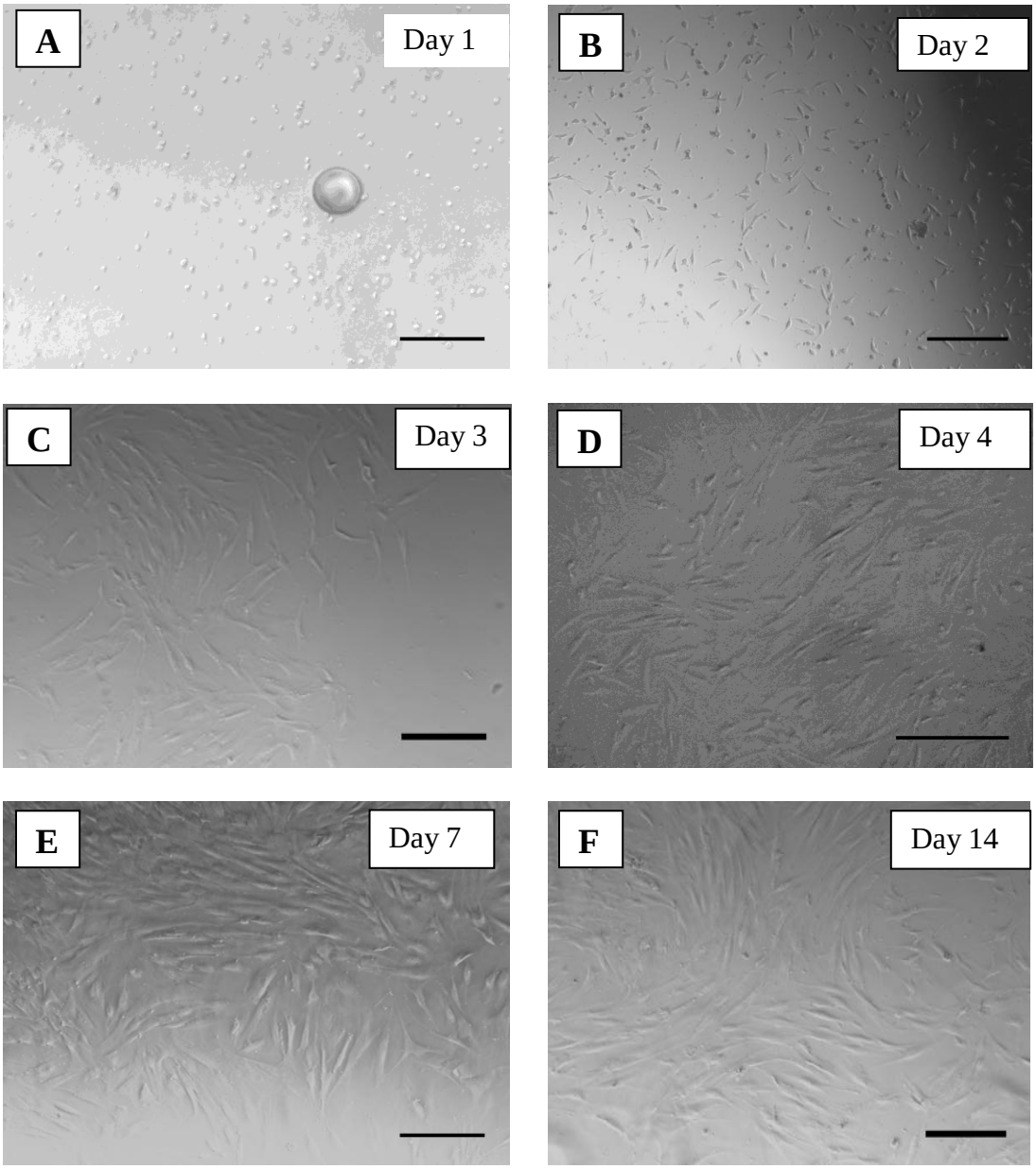


Figure 15: Phase contrast microscopy images showing morphological characteristics of cultured hMSCs A) Initially the cells were round in shape B) Adherent cells after 48 h of culture C) Cells became elongated as spindle fibroblastic shape after 4 days of culture D) hMSCs became confluent in 14 days E) hMSCs after passage 1 and F) after passage 2. (Scale bar 100 µm)

Further, cells became elongated as spindle fibroblastic shape after 2 days of culture (Figure 15C) and cultured cells reached confluence after 4 days (Figure 15D). Cells attains the fibroblastic morphology after 1st passage (Figure 15 E & F)

5.2.3 Immunophenotypic characterization of MSCs

Isolated MSCs were cultured and colonies were found to appear within 5 to 7 days after initial culturing. MSCs were harvested using 0.25% Trypsin/EDTA solution and cells were expanded upto passage 4. The cells were then subjected to immunophenotypic characterization by flow cytometry analysis, the data (dot plot) of which is shown in Figure 16. The immunophenotypic characterizations are shown to be positive for CD90 (99.01%), CD73 (99.5%), CD105 (98%) and CD44 (98.5%) expression and negative for CD34 (1.0%), CD45 (0.5%), and HLA-DR (1.2%) indicating the cells are mesenchymal stem cells (Figure 16). Immnuofluorescence also found to express CD90, and CD105, but not the hematopoietic origin marker CD34 as shown in Figure 17.

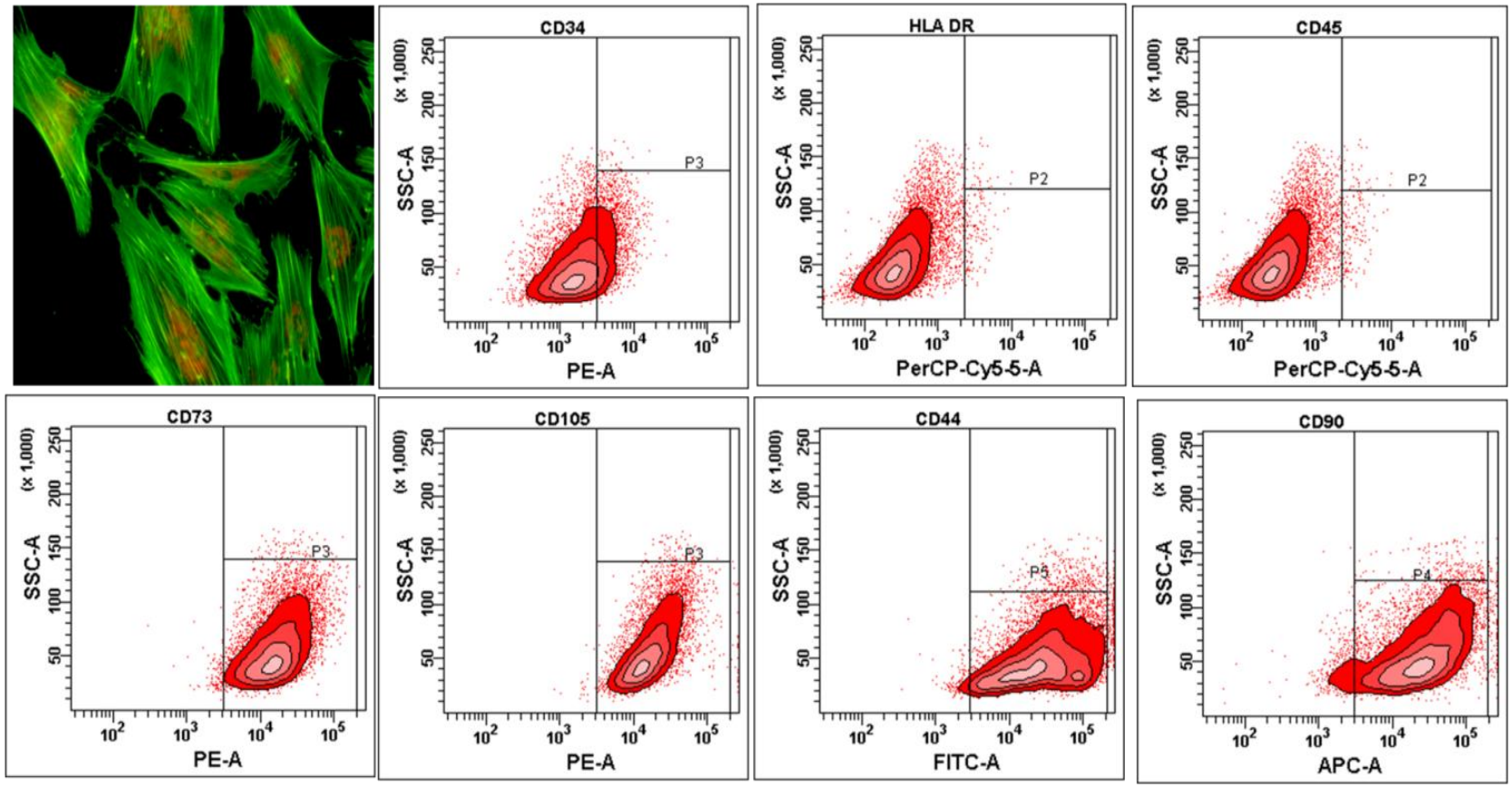


Figure 16: Flowcytometric analysis on the expression of MSC markers CD90, CD105, CD44, and CD73 as well as hematopoietic markers CD34, HLA-DR and CD45

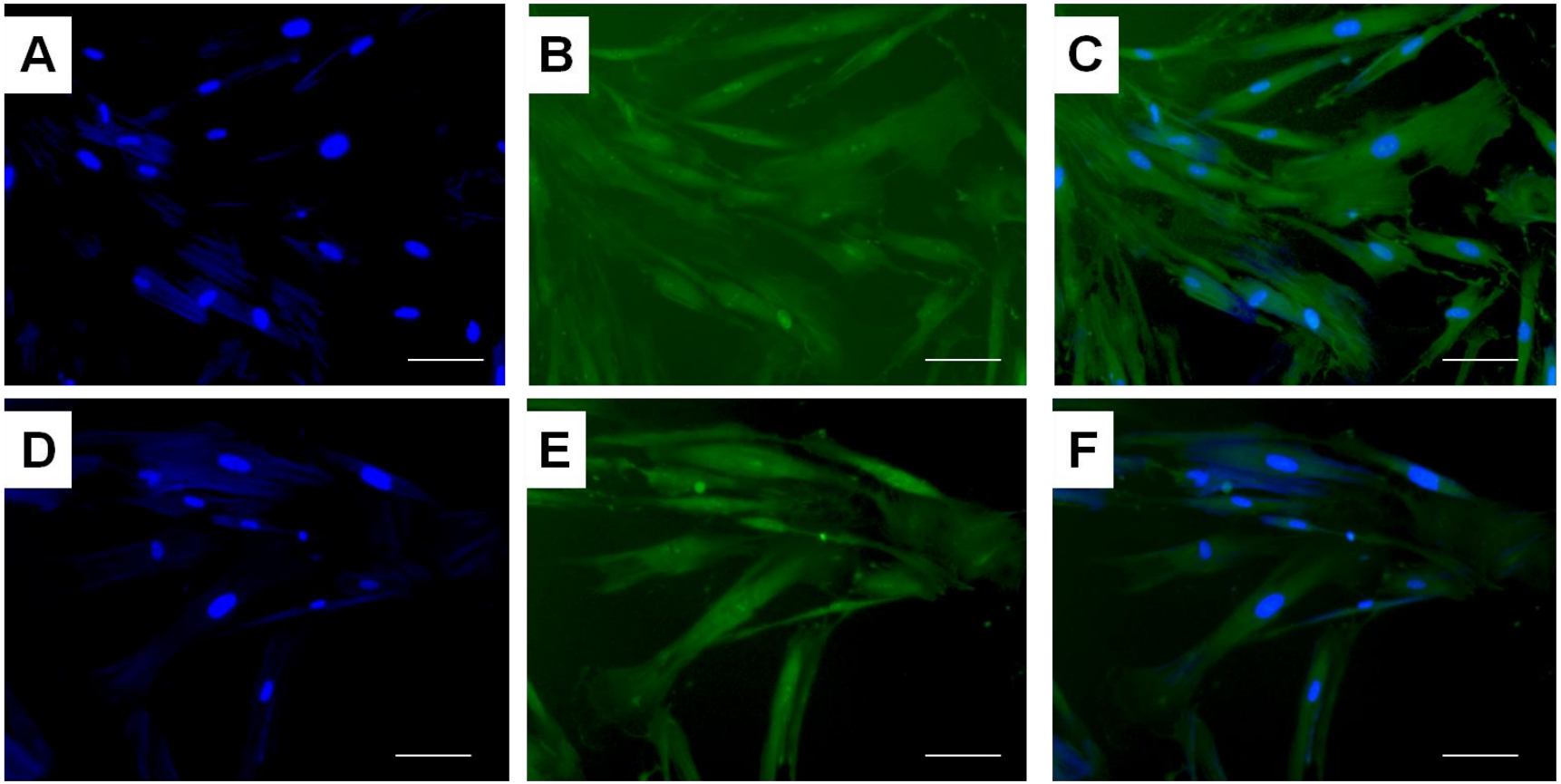


Figure 17: Immnuofluorescence observation of passage 4 post thaw MSCs. (A, D) Nuclear staining with 4, 6-diamidino-2-phenylindole (DAPI). (B, C) Cell-surface marker CD90 and (E, F) Cell-surface marker CD105. (Scale bar 100 µm)

5.2.4 Effect of CPAs on post-thaw MSCs viability

A series of cryopreservation experiments were conducted in controlled rate freezing equipment for the evaluation of the selected CPAs in single as well as in combination for their ability to preserve MSCs. The cell viability with post thawed MSCs cryopreserved in various freezing solutions prepared from trehalose, ectoin and catalase were measured and the experimental results are shown in Figure 18. The result shows that the higher cell viability is observed when CPAs are used in combination than the single one. Furthermore, the highest cell viability of 82% is obtained with freezing solution that consists of 30mM trehalose, 30mM ectoin and 100µM catalase. This freezing solution is designated as $T_{30}/E_{30}/C_{100}$. No further improvement in cell viability is observed with increase in either trehalose or ectoin in the freezing solution. It is also demonstrated that catalase has a positive impact on the freezing solution facilitating improved cell viability Therefore, it is established that $T_{30}/E_{30}/C_{100}$ is the most favorable freezing solution achieving maximum MSCs viability.

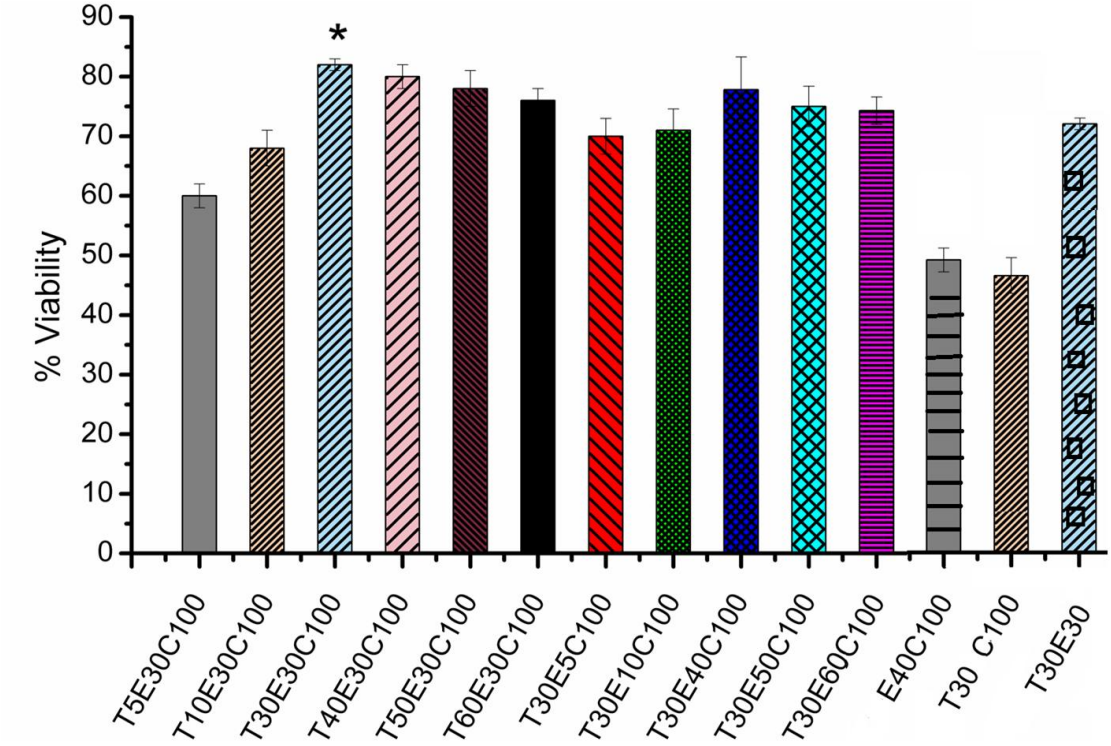


Figure 18: Effect of CPAs on cell viability of cryopreserved MSCs

5.2.5 Effect of addition of Me_2SO in $T_{30}/E_{30}/C_{100}$ freezing solution

The effect of $T_{30}/E_{30}/C_{100}$ $_0$ freezing solution with and without Me_2SO was also studied to investigate any influence of Me_2SO on cryopreservation of MSCs. The experimental data is shown in table 7. It is observed that the cell viability of $T_{30}/E_{30}/C_{100}$ is higher than the cell viability obtained with conventional 10% v/v Me_2SO. The highest cell viability of 84% is shown by $T_{30}E_{30}C_{100}$ with addition of 2.5%v/v Me_2SO. The cell viability (82%) is comparable to the cell viability obtained with $T_{30}E_{30}C_{100}$ without adding Me_2SO. However, keeping in view the harmful effect of Me_2SO, $T_{30}E_{30}C_{100}$ is established as the most favorable freezing medium.

Table 7: Effect of Me_2SO as an additive in freezing solution on cell viability

Solution	Me_2SO (%)	Trehalose (mMol/l)	Ectoin (mMol/l)	Catalase (µg/ml)	Viability (%)
1	0	30	30	100	82
2	2.5	30	30	100	84
3	5	30	30	100	75
4	10	30	30	100	70
5	10	0	0	0	61
6	**2.5**	**0**	**0**	**0**	**24**

5.2.6. MTT Assay

The cell viability of cryopreserved MSCs was also evaluated by MTT assay. The assay result is shown in Figure 19. It is indicated from Figure that solution containing $T_{30}/E_{30}/C_{100}$ have achieved better cell viability as compared to other freezing solutions tested after 24 h of post thaw, though a variation in cell viability is observed among the freezing solutions. The maximum cell viability as indicated by slightly higher OD value is achieved with cryopreserved solution consisting of a combination of $T_{30}/E_{30}/C_{100}$ with 2.5% v/v of Me_2SO. A comparable cell viability is also achieved with $T_{30}/E_{30}/C_{100}$ freezing solution. Keeping in view the detrimental effect of Me_2SO, $T_{30}/E_{30}/C_{100}$ is established as the most favorable freezing medium which is also well

supported by the cell viability result obtained by flowcytometry analysis as described in previous section.

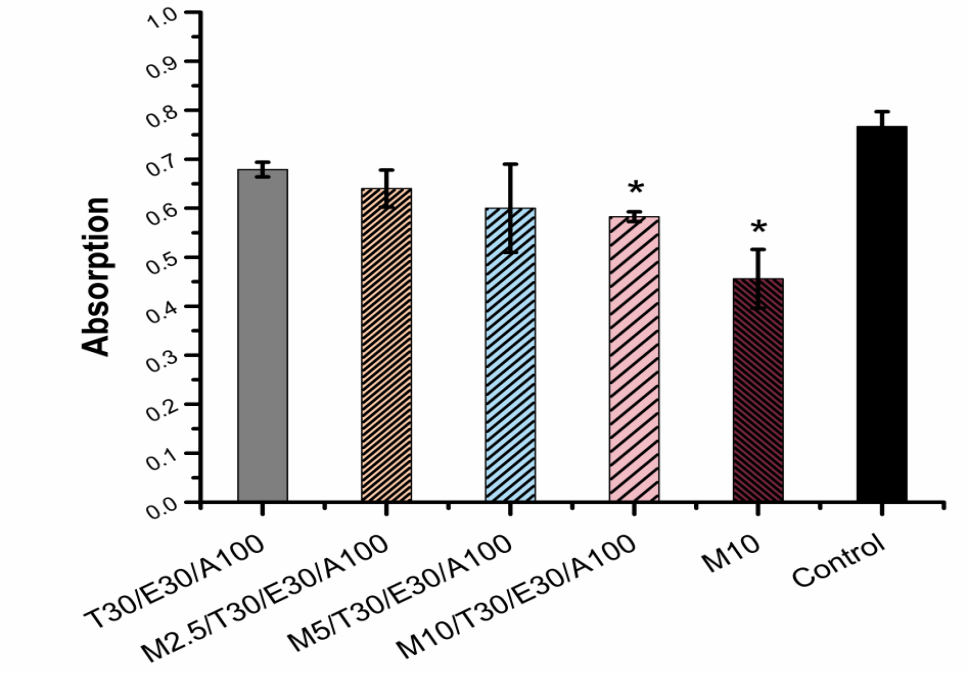


Figure 19: MTT assay of post-thawed MSCs cryopreserved in different compositions of extracellular cryoprotectant, intracellular, antioxidant with and without Me_2SO. MSCs cryopreserved in 10% Me_2SO taken as control. All the data are compared with control using student t-test

5.2.7 Post-thaw phenotyping characterisation of cryopreserved MSCs

The phenotyping characterisation of MSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ with and without Me_2SO has been done by flow cytometry analysis. CD90, CD44, CD45, CD105, CD73 and CD34 expressions of cryopreserved MSCs were measured and compared with control. The results are tabulated in table 8. As expected, both cryopreserved and noncryopreserved MSCs have shown low expression of hematopoietic stem cell markers CD34, HLA-DR and CD45 and high expression of mesenchymal-associated marker CD90, CD73, CD105 and CD44. The corresponding positive CD expressions were in the range of 98-99%. Furthermore, there is no significant variation in phenotype characteristics are observed with MSCs crypreserved in traditional Me_2SO.

Table-8: Immunophenotypic characterisation of MSCs after cryopreservation

Cryoprotectant	CD45	CD34	HLA-DR	CD90	CD73	CD105
$T_{30}E_{30}C_{100}$	0.60±0.41	0.33±0.26	1.73±37	98.77±31	97.66±78	98.94±37
$T_{30}E_{30}$ $M_{10}C_{100}$	1.36±0.08	0.92±0.48	1.55±33	97.22±71	98.79±24	99.77±66
M_{10}	0.92±0.22	0.22±0.04	1.49±71	98.88±59	99.06±97	98.45±46
$T_{30}E_{30}M_{2.5}C_{100}$	0.60±0.41	0.33±0.26	1.73±37	98.77±31	97.66±78	98.94±37
$T_{30}E_{30}M_5C_{100}$	1.36±0.08	0.92±0.48	1.55±33	97.22±71	98.79±24	99.77±66

5.2.8 Cell proliferation assessment in cryopreserved MSCs

As it is expected, non-cryopreserved MSCs are found to proliferate rapidly than cryopreserved MSCs [69]. The growth curve of post-thaw MSCs in all freezing solutions used in this as well as non-cryopreserved MSCs have shown similar trend i.e. In first 2 days after inoculation, no variation in cell number is observed that may be attributed to the adherence of cell to the tissue culture plates, and on day 3, the cells entered the logarithmic growth stage representing an exponential increase in cell number. The doubling time of MSCs in freezing solutions are in the range of 29.0 to 31.9 h (Figure 20 and Table 9). Thus it has been demonstrated that MSCs cryopreserved in the newly formulated freezing solution $T_{30}/E_{30}/C_{100}$ has retained their cell proliferation ability.

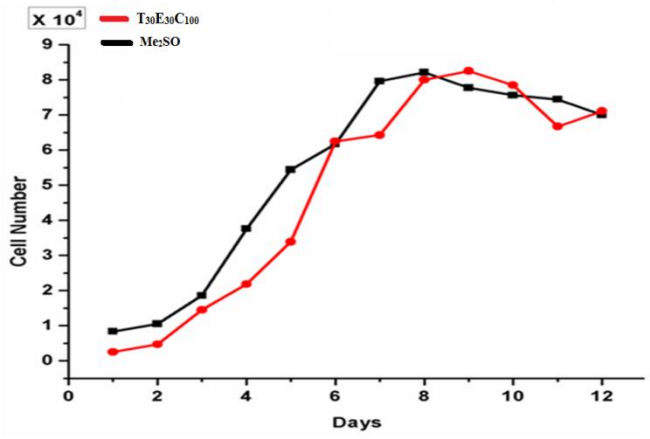


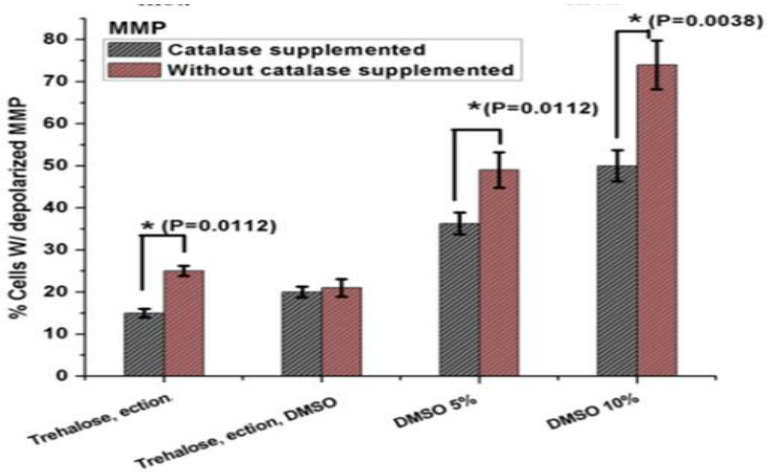
Figure 20: Growth curves of post thaw hMSCs cryopreserved with $T_{30}/E_{30}/C_{100}$ and Me_2SO (10%v/v)

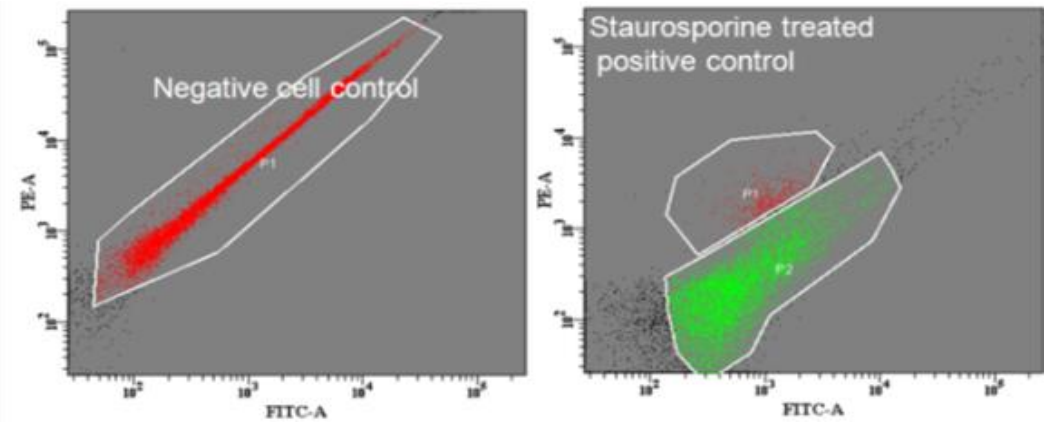
Table 9: Population doubling time of MSCs

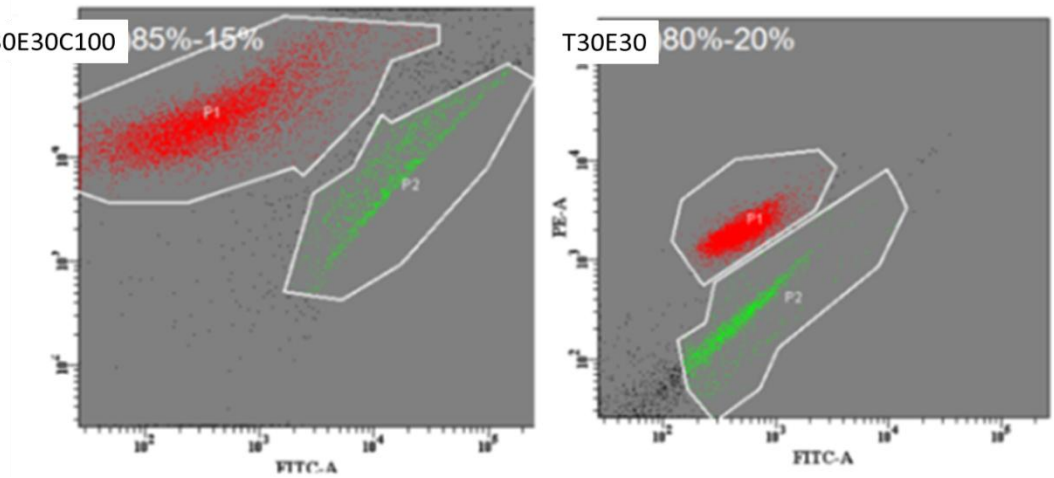
Cryoprotectant	Population doubling time (h)
$T_{30}E_{30}C_{100}$	29.7
$T_{30}E_{30}$ $M_{10}C_{100}$	31
M_{10}	30
$T_{30}E_{30}M_{2.5}C_{100}$	31
$T_{30}E_{30}M_5C_{100}$	31.6
Fresh	29.0

5.2.9 Change of mitochondrial membrane potential in cryopreserved MSCs

Recent studies have reported a rise in reactive oxygen species (ROS) like superoxide anion and hydrogen peroxide during cryopreservation procedure [23]. Mitochondria are considered as the main source of this ROS. This superoxide anion induces the release of cytochrome C from the intermembrane of mitochondria into the cytoplasm that eventually leads to apoptosis. Therefore, ROS suppression using strong antioxidants can be an effective agent for improving cell recovery from cryopreserved cells. Furthermore it is reported that the generation of ROS and mitochondrial potential are directly correlated [61]. Hence, it is important to understand the changes in mitochondria during long term cryopreservation of hMSCs. Therefore, the mitochondrial membrane potential (MMP) of MSCs was studied in presence or absence of antioxidant using a mitochondrial membrane potential-sensitive dye, JC-1. After thawing, cryopreserved cells with and without antioxidant (catalase 100µg) were divided into two groups for analysis. The MMP rate was measured as 14±1.2% and 26±2.0% for the combination of trehalose and ectoin with or without catalase which is stastically significant as shown in Figure 21. Similarly the conventional 10%v/v Me_2SO as sole cryoprotectant has shown significant variation in MPP with (49±4%) or without (71±10%) supplement of catalase. From this study, it has been established that the combination of trehalose, ectoin, and antioxidant has very low level of depolarization and 10% v/v Me_2SO has shown comparatively higher depolarization rate.


MMP
Catalase supplemented
Without catalase supplemented
% Cells W/ depolarized MMP
* (P=0.0112)
*(P=0.0112)
* (P=0.0038)
Trehalose, ection
Trehalose, ection, DMSO
DMSO 5%
DMSO 10%


Negative cell control
Staurosporine treated
positive control
FE-A
FITC-A
P1
P2


T30E30C100
85%-15%
T30E30
80%-20%
FE-A
FITC-A
P1
P2

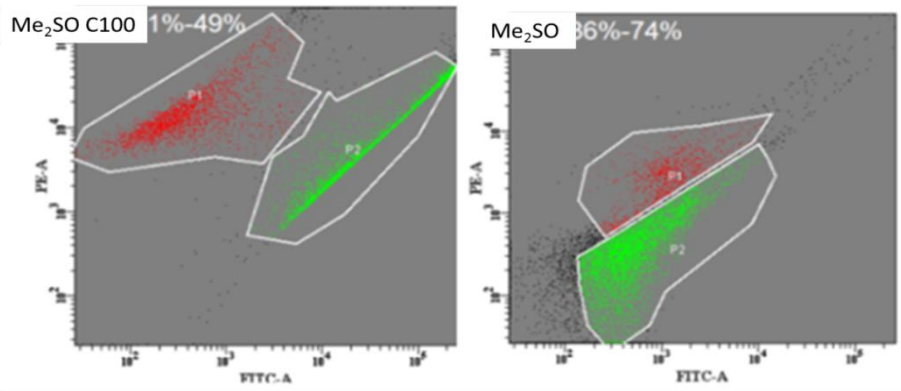


Figure 21: Analysis of mitochondrial membrane potential (MMP) of post thaw MSCs. At indicated time points, cell samples were stained with MMP sensitive dye JC-1. Change in red (polarized) to green (depolarized) fluorescence of MSCs was analyzed by FACS, and the data present as dot plots to depict percentage of MMP. Staurosporin was added to fresh MSCs as a positive control to induce maximum MMP

5.2.10 Cytoskeleton analysis of post-thaw MSCs

As it has already been mentioned that F-actin a main constituent of cytoskeleton has a key role in the maintenance of cellular function. However, F-actin depolymerises and accumulates during low temperature freezing that lead to cell damage [225]. Therefore, it has been investigated the effect of cryopreservation on the organization of F-actin. Fluorescence image analysis for change in cytoskeleton was done with MSCs treated with different cryoprotectant solutions. However, the result obtained with MSCs cryopreserved in most effective freezing solution consisting of trehalose, ectoin, and catalase (**$T_{30}/E_{30}/C_{100}$**) and traditional 10% v/v Me_2SO are shown in Figure 22. It clearly shows that MSCs frozen in $T_{30}/E_{30}/C_{100}$ solution retain their original shape without undergoing any deformation or damage in F-actin ultra structure after 48 h of post thaw culture (Figure 22 C) and similar type of morphology is also shown by control (Figure 22A). Moreover, cells have shown normal cell behavior i.e. fibroblast like morphology during post thaw culturing which is evident from Figure 22F. In contrast, the distortion in F-actin is observed when MSCs are cryopreserved in 10%v/v Me_2SO as it is indicated from Figure 22B. A similar type of morphology is observed, after 72h of post thaw cryopreserved cells in both 10% v/v Me_2SO and $T_{30}E_{30}C_{100}$ as shown in Figure 22D and E. It is further noticed (Figure 22F) that during the progress of culture, MSCs frozen in $T_{30}/E_{30}/C_{100}$ became long shuttle-shaped and on 7 days, the occurrence of confluence indicates the start of passaging. Furthermore, cells have shown parallel or whirlpool-shaped arrangement on prolonged culturing upto 2, 4 and 8 passages.

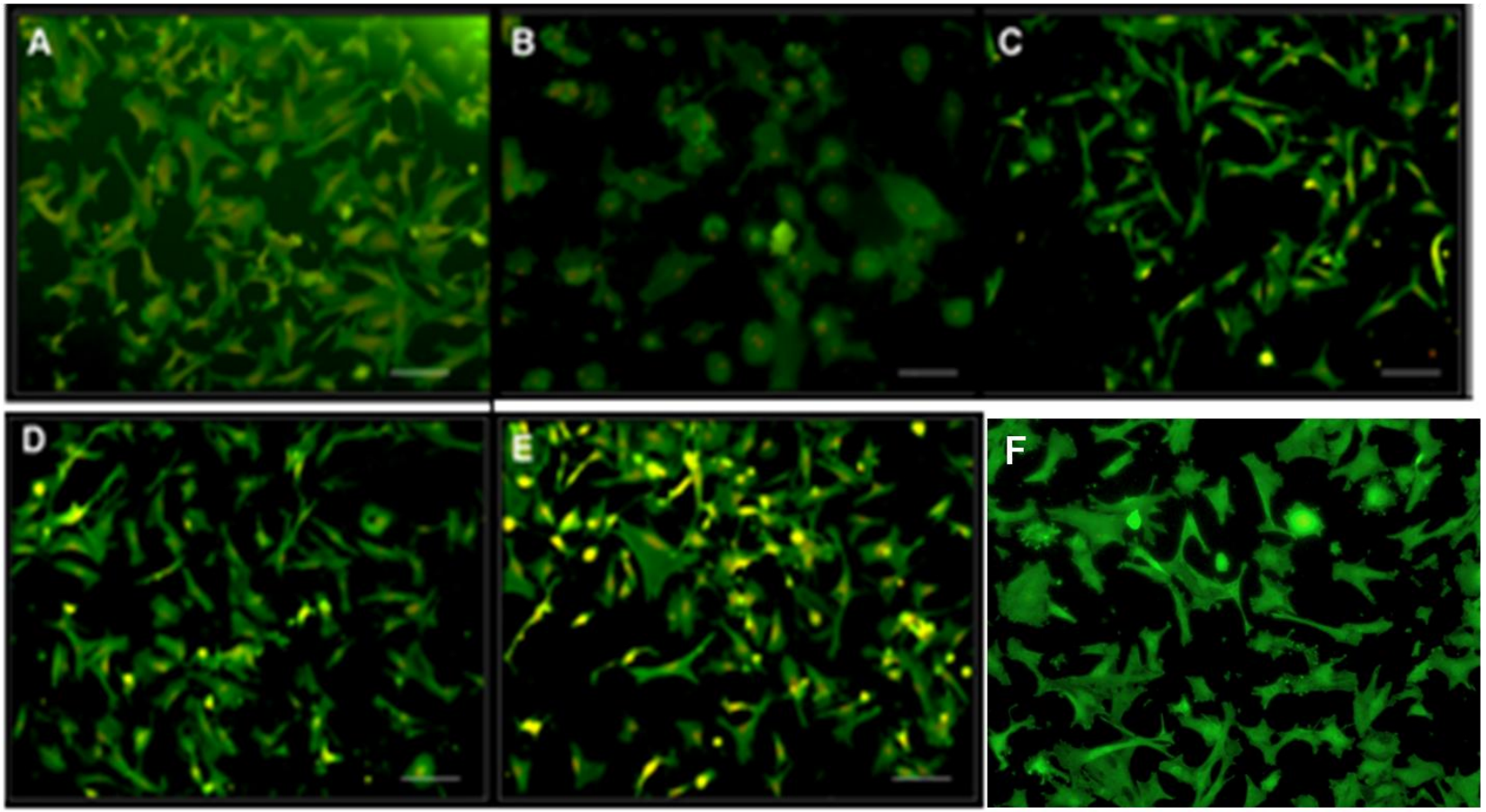


Figure 22: F-actin morphology and distribution in cryopreserved hMSCs. (A) Fresh 4th passaged MSCs (B) hMSCs cryopreserved in 10%Me_2SO after 48h post thaw (C) hMSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ after 48h post thaw, (D) hMSCs cryopreserved in 10%Me_2SO after 72h post thaw (E) hMSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ after 72h post thaw, (F) hMSCs cryopreserved in $T_{30}/E_{30}/C_{1005}$ after 7 days of post thaw. All the cells were labeled with FITC-phalloidin and visualized by fluorescence microscopy (Scale bar 50 µm

5.2.11 Effect of storage duration on cell viability

It is evident from literature that the cell viability immediately after thawing cannot be reliable criteria for the evaluation of cryopreservation efficacy. It is reported that the decrease in cell viability observed with post thaw sample is related to apoptotic and necrotic processes which occur within first 24 hr after thawing process [216]. Therefore the apoptosis assay was done with 24 hr after post thaw MSCs at an interval of 1, 6, and 12 months of freezing and was compared with fresh MSCs using Annexin/7ADD kit. Freshly isolated MSCs from cord blood were used as an experimental control. Results (table 7 and Figure 23) indicate that after 1 month, the freezing medium $T_{30}E_{30}C_{100}$ has shown maximum cell viability of 84.90%, 4.17% early apoptotic cells (annexin-V^+), 5.69% late apoptotic cells (annexin-V^+ and 7ADD+) and 5.24% necrotic cells (annexin-V^+ and 7ADD+)). The corresponding results obtained after six months of cryopreservation are 75.74% cell viability, 20.87% early apoptotic, 0.50% late apoptotic and 2.89% necrotic cells. Comparable result is also obtained with cryopreserved MSCs after 12 months of storage. The cell viability achieved in later case is 73%. Apoptosis results with $T_{30}E_{30}C_{100}$ are also shown to be encouraging as compared to the result achieved using conventional 10%(v/v) Me_2SO as freezing medium (Table 10) and a representive data is shown in Figure 23.

Table 10: Effect of storage time on MSCs viability

Freezing medium	Storage duration	%Viable cells	%Necrotic cells	%Early apoptosis cells	%Late apoptosis cells
	Freshly isolated MSCs	98.04±1.1	0.02±0.005	1.70±0.8	0.24±0.06
	1 month	84.90±2.5	5.69±0.9	4.17±0.5	5.24±1.7
$T_{30}E_{30}$, C_{100}	6 months	75.74±1.1	2.89±0.12	20.87±0.21	0.50±0.1
	12 months	73.9±2.3	3.93±1.4	17.07±2.2	3.93±0.3
	1 month	61.57±3.2	4.54±1.3	24.23±2.1	9.57±1.1
10% Me_2SO	6 months	51.74±1.7	1.42±0.5	35.86±3.2	10.98±2.5
	12 months	43.76±2.3	17.37±1.7	20.14±2.6	18.73±1.9

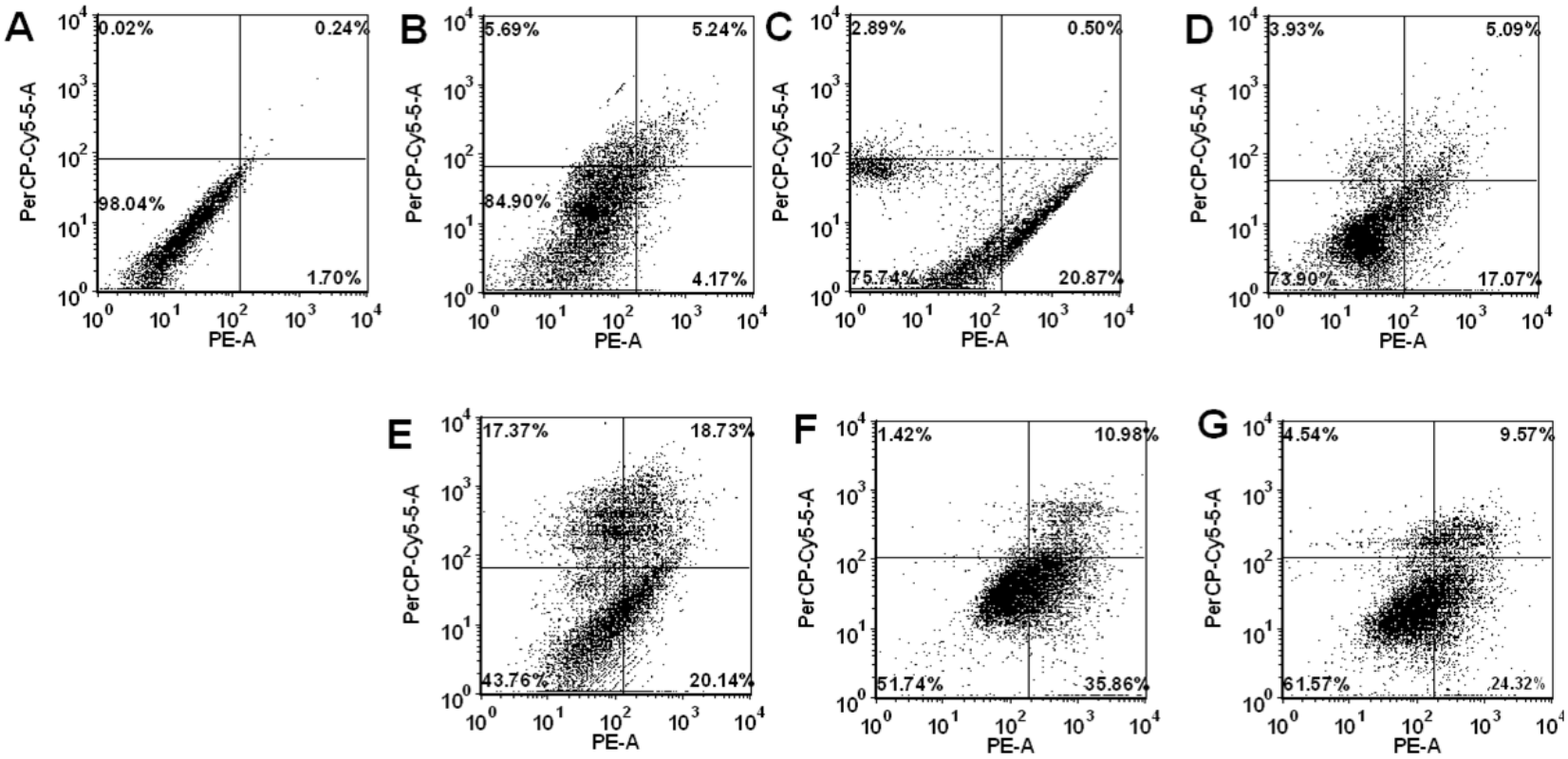


Figure 23: Flowcytometric analysis of MSCs apoptosis performed using 7AAD and annexin. The image shows the level of viability of MSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ after (B) 1 month (C) 6 months and (D) 12 months. Similarly, the image shows the level of viability of MSCs cryopreserved in 10%Me_2SO after (E) 1 month, (F) 6 months and (G) 12 months. Viability of all the samples are compared with freshly isolated MSCs (A)

5.2.12 Effect of cryopreservation on differentiation potential of hMSCs

hMSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ freezing medium were induced into adipogenic, osteogenic and chondrogenic differentiation to examine whether the thawed hMSCs retained their ability to differentiate into three specific lineages. The experimental observations are described here-

Osteogenic potential

The histological results of the osteogenic differentiation of hMSCs are shown in Figure 24(D, E). hMSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ for 12 months is observed to be sufficiently differentiated into osteoblasts as shown by the deposition of calcium on 14 days of culture (Figure 24D). The differentiation is stronger on 21 days as it is evident from the deposition of more calcium when major portion of the MSCs was mineralized (Figure 24E).

Chondrogenic potential

The chondrogenic differentiation of hMSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ and non cryopreserved hMSCs is depicted in Figure 24 (A, B). The cells were stained positive with safranin O indicates the formation of cartilage matrix on 14days of culture. The entire well plate is observed to be covered with differentiated extracellular cartilage matrix on 21 days (Figure 24B).

Adipogenic potential

The histological results of adipogenic differentiation of cryopreserved hMSCs are shown in Figure 24 (G, H). When the post thaw MSCs were exposed to the adipogenic medium, the cell morphology underwent transition from elongated fibroblast shape into a round or polygonal shape. After 14 days of induction, a consistent cell vacuolation is evident in the induced cells (Figure 24G). Most of the differentiated cells showed red lipid droplets throughout the cytoplasm when stained with 0.36% Oil red O stain as depicted in Figure 24H [64],[36].

Thus it has been demonstrated that the MSCs cryopreserved in $T_{30}/E_{30}/C_{100}$ freezing solution has retained its adipogenic, osteogenic and chondrogenic differentiation ability even after 12 months of cryopreservation.

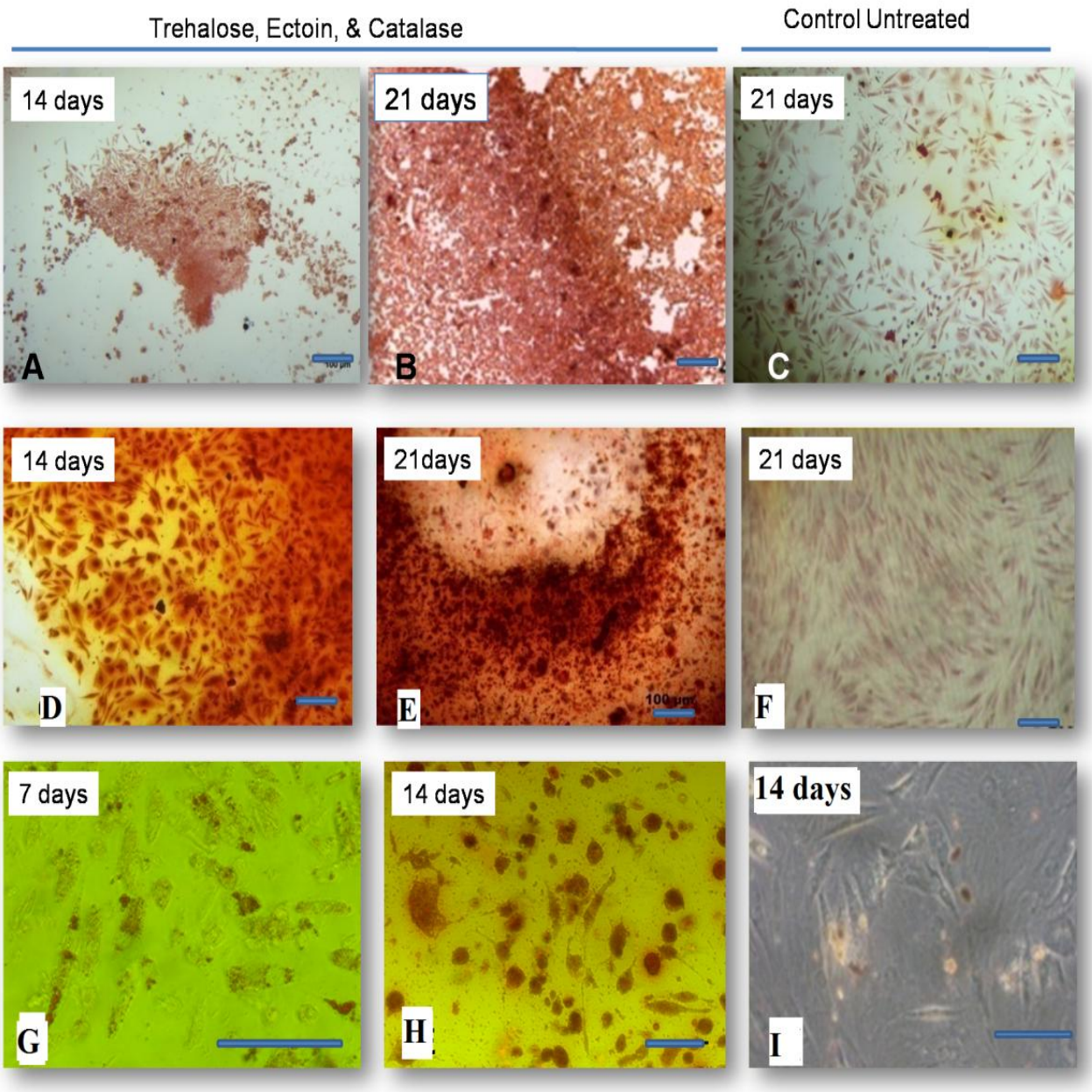


Figure 24: Representative phase contrast images of differentiated and undifferentiated hMSCs. Images in first and second column are of differentiated post thaw MSCs cryopreserved in freezing medium containing trehalose, ectoin & catalase ($T_{30}E_{30}C_{100}$). (A, B) shows differentiation of MSCs to osteocytes, (stained with alizarin Red S stain (D, E) differentiation of MSCs to chondrocytes (stained with Saffranin O and (G, H) differentiation of MSCs to adipocytes (stained with oil red O) (Scale bar 100 µm), Images in third column are for control. The images show the adipogenic, osteogenic and chondriogenic differentiation ability of cryopreserved MSCs in new freezing medium.)

4.2.13 Effect of cryopreservation on clonogenic potential of MSCs:

Cryopreservation may affect colony forming capacity of stem cells that results in the reduction of cell differentiation. In a study on controlled rate cryopreservation of UCB, it is observed that cryopreserved CD34+ cells contain a significant population of non-clonogenic early apoptotic cell which cannot be differentiated[65]. Therefore, colony-forming unit-fibroblast (CFU-F) assay was performed to investigate the effect of cryopreservation solution $T_{30}E_{30}C_{100}$ on the clonogenic capacity of post thawed hMSCs [36]. As shown in Figure 25, non-cryopreserved MSCs displayed a similar CFU-F potential as observed with post thaw MSCs cryopreserved in $T_{30}E_{30}C_{100}$ freezing media. The corresponding CFU-F values are measured as 11± 0.7 and 10 ± 0.5 (SD) respectively. Furthermore, hMSCs cryopreserved in conventional 10% v/v Me_2SO is shown to have lower colony forming capacity in comparison to hMSCs cryopreserved in $T_{30}E_{30}C_{100}$ indicating the superiority of new freezing solution than the conventional one.

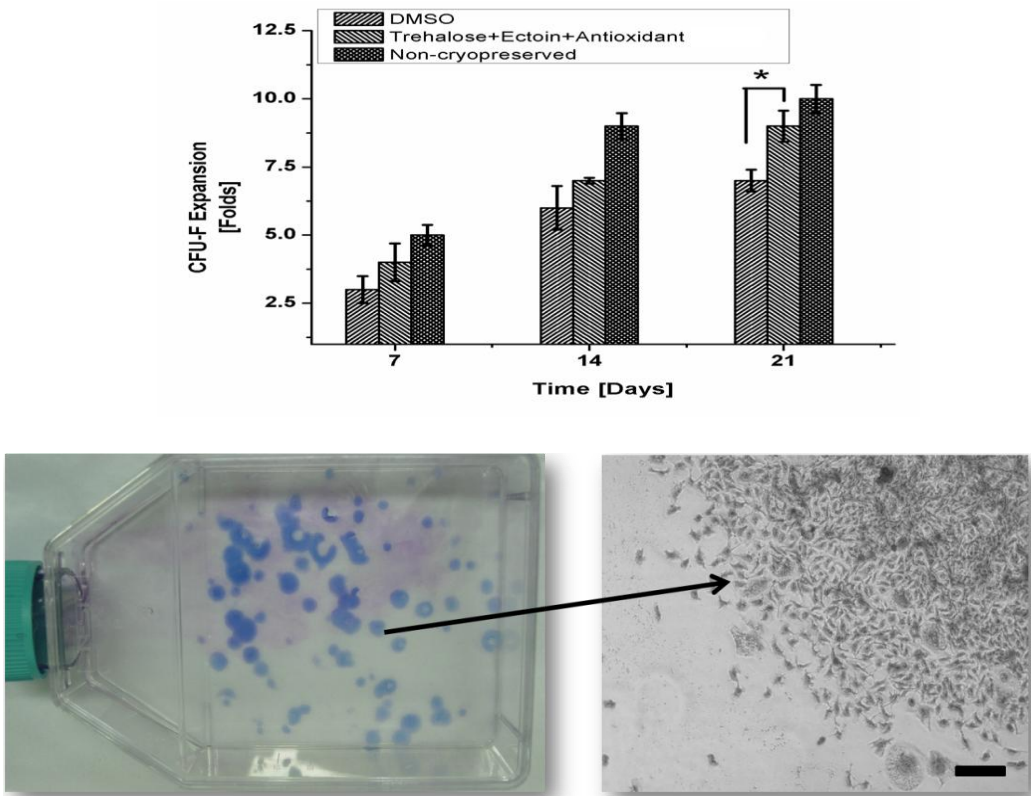


Figure 25: Effect of cryopreservation on CFU-F ability of MSCs. Data represents the mean ± SD per 1×10^3 cells of three separate experiments

5.2.14 Study on apoptosis pathways in cryopreserved induced cell death

As already mentioned that, cryopreservation plays an important role in long term storage or banking of MSCs. MSCs derived from various sources are cryopreserved by slow freezing protocols utilizing Me_2SO as cryoprotectant in freezing medium. However, it is reported that Me_2SO fails to protect hMSCs from cryopreserved induced cell death (CICD) due to external stress developed during cryopreservation process. It is, therefore, essential to understand the mechanism of cryopreserved induced cell death using cryoprotective agents, which may be helpful in developing an effective cryopreservation protocol. It is further reported that the event of CICD is largely due to apoptosis [228]. Cryopreservation induced apoptosis is significant in a wide variety of cell systems including fibroblasts, hepatocytes [232], cord blood [231-232], peripheral blood mononuclear cells [229] renal cells [235], spermatozoa [236], oocytes and ovarian tissue [234]. These studies have thrown light on the molecular based cell death following cryopreservation and thus affecting cryopreservation outcome. It is also reported that manifestation of cryopreservation induced cell death may take few h to days. However, the maximum apoptotic and necrotic activity occur at 24 h post thaw, following a decline in cell survival upto 48 h post thaw. This study of the timing of cell death following cryopreservation is termed as cryopreservation-induced Delayed-Onset Cell Death (CIDOCD). Previously, it has been observed [235] that cryoinjury induced cell death is initiated by the intrinsic or mitochondrial apoptotic pathway. However, according to recent report [236], both extrinsic and intrinsic pathways are activated after post thaw. Initiation of apoptosis after-cryopreservation has been shown via membrane-mediated Fas-receptor initiation by caspase-8 and caspase-9 activation (237, 238). Studies have also shown that apoptotic pathways are activated after thawing that in turn activates caspases 3, 8 and 9 [239].

All these studies suggest that programmed cell death following cryopreservation is important to characterize and study. The strategy to control both membrane and mitochondrial mediated apoptosis pathway is essential to improve the outcome of cryopreservation procedure. The increase in calpain-like activity during cryopreservation is another important factor that influences post thaw cell survival [238-239] and the addition of calpain inhibitor results in higher viability rate. However, the specific role of calpain in cryopreservation induced apoptosis and interrelation between calpain and caspase mediated apoptosis so far remains unclear.

In this part of research, an attempt has been made to study the apoptosis pathways and mechanism for cryopreserved induced cell death. The study also investigates the extrinsic and intrinsic pathways as well as the involvement of calpain in the MSCs cryopreservation process.

5.2.14.1 Post thaw cell viability

To identify and characterize the cryopreserved induced apoptosis in MSCs, cells were cryopreserved with inhibitors and analyzed using flowcytometer immediately after post thaw. Result shows (Figure 26) that there is no significant difference in cell viability observed immediately after post-thaw. It is further indicated that 10% cells died during cryopreservation process which is independent of apoptosis. This cell death may be due to physical stress produced during cryopreservation process [237]. Initial cell viability result shows that all cryopreserved MSCs with or without pre-treatment of inhibitor have the same cell viability range of 85±5%. Thus the result suggests that initial cell death cannot attribute to the activity of caspase 3, 8, 9 or calpain.

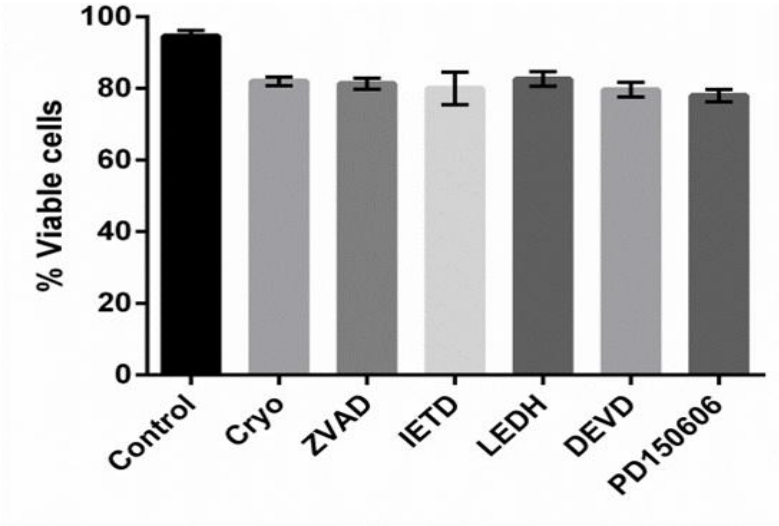


Figure 26: Effect of cryopreservation on cell viability in presence of caspase and calpain inhibitors. Bars represent the data from at least three experiments – standard error of the mean. 'Cryo' means cryopreserved sample without inhibitor. The result shows that all the cryopreserved cells with or without inhibitor have same viability range of 85%±5.

5.2.14.2 Caspase-mediated apoptotic signaling pathways

One of the important characteristics of apoptosis is DNA degradation, which is thought to be a caspase dependent process. To compare the effect of caspase on cryopreservation induced

apoptosis, MSCs were incubated with various caspase inhibitors. In this study, MSCs cryopreserved in freezing solution shows a large population of cells with sub G0 phase (degraded DNA). Pretreatment of MSCs with general caspase inhibitor (z-VAD-fmk) has shown its ability to block the degradation of DNA, whereas pretreatment with z-IETD-fmk, z-LEHD-fmk, or z-DEVD-fmk are not able to block DNA degradation (Figure-27 B) significantly. Furthermore to investigate the kinetics of cell death, cells were subjected to Annexin V/7-AAD staining to detect early apoptotic (Annexin V), late apoptotic/necrotic (Annexin V/7-AAD), and necrotic (7-AAD) cells (Figure 27). In comparison to non-cryopreserved MSCs, a significant percentage of early apoptotic and late apoptotic/necrotic cells are observed when MSCs were cryopreserved in 10% Me_2SO. However, within 24 h of post thaw, there is a significant amount (approximately 35%) of cell death occurs even with caspase inhibition by z-VAD-fmk.

The kinetics of early, late apoptotic and necrotic cells in presence of different inhibitor treatment after 24h of post thaw are summarized in Figure 27. Although the pretreatment of MSCs with z-VAD-fmk before cryopreservation is not observed to be completely protective after 24 h of post thaw, it inhibits the loss of cell viability significantly as compared to cryopreserved MSCs without inhibitor pretreatment. In marked contrast, pretreatment of these cells with selective caspase inhibitors such as z-IETD-fmk, z-LEHD-fmk, and z-DEVD-fmk did not have any effect on the loss of cell viability induced by cryopreservation.

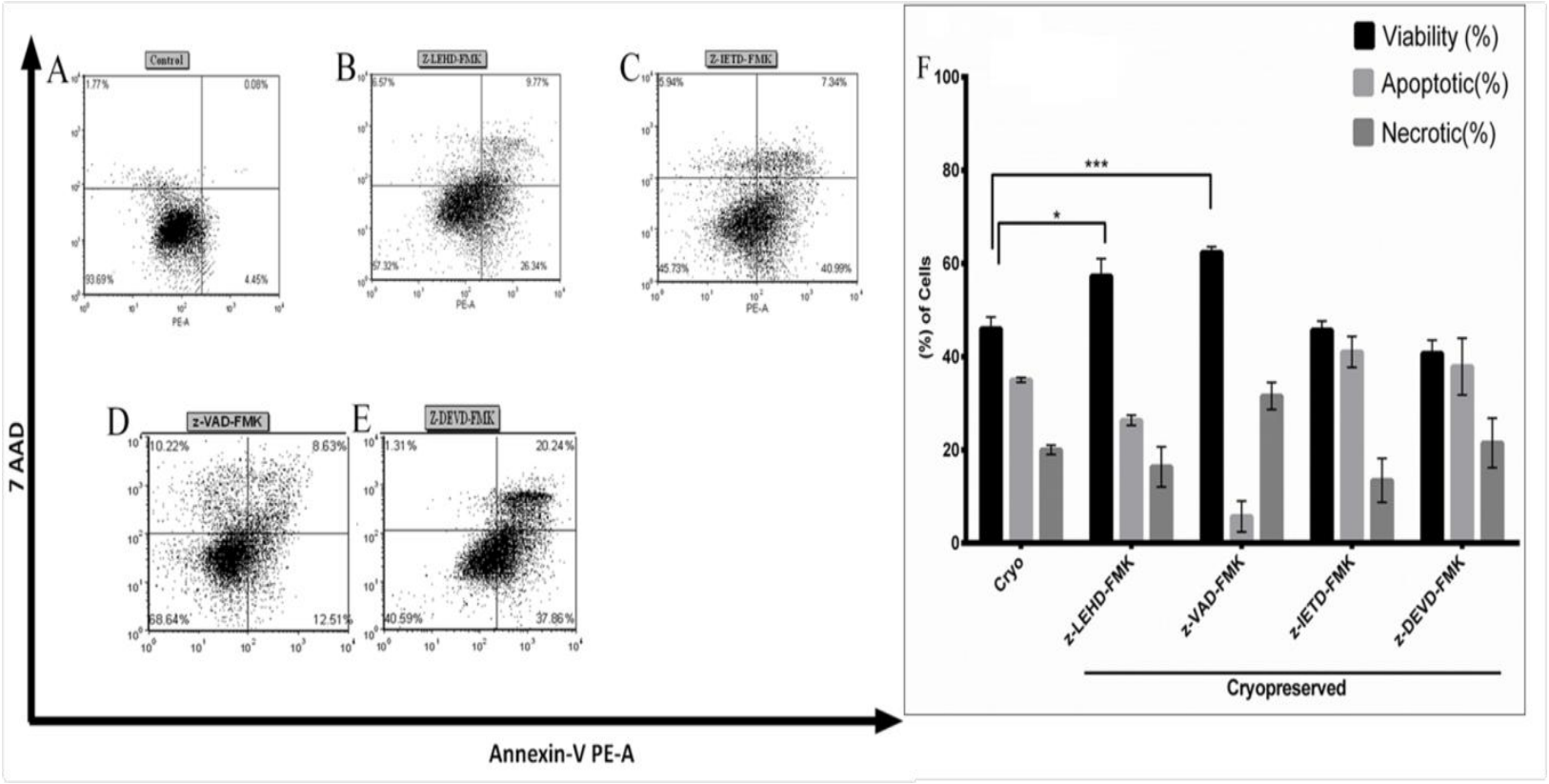


Figure 27: Effect of caspase inhibitors on cryopreservation-induced apoptosis of MSCs. (A) Untreated MSCs; (B) Cryopreserved sample treated with 50 mM z-LEHD-FMK; (C) Cryopreserved sample treated with 50 mM z-VAD-FMK; (D) Cryopreserved sample treated with 50 mM z-IETD-FMK; (E) Cryopreserved sample treated with 50 mM z-DEVD-FMK; (F) The % of viable, apoptotic, and necrotic cells. Bars represent the data from at least three experiments ± standard error of the mean [* indicates difference from control (i.e., cryopreserved MSCs without pretreatment with caspase inhibitor)]. Cryo = cryopreserved sample without inhibitor pretreatment

5.2.14.3 Western blotting analysis of caspase proteins

To dissect the potential role of caspases in cryopreservation-mediated MSCs apoptosis, MSCs were pretreated with various caspase inhibitors before cryopreservation. The aim of investigation is whether these caspase proteins are cleaved during cryopreservation induced apoptosis of MSCs, and whether this process is selectively inhibited by z-VAD-fmk, z-IETD-fmk, z-LEHD-fmk, or z-DEVD-fmk. MSCs with or without pretreatment with caspase inhibitors were cryopreserved and 24h post thaw cells were harvested for Western blot analysis using antibodies specific to caspases 8, 9, and 3.

The Western blot analysis result is shown in Figure 23. The Figure shows the appearance of typical cleavage fragments of caspases 8, 9, and 3 proteins in cryopreserved MSCs with and without treatment with caspase inhibitors except z-VAD-fmk. To determine the potential involvement of the death receptor pathway in cryopreserved-induced apoptosis, we also examined the activity of Bid protein. It has been reported that Bid relays the apoptotic signal from the cell surface to the mitochondria. Furthermore, after cleavage due to apoptosis, truncated Bid translocates into mitochondria and disrupts the mitochondrial membrane potential, thereby triggers cytochrome c release [255]. Cryopreservation also induces activation of Bid as evident from Figure 28. It is observed that 32 kDa-band represents the precursor of caspase-3 while two smaller bands of 17 kDa and 12 kDa indicate the active subunits. A significant decrease in amount of caspase-3 precursor (32 kDa) in cryopreserved MSCs is observed in presence of z-DEV-fmk caspase inhibitor. Whereas a significant increase in caspase-8 (18 kDa) activity is observed in MSCs cryopreserved in presence of other inhibitors. Moreover, the general caspase inhibitor (z-VAD-fmk) tends to decrease the expression of caspase proteins to a non-detectable level. Result also shows that z-VAD-fmk is able to significantly decrease the level of caspase-9 precursor. Other than z-VAD-fmk, there is no significant alteration of caspase-9 precursor in MSCs cryopreserved with other apoptotic inhibitors observed. Cryopreservation induces the activation of the initiator caspases 8, the effector caspase 3 and the pro-apoptotic protein Bid. Caspase 9 is activated by release of cytochrome *c* from the mitochondria, which causes the formation of the apaf-1/caspase 9 apoptosome. Caspase 8 is cleaved and activated during the formation of the DISC at the plasma membrane. Caspase 3 is normally in the cell as an inactive zymogen, where it is cleaved by initiator caspases such as 8 and 9 [26].

However, the result shows that neither caspase 8/caspase 3 nor caspase/tBid/mitochondria pathway is solely responsible for cryopreserved induced MSCs cell death as both proteins from both the pathways are activated.

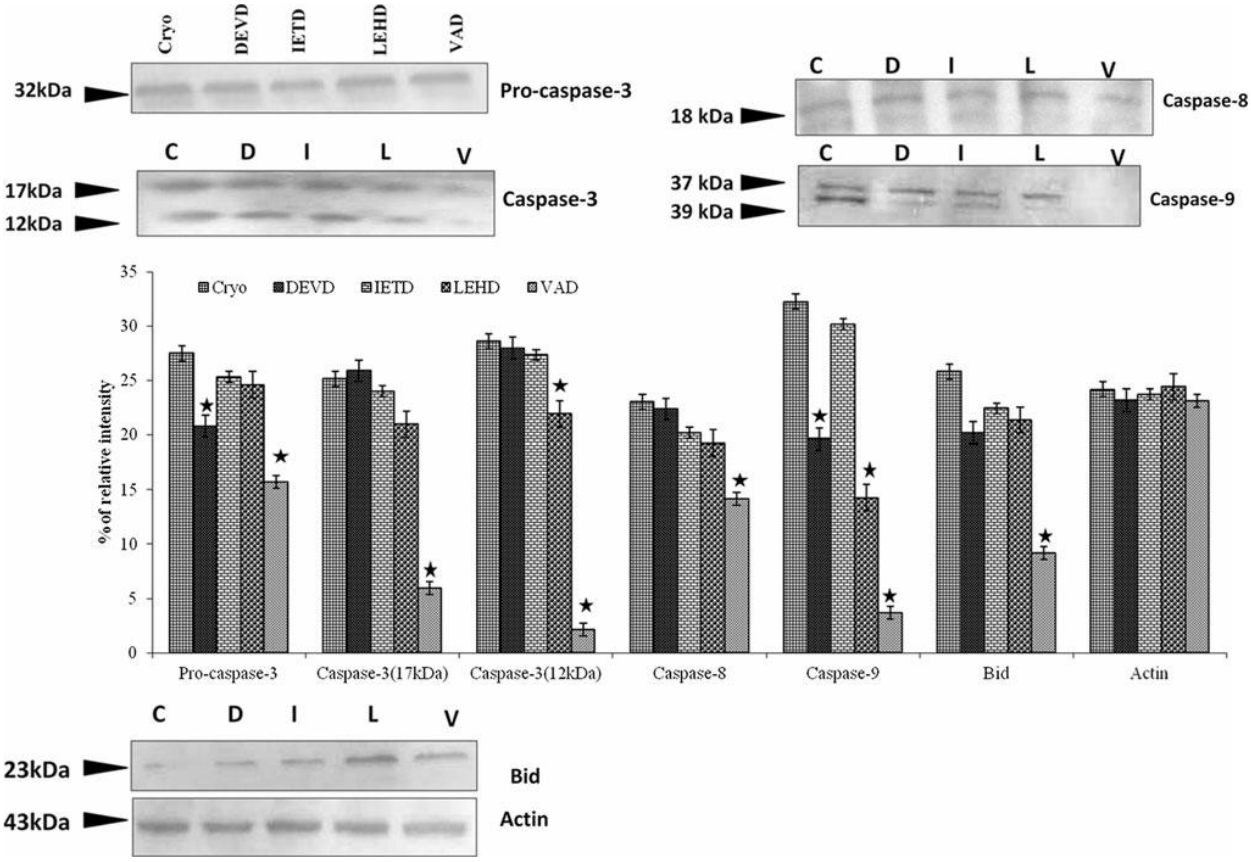


Figure 28: Effect of cryopreservation on activation of caspase and Bid proteins. Actin has been used as housekeeping marker protein. Bars represent data from at least three experiments – standard error of the mean. C = cryopreserved sample without inhibitor pretreatment; D= cryopreserved sample treated with 50 mM z-DEVD-FMK inhibitor; I = cryopreserved sample treated with 50 mM z-IETD-FMK; L = cryopreserved sample treated with 50 mM z-LEHD-FMK; V= cryopreserved sample treated with 50 mM z-VAD-FMK.

5.2.14.4 Mitochondrial membrane potential

The mitochondrial or intrinsic pathway is considered to be one of the two predominant signaling cascades leading to apoptosis and many studies have described the depolarization of the mitochondrial membrane as an important characteristic of this pathway [245, 246]. Further, mitochondrial membrane depolarization is associated with release of cytochrome c and subsequent activation of caspases 9 and 3 [247]. To examine whether cryopreservation induced

apoptosis of MSCs involves any loss of mitochondrial membrane potential, a dual-fluorescent dye JC-1 was used. When the mitochondrial membrane depolarizes during cell death the dye dissociates into its monomeric form, which fluoresces at 530nm. In this study, the monomer vs. aggregate fluorescence plots (Figure 29 (A)) indicates a loss in aggregate fluorescence and an increase in monomer fluorescence representing a decrease in mitochondrial membrane potential. Interestingly, z-VAD-fmk, z-IETD- fmk, z-LEHD- fmk, z-DEVD-fmk caspase inhibitors has shown to significantly inhibit the loss of mitochondrial membrane potential caused by cryopreservation.

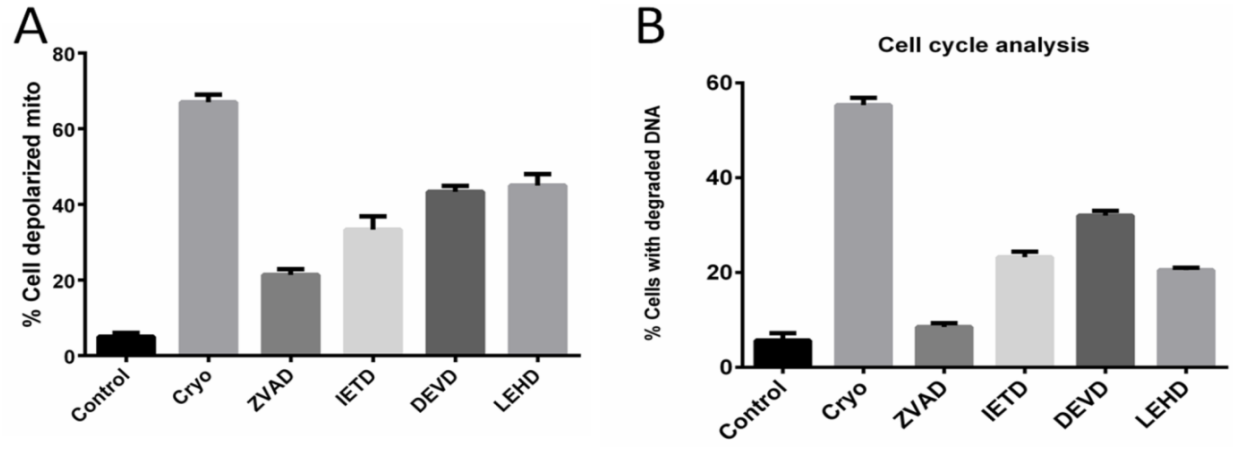


Figure 29: Effect of caspase inhibition on cryopreserved-induced mitochondrial membrane potential and DNA degradation in MSCs after 24 h of post thaw (A) Bar graphs showing the percentage of depolarized MSCs for cryopreserved MSCs with and without caspase inhibitor and (B) the corresponding percentage of MSCs with degraded DNA. "Cryo" means cryopreserved sample without pre-treatment with any inhibitor. Bars represent data from three separate experiments ± standard error of the mean

5.2.14.5 Calpain activity

Calpain is a calcium-dependent neutral protease and is reported in initiating or progressing the events associated with apoptosis causing cell death during cryopreservation [251]. So in this study, the effect of calpain with and without calpain specific inhibitor (PD150606)) were investigated in apoptosis induced cell death during cryopreservation of MSCs. The choice of PD150606) as an effective calpain inhibitor is based on the published literature [252].

To study the effect of calpain inhibitor on cell cycle, MSCs were pretreated with calpain inhibitor before cryopreservation. Result shows the significant inhibition of DNA degradation by the addition of calpain inhibitor (Figure 30 B). To examine the effect of calpain inhibitor on post thaw, MSCs were assessed by Annexin V assay for determining viable cells, apoptotic cells and necrotic cells. From the results it is indicated that, the presence of calpain inhibitor does not show any significant improvement in cell viability. However the addition of general caspase inhibitor (z-VAD-fmk) and calpain inhibitor (PD150606) in combination improve the cell viability upto 80±2%. Thus the result suggests that cryopreserved induced apoptosis is attributed by both caspase dependent and caspase independent.

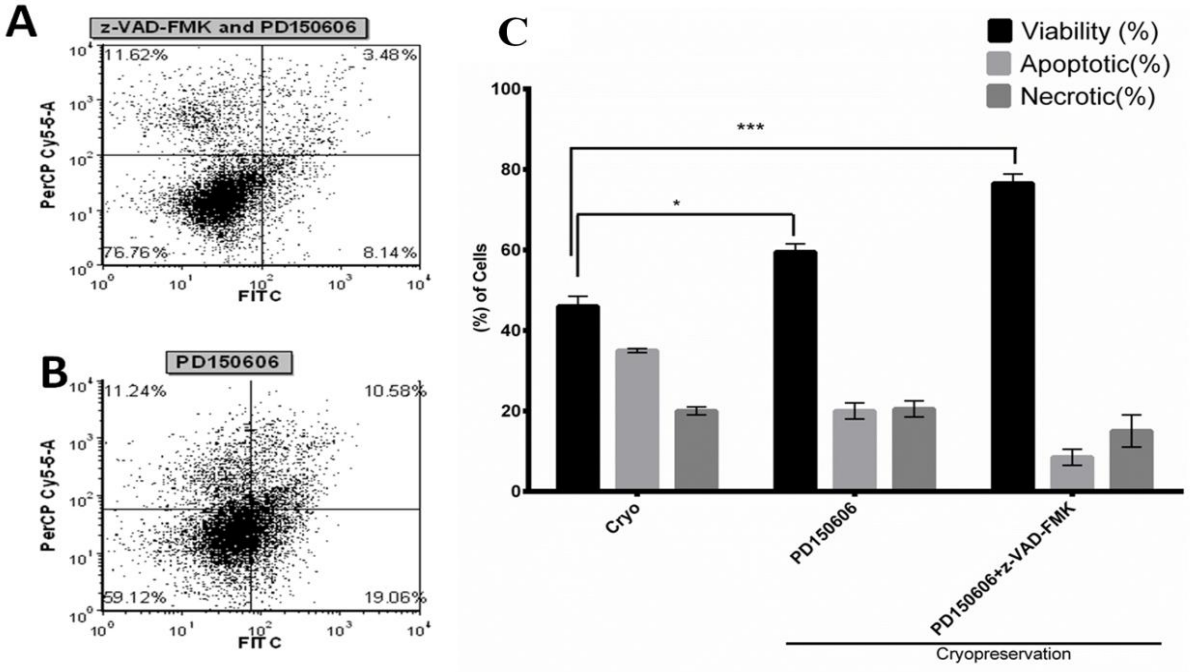


Figure 30: Effect of caspase inhibitors on cryopreservation-induced apoptosis of MSCs. (A) Untreated MSCs; (B) Cryopreserved MSCs treated with 50 mM z-LEHD-FMK; (C) Cryopreserved MSCs treated with 50 mM z-VAD-FMK; (D) Cryopreserved MSCs treated with 50 mM z-IETD-FMK; (E) Cryopreserved MSCs treated with 50 mM z-DEVD-FMK; (F) denotes % viable, apoptotic, and necrotic cells. Bars represent the data from at least three experiments – standard error of the mean, [* indicates difference from control (i.e., cryopreserved MSCs without pretreatment with caspase inhibitor)]. 'Cryo' means cryopreserved sample without inhibitor.The flowcytometric analysis shows that cryopreserved induced apoptosis is attributed bt both caspase dependant and caspase independent.

5.2.14.6 Western blotting analysis for calpain protein

The Western blot analysis has revealed that (Figure 31) the expression of calpain increases in cryopreserved MSCs and addition of calpain inhibitor decreases the cleavage of calpain protein. It is also observed that the addition of pan caspase inhibitor does not have any effect on expression of calpain protein. Interestingly, the addition of both calpain and general caspase inhibitor (z-VAD-fmk) has shown synergistic effect in lowering the expression of calpain. This result suggests that the general caspase inhibitor is not able to inhibit calpain dependant pathway. Also caspase and calpain mediated cell death pathways are interconnected.

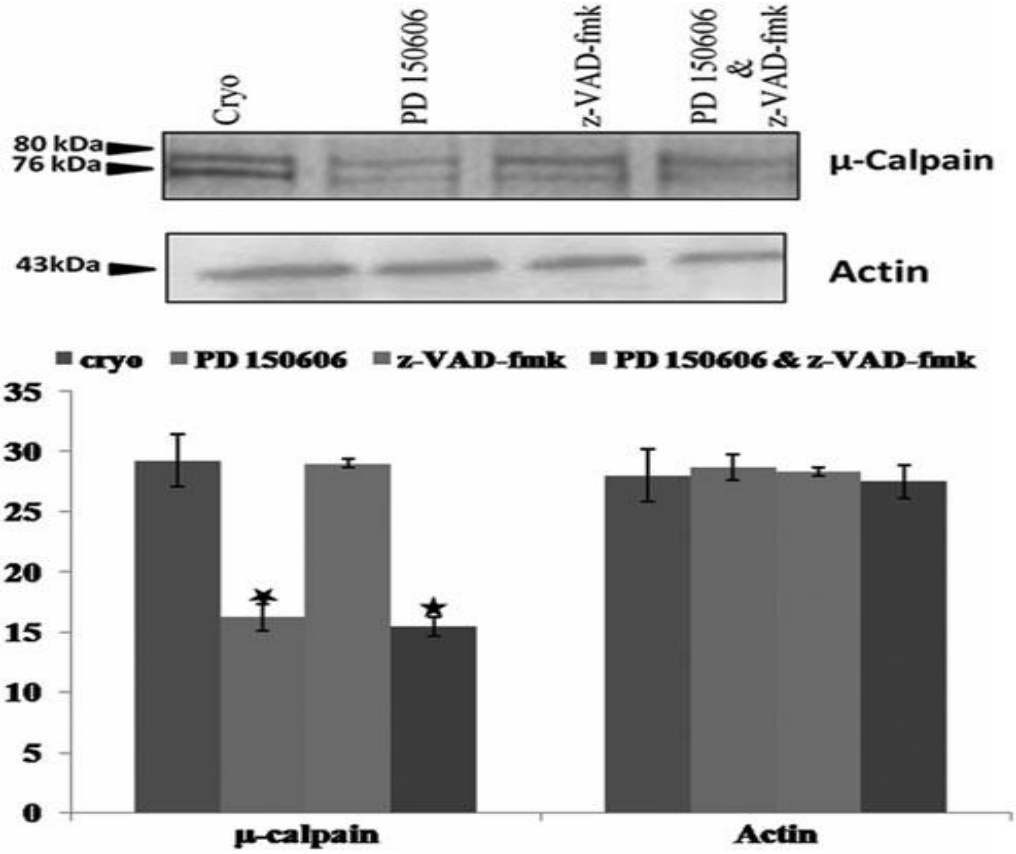


Figure 31: Effect of calpain inhibitor PD150606 on activation of m-calpain proteins. Cryopreserved MSCs were investigated after pretreatment with PD150606 with or without z-VAD-FMK. Bars represent data from three separate experiments– standard error of the mean. 'Cryo' means cryopreserved sample without inhibitor. PD150606 =MSCs pretreated with 50 mM PD 150606; z-VAD-FMK=MSCs pretreated with 50 mM z-VAD-FMK; PD150606 and z-VADFMK=MSCs pretreated with a combination of 50 mM PD150606 and 50 mM z-VAD-FMK.The result shows that the general caspase inhibitor is not able to inhibit calpain dependent pathway

5.2.14.7 Effect of calpain inhibitor on mitochondrial membrane potential and DNA degradation

The experimental result of the influence of calpain inhibitor is shown in Figure 32A. Calpain inhibitor (PD150606) did not show any significant effect in mitochondrial membrane potential (MMP) during cryopreservation. However, the combination of PD150606 and z-VAD-fmk significantly maintained the mitochondrial membrane potential (MMP) and decreased the amount of DNA degradation in post thaw MSCs. These data suggest that the apoptotic signaling to the mitochondria has both caspase dependent and independent components. And the calpain dependent pathway also works through mitochondrial pathway.

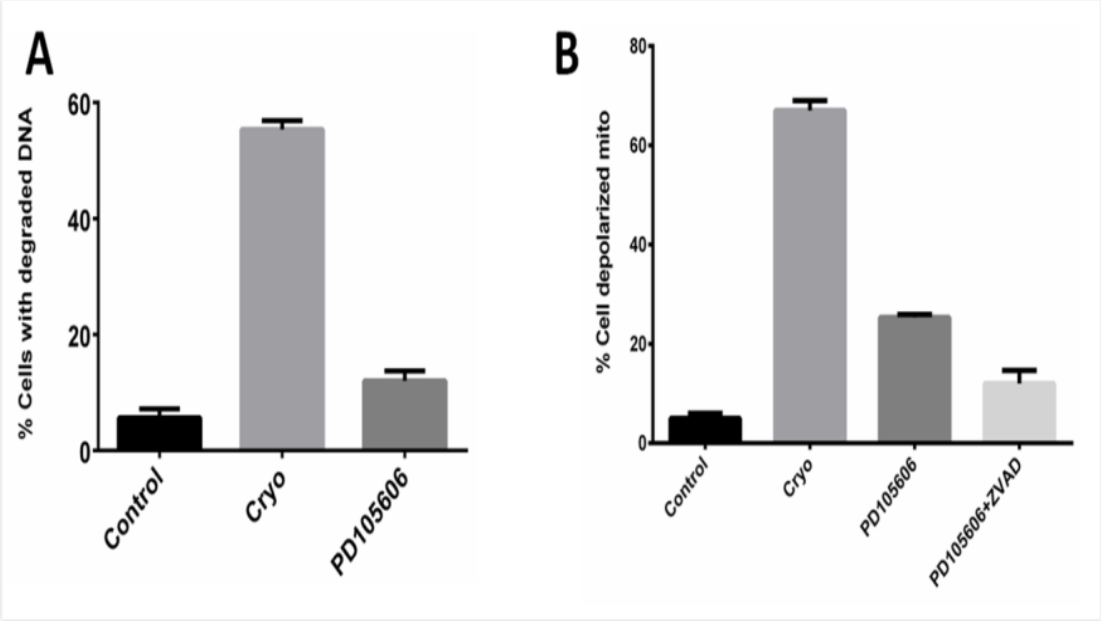


Figure 32: Comparative confocal images (F-actin) of cryopreserved and non-cryopreserved silk nanofiber TECs. (A) (i) Shows noncryopreserved TECs (control), (ii and iii) shows TECs immediately after cryopreservation with scale bar 5µm, (B) 24 h after cryopreservation with scale bar 5 µm and (C) close-up images of actin cytoskeleton 48 h after cryopreservation (ii) with scale bar 40 µm. In both Me_2SO and $T_{30}/E_{30}/A_{100}/I$ cryopreserved groups, the cytoskeleton in the frozen/thawed regions is damaged, with shortened extensions and less number of cells compare to noncryopreserved cells. In Me_2SO frozen group, the cells have condensed, distorted after freezing, and less damage is observed in $T_{30}/E_{30}/A_{100}/I$ groups.

5.2.15Optimization of Controlled rate freezing parameters for cryopreservation of hMSCs using $T_{30}E_{30}C_{100}$ freezing medium

It is fact that depending on the type of cells and freezing media, there is need to maintain an optimal condition that offers maximum cell recovery following cryopreservation. In this context, incubation temperature, freezing medium, cooling rate, storage temperature, cell concentration during freezing, and duration of storage, are some of the key factors that has great influence on the cryopreservation outcome. while a few studies have examined the influence of freezing conditions on post thaw viability of MSCs using Me_2SO as cryoprotectant [41, 44], no such study has been reported so far for optimization of cryopreservation conditions of MSCs using combination of cryoprotectants. Keeping this in view, efforts has been given in this section to investigate the effect of key controlled rate freezing parameters on cryopreservation of MSCs and establish the optimum cryopreservation condition using the most effective freezing medium $T_{30}E_{30}C_{100}$ in presence of general caspase and calpain inhibitors.

5.2.15.1 Effect of cooling profile on MSCs viability

The cryopreservation experiment was carried out at different prenucleation cooling rate such as- 1ºC/min, 2.5ºC/min, 5ºC/min and 10ºC/min to examine the effect of prenucleation cooling rate on cell viability. The others parameters such as: nucleation temperature (-7.5°C), cold spike temperature (-40°C), hold time (10min), and post nucleation cooling rate (1°C) were maintained constant. The parameters were chosen based on published literature [143]. As shown in Figure 33a, the effect of prenucleation temperature is shown to have a significant influence on cell viability. The cell viability increases with decrease in cooling rate. The maximum cell viability of 82% is obtained at prenucleation cooling rate of -1.0°C/min. Comparable cell viability is also achieved at -2.5°C/min.

Similarly, the effect of nucleation temperature (-2.5°C, -5°C, -7.5°C and -10°C) on cell viability was studied and the experimental result is shown in Figure 33b. The highest cell viability of 78% is achieved at nucleation temperature of –7.5°C and a decline in cell viability is observed with either higher or lower than –7.5°C nucleation temperature. The others parameters such as:

prenucleation cooling rate 1°C/min, cold spike temperature (-40°C), hold time (10min), and post nucleation cooling rate (1°C) were maintained constant.

The effect of shock cooling or so called cold-spike temperature on cell viability was investigated by performing cryopreservation experiment at varying shock cooling temperature in the range of -20°C, -40°C, -60°C and -80°C. Figure 28c shows the effect of shock cooling temperature on MSCs recovery. The optimal cell viability was recorded as 85% at -80°C. The others parameters such as: prenucleation cooling rate 1°C/min, nucleation temperature (-7.5°C), hold time (10min), and post nucleation cooling rate (1°C) were maintained constant.

Figure 33d shows the effect of different holding times at post-nucleation temperature of -30°C. An upward trend of cell viability is observed till holding time 10min is reached and there is decrease in cell viability observed beyond this holding time. The optimal cell recovery of more than 80% is obtained with 10min holding time.

Finally, Figure 33e shows the effect of post nucleation cooling rate on post thaw cell viability. A decrease in cell viability is observed over the range of cooling rate tested and the post nucleation cooling rate of 1°C/min offers the maximum cell viability (84%) results. The others parameters such as: prenucleation cooling rate 1°C/min, nucleation temperature (-7.5°C), and holding time (10min) were maintained constant.

Our results demonstrate the sensitivity of cell viability to the cooling profile used during cryopreservation in controlled rate freezing. However, the optimized cooling profile is establishes as prenucleation cooling rate 1^{o}C/min, nucleation temperature -7.5^{o}C, cold spike of 80^{o}C/min, post nucleation holding time of 10min and post nucleation cooling rate of 1^{o}C/min. The resultant optimum cooling profile is shown in Figure 34. The results from the controlled rate freezer (CRF) temperature control study, illustrating the process temperature profiles for sample in the CRF chamber with cold-spike nucleation control over the period of a cryopreservation study. A blue line indicates the temperature profile of the chamber in response to a programmed profile for freezing hMSCs. The typical cooling profile included five steps: (1) equilibrate temperature, (2) induce ice formation with a cold spike, (3) warming rate, (4) hold at –35 °C for 10 minutes, and (5) cool to –40 °C at a cooling rate of 1.0 °C/min. The uniformity of nucleation is apparent right after the cold gas was plunged to –80 °C, as shown in the figure

temperature profiles of sample and chamber were tightly aligned together through the whole freezing process. Optimization of cooling profile has been carried out based on the sample temperature.

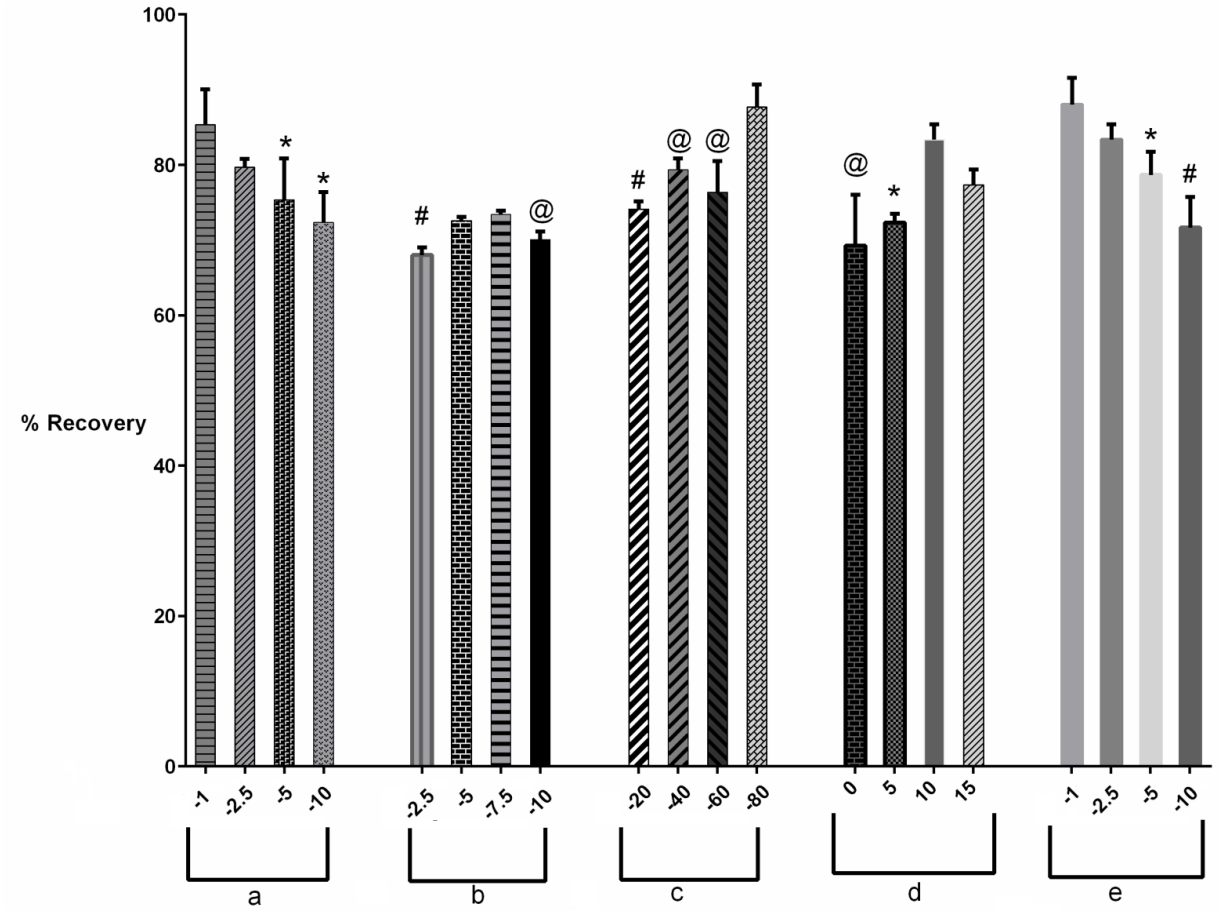


Figure 33: Percentage recovery of MSCs after cryopreservation in controlled rate freezer under different cooling variables (a) pre-nucleation cooling rate in °C/min (b) prenucleation temperature in °C; (c) cold-spike temperature in °C/min; (d) holding time in min(e) post nucleation cooling rate in °C/min

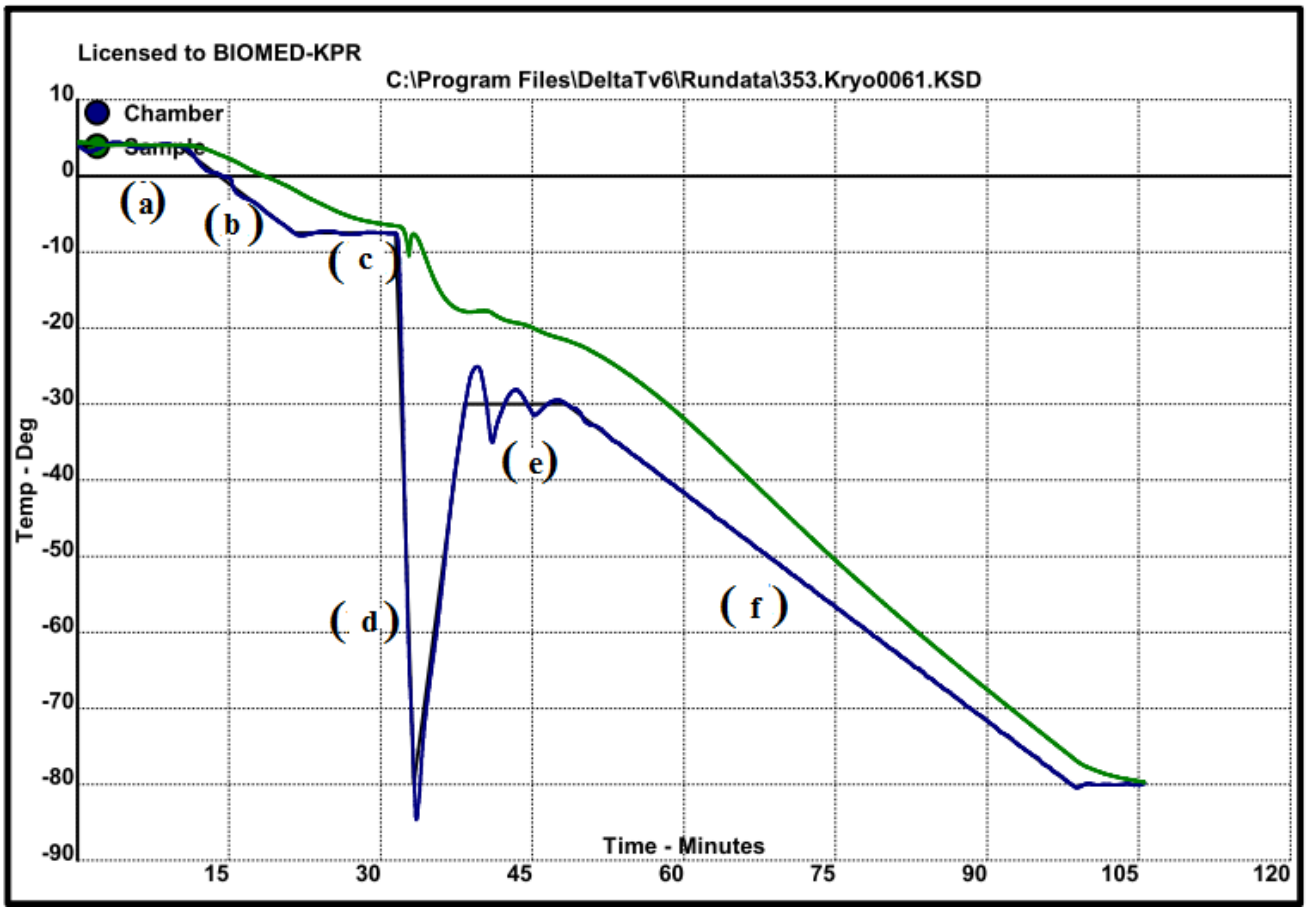


Figure 34: Temperature profile of cryochamber and sample with time; blue line represents the chamber temprature, green line represents the sample temperature. (a) equilibrium temperature; (b) a pre-nucleation cooling step; (c) a pre-nucleation temperature step; (d) a cold spike step; (e) a post-nucleation temperature hold step and (f) a final cooling step.

5.2.15.2 Effect of cell density

The cryopreservation experiment was designed to test whether cell concentration has any effect on cell viability following cryopreservation. Study has shown that increase in cell concentration decreases cell viability and metabolic activity in hepatocytes [70]. During slow cooling, ice formation mainly occurs on extracellular spaces but as the cell concentration increases extracellular spaces are decreased by cell packing due to which intracellular ice formation occurs more readily. In this study the varying concentration of MSCs such as 0.5, 1, 2, 3, 4 and 5×10^6/ml were investigated to establish optimal concentration to maximize cell viability. The cell viability results of post thawed MSCs are shown in Figure 35. Each set of experiment was performed in triplicate. An unpaired t-test was conducted taking 3×10^6/ml MSCs as control. The

result indicates that the maximum cell recovery is achieved with 3×10^6/ml followed by 2×10^6/ml. Furthermore, a decrease in cell viability is observed with increase in cell concentration beyond 3×10^6/ml.

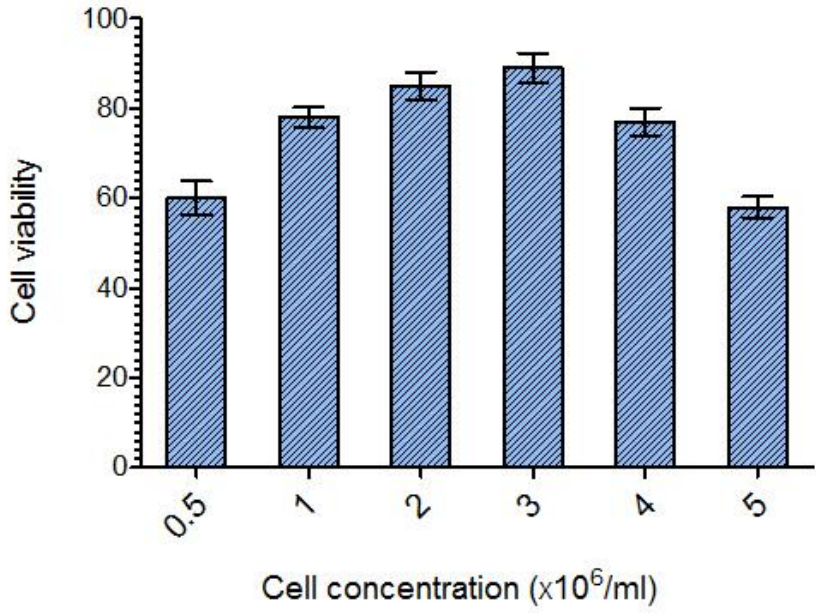


Figure 35: Effect of cell concentration on cell viability

5.2.15.3 Effect of storage temperature

Recent study suggests that storage temperature plays an important role in cell viability [91-102]. So, the effect of different storage temperature such as −80°C, −150°C and −180°C has been studied on the cell viability. The different storage temperatures were chosen based on the published literature [66-69]. The experimental data of MSCs viability after cryopreservation is shown in Figure 36. Result shows that cell viability significantly varies with storage temperature from -80°C to -150°C. However the maximum cell viability is achieved at -150°C and then a decline in cell viability. is observed

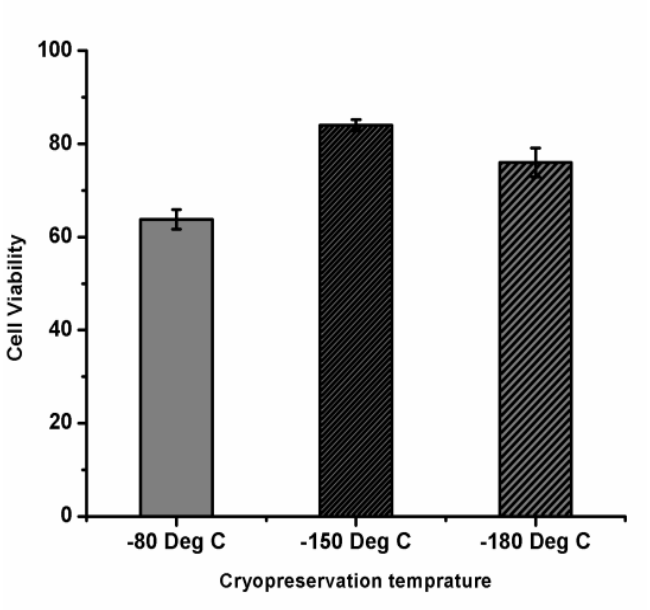


Figure 36: Effect of different storage temperature on cryopreserved MSCs viability

5.2.15.4 Verification of cryopreservation protocol

5.2.15.4.1 Effect of cryopreservation on cytoskeleton and mitochondria distribution

As it is mentioned earlier, F-actin has a role in controlling cell morphology and surface movement [120] as well as cellular functions [121-124]. It is reported that the critical factor that changes F-actin morphology and distribution is not associated with addition or removal cryoprotectant but freezing profile [123]. Result shows (Figure 37) that at the optimum controlled rate of freezing (CRF) condition F-actin shows a better maintenance recovery in morphology and distribution of cytoskeleton in cryopreserved MSCs than that of the cells cryopreserved using uncontrolled rate of freezing using mechanical freezer. The result also confirms the organization of cytoskeletons of cryopreserved MSCs is similar to the non-cryopreserved MSCs. The quantitative and morphological alterations in the mitochondrial potential were detected using JC-1, a fluorescent probe [125]. Result shows that there is no significant changes in mitochondria fluorescence in cryopreserved MSCs at optimum condition compared to non cryopreserved MSCs (as negative control) shown in Figure 37.

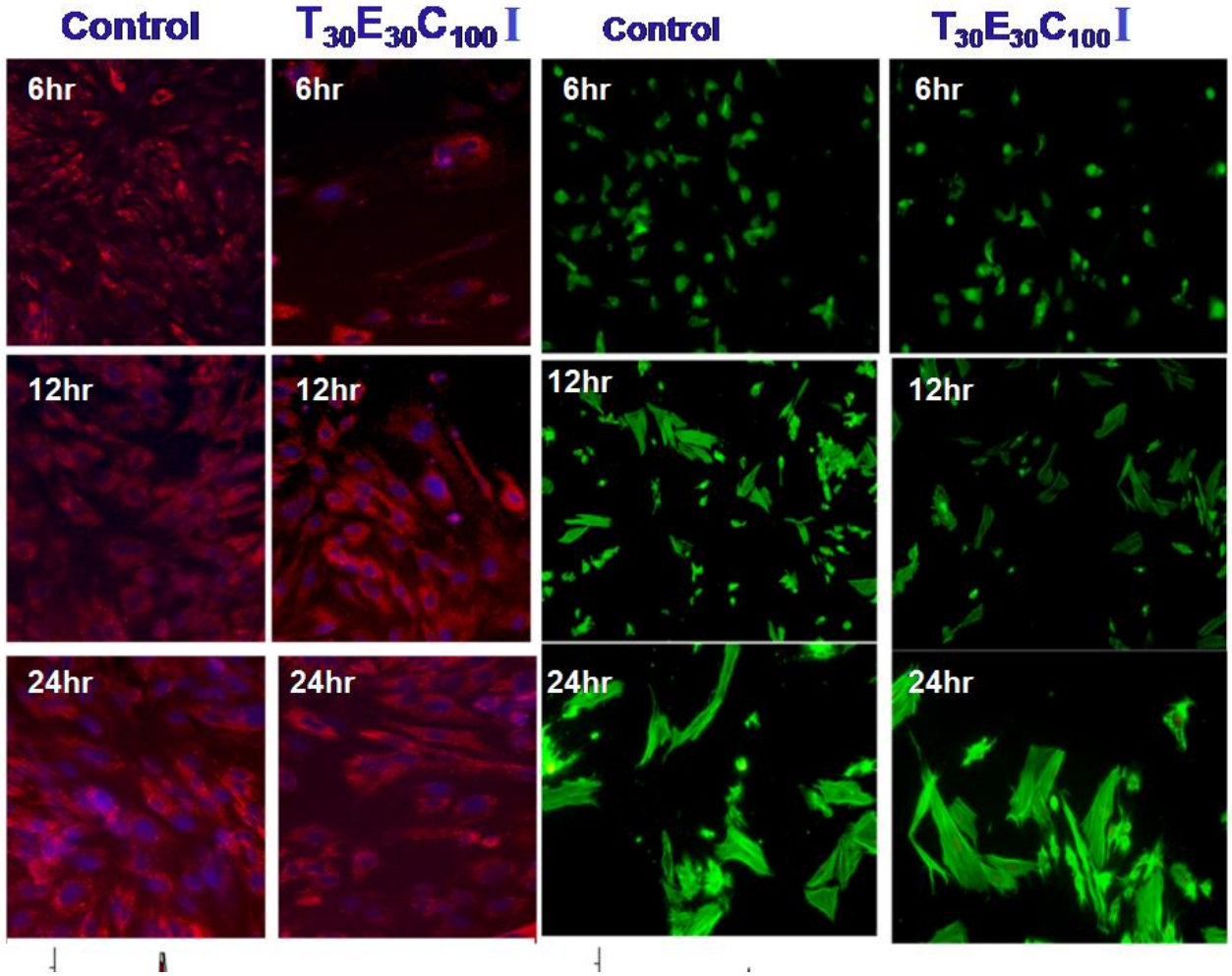


Figure 37: Fluorescence images of F-actin and mitochondria distribution in fresh and cryopreserved hMSCs. Scale bar: 100µm

5.2.15.4.2 Cell cycle and viability analysis

Cell cycle analysis was performed to determine the cell phase after cryopreservation (Figure 38). The results indicate that both non cryopreserved and cryopreserved MSCs at optimum controlled rate freezing condition show similar trend with highest percentage of cells in G_0/G_1 as compared to, S and G_2/M which signify that most of the cells are active and proliferating (Figure 38). Analysis of cell cycle indicates that the cryopreserved MSCs are in 78% G_0/G_1; 4.67%G_2/M; and 6.43% S phases (Figure 38B). The average viability is calculated based on flowcytometry is 85%.

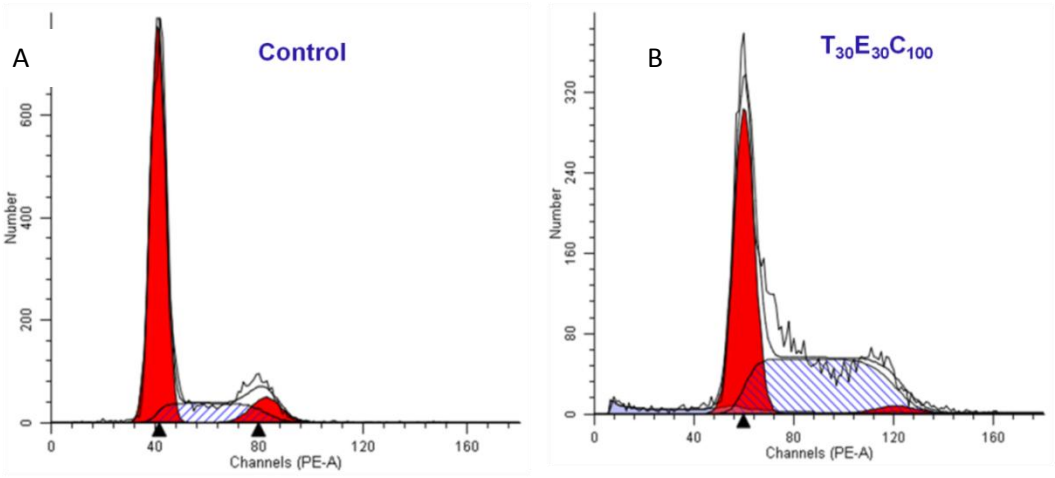


Figure 38: Cell cycle analysis of (A) non-cryopreserved MSCs and (B) Cryopreserved MSCs

Comparison of the freezing solution

A comparison of the efficiency of freezing solutions for the cryopreservation of MSCs has been shown in Table 11. It is indicated that after 12 months of cryopreservation 10% v/v Me_2SO shows the lowest viability. Further an increased viability of $T_{30}/E_{30}/C_{100}$/I was obtained with addition of apoptotic inhibitors .and the highest MSCs viability is shown by $T_{30}/E_{30}/C_{100}$/I at optimum controlled rate freezing condition

Table 11: Comparison of cell viability of cryopreserved MSCs in $T_{30}E_{30}C_{100}$ solutions and Me_2SO after 12months of storage

Sl. No.	Freezing solution	Conditions	Viability
1	10% v/v Me_2SO	Control rate freezing (1°C/min)	61%
2	Trehalose (30mM), ectoin (30mM), catalase (100µg)	Control rate freezing (1°C/min)	73.6%
3	Trehalose (10mM), ectoin(5mM), catalase (100µg) with pan caspase (50µM) and calpain inhibitor (50µM)	Control rate freezing (1°C/min)	80%
4	Trehalose (10 mM), ectoin (5mM), catalase (100µg) with pan caspase (50µM) and calpain inhibitor (50µM)	Control rate freezing at optimized condition	85%

Conclusion

The main aim of the study in this phase was to explore potential natural CPA or a combination these CPAs to formulate an effective freezing solution towards preservation of MSCs by a controlled rate freezing method. The combination of CPAs used under study has shown better performance than the individual one as well as the traditional 10%v/v DMSO. Among the CPAs combinations, freezing solution consisting of trehalose, ectoine and catalase is found to be the most effective. Furthermore, from the cryopreservation study on the freezing solutions prepared from a different composition of trehalose, ectoin and catalase, the freezing solution with trehalose (30mM ectoin (30mM) and catalase (100µg) has shown optimum composition achieving maximum cell viability. The viability of cryopreserved MSCs was further improved by the addition of apoptotic inhibitors, the most effective of which are calpain PD150606 (50µM) and caspase z-VAD-fmk (50µM) inhibitors. This freezing solution has exhibited maximum post thaw survival with retention of cytoskeleton, membrane potential, rate of proliferation and differentiation ability. Furthermore, the optimum condition for the controlled rate freezing of MSCs was established as prenucleation cooling rate 1°C/min, nucleation temperature -7.5°C, cold spike 80°C/min, post nucleation holding time 10min and post nucleation cooling rate 1°C/min. and the maximum cell viability obtained at optimum condition is 85% as shown in Table 11.

PART II

Cryopreservation of hMSCs seeded silk nanofibers based tissue engineered constructs

As it is described in previous chapter that because of the possibility of autologous and non-tumorogenic characteristics, MSCs has been used as an attractive cell source in recent research focusing on the development of various tissue engineered constructs (TECs) to repair defect and/or diseased tissues and organs. In view of the emerging demand of these tissue engineered products, the development of an effective preservation technique is of paramount importance for the potential future applications of these tissue constructs clinically.

Besides cell source, the development of an artificial extra cellular matrix so called scaffold from biodegradable and biocompatible biopolymers is another important aspect in tissue engineering. Among the various biomaterials, silk fibroin (SF) extracted from silkworm cocoons possesses suitable surface, remarkable mechanical and desired biological properties that can mimic the human bone and other tissue and thus is considered as a potential candidate for the development of TECs using MSCs [71] .

Therefore, in this phase of dissertation work, the performance of the best freezing medium consisting of the combination of trehalose, ectoin and catalase ($T_{30}/E_{30}/C_{100}/I$) was evaluated towards the preservation of MSCs seeded SF scaffold (TECs) and the optimum control rate freezing condition was established.

5.3.1 Structural integrity and cell morphology in TECs

The crypreservation strategy has to be designed in such a way that it must be able to preserve the constructs on long term basis without affecting any structural integrity of the scaffold and maintain the membrane integrity of the cells in TEC. It is reported that the maintenance of structural integrity of the scaffold is a prerequisite in cryopreservation of cell–biomaterial constructs. Any deformation in the scaffold during cryopreservation may affect cell functionality. It was, therefore, investigated whether the cryopreservation had any adverse effect on the scaffold structure and morphology of MSCs in TECs by SEM study. SEM images (Figure 39, 40) of scaffolds and TECs before and after cryopreservation were generated on 1 and 14 days of culture. As indicated in Figure 37, no visible structural deformity on the surface of the nanofibrous scaffolds with (Figure 39) and without seeded MSCs (Figure 39) as well as before and after cryopreservation are observed. Furthermore, while comparing the type of freezing medium used for cryopreservation such as Me_2SO and combination of natural osmolytes ($T_{30}/E_{30}/C_{100}/I$), TECs cryopreserved in $T_{30}/E_{30}/C_{100}/I$ freezing solution have shown better MSCs attachment and spreading than Me_2SO as evident from Figure 40. This enhanced

cell attachment may be due to less mass transfer impedance, uniform heat distribution and desired pore size of scaffold that facilitated the penetration of cryoprotectant as reported earlier [256]. Furthermore, no significant morphological difference of MSCs was observed between non-cryopreserved and cryopreserved TECs. This signifies that the microcellular environment of the MSCs in nanofiber scaffold used in this study remain unaltered in post thaw TECs. Thus it has been established that $T_{30}/E_{30}/C_{100}/I$ is an effective freezing medium for TECs preservation in terms of maintenance of structural integrity of scaffold and retention of cell morphology during cryopreservation process.

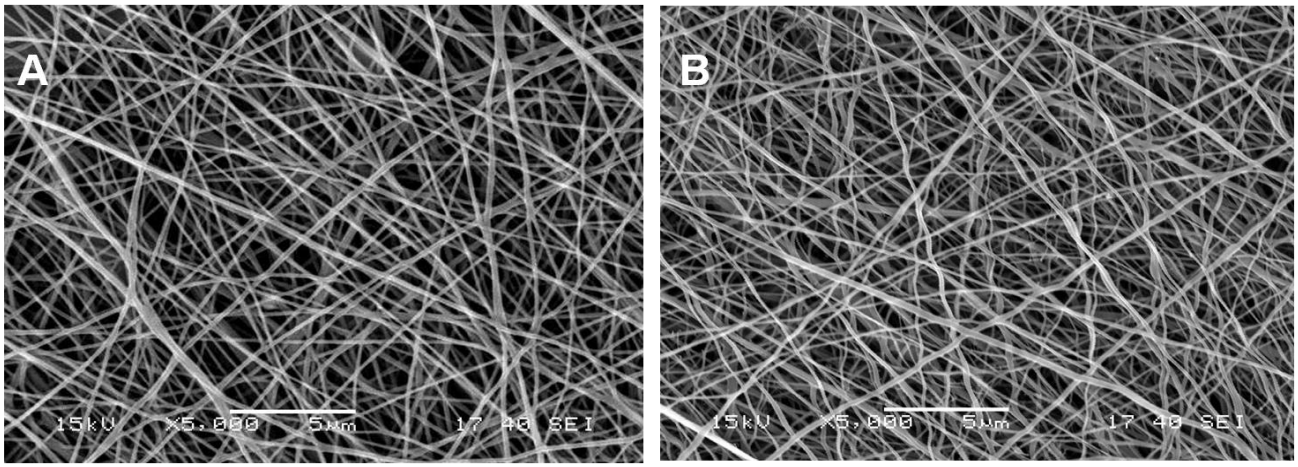


Figure 39:SEM images of (A) noncryopreserved and (B) cryopreserved scaffold. Scale bar: 5µm

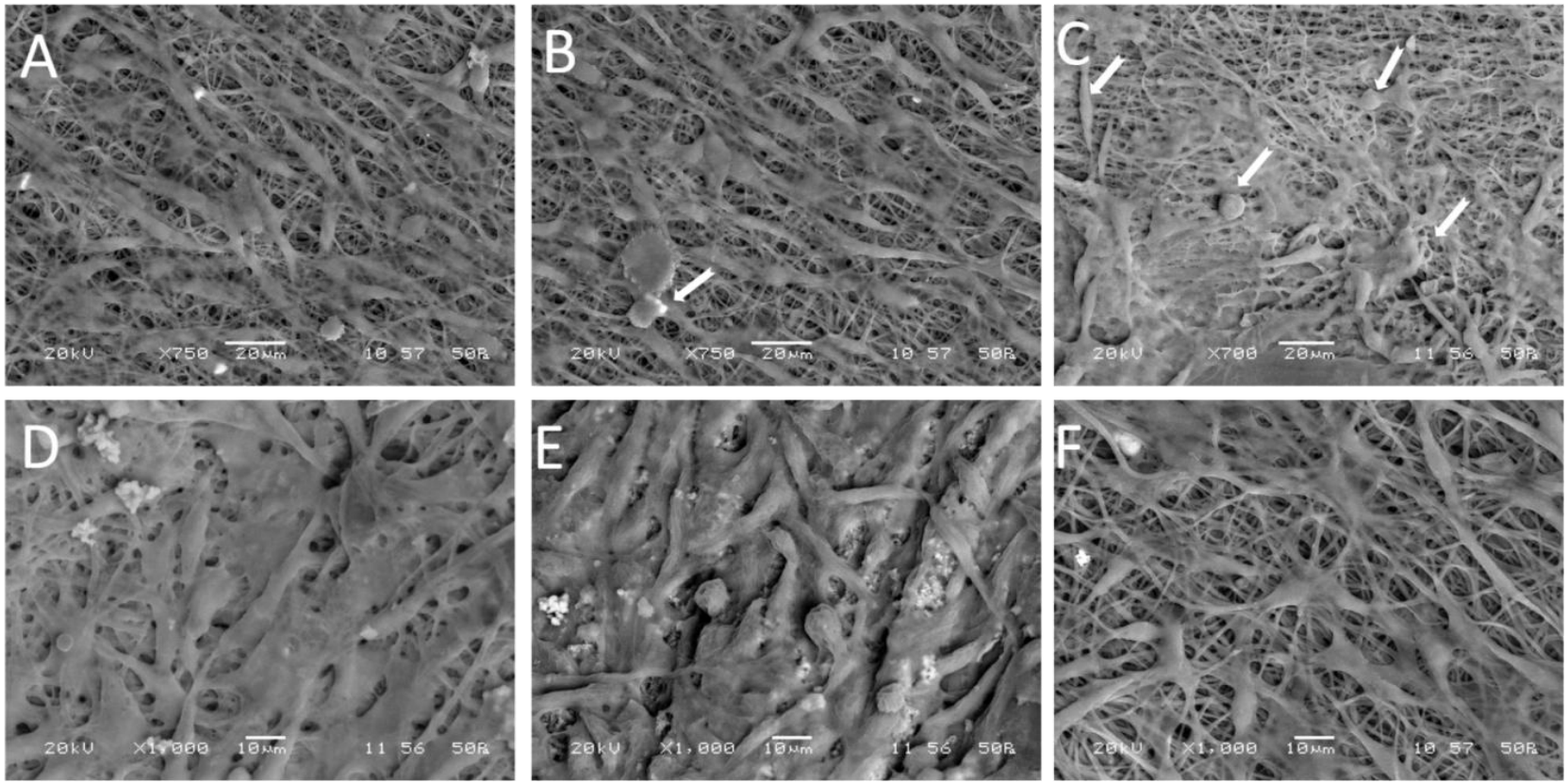


Figure 40: SEM images of cryopreserved scaffold (A) precryopreserved constructs after 1 day, (D) precryopreserved constructs after 14th days , (B) Cryopreserved constructs using freezing solution $T_{30}/E_{30}/C_{100}/I$ after 1 day, (E) Cryopreserved constructs using freezing solution $T_{30}/E_{30}/C_{100}/I$ after 14days, (C) Cryopreserved constructs using freezing solution Me_2SO after 1 day and (F) Cryopreserved constructs using freezing solution Me_2SO after 14 days. Scale bar: 10µm

5.3.2 MSCs viability

The MSCs viability in cryopreserved TECs was assessed using flowcytometry analysis by determining the percentage of post thaw necrotic cells. As shown in Figure 41, the cell viability in TECs is obtained as 54.37±2.1% using 10% Me_2SO, 72.5± 1.8% with $T_{30}/E_{30}/A_{100}/I$ freezing solutions and 95±2% with non-cryopreserved MSCs. Among the various freezing medium, the maximum cell viability was achieved with $T_{30}/E_{30}/C_{100}/I$ freezing solution. Furthermore, result shows that CPAs containing trehalose/ ectoin/catalase with or without apoptotic inhibitors have better cell viability than the standard 10% Me_2SO solution which is statistically significant (p=0.0003 and 0.0021). Furthermore, the cell viability of non-cryopreserved and cryopreserved TECs groups showed statistically significant differences, the higher MSCs viability is obtained with the former, which is obvious. The cell viability results were further confirmed by MTT assay.

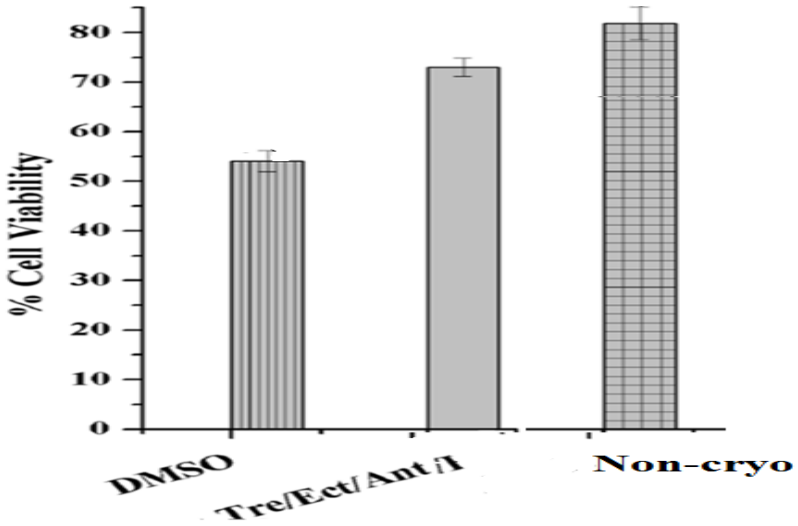


Figure 41: Cell viability after 24h of post thaw TECs in different freezing solution and noncryopreserved MSCS. Bar represents standard deviation (n = 3)

5.3.3 Proliferation assay

The metabolic activity of MSCs seeded on SF scaffolds after post thaw was evaluated by MTT assay and the result is shown in Figure 42. It is observed that the metabolic activity of the cells on the scaffold cryopreserved in $T_{30}/E_{30}/A_{100}/I$ is significantly higher than the TECs cryopreserved in 10% Me_2SO (*p value > 0.05and **p value > 0.005). The result has shown similar trend as the cell viability results obtained by flowcytometry analysis as presented in previous section.

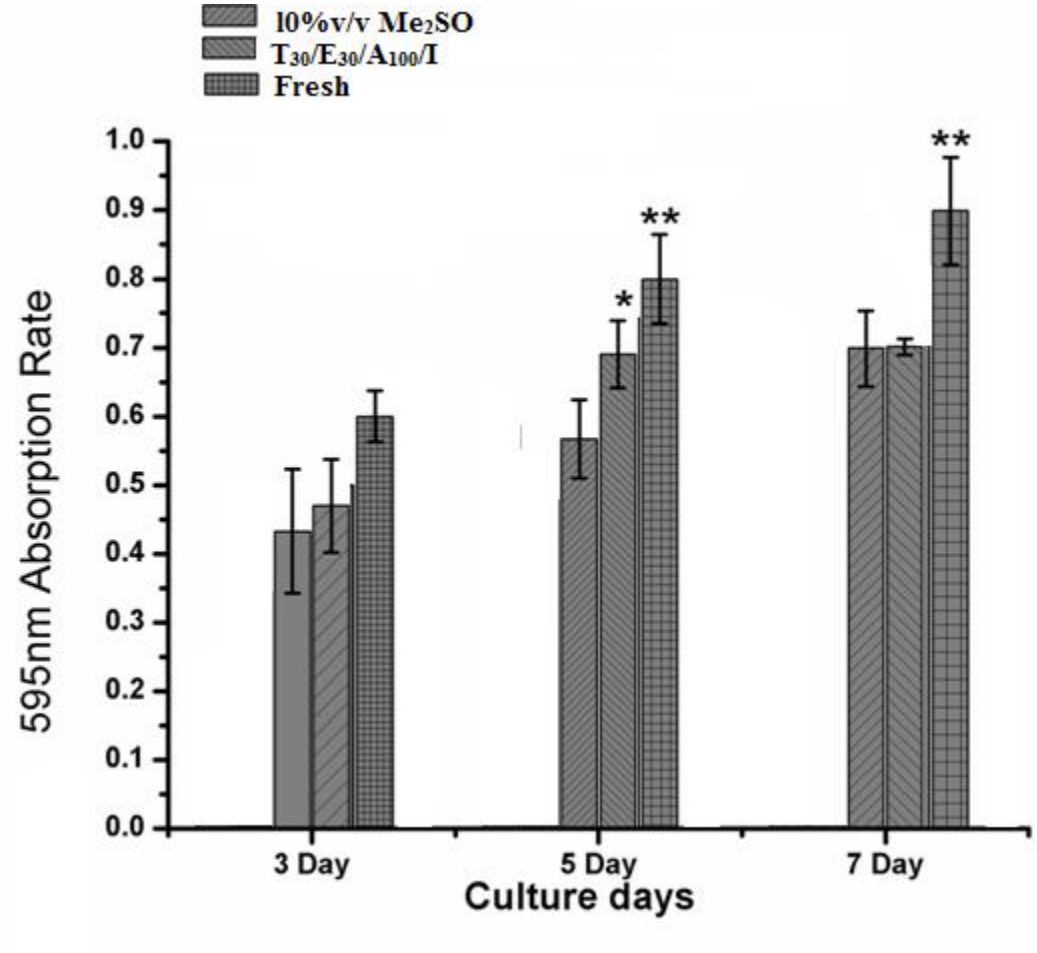


Figure 42: MTT assay of the metabolic activities of post thaw MSCs seeded on SF scaffolds. Result demonstrates statistical differences between l0%v/v Me_2SO (control) and $T_{30}/E_{30}/C_{100}/I$ freezing solution after 3, 5 and 7 days (n = 3; ∗p < 0.05; ∗∗p < 0.01)

5.3.4 Cytoskeleton analysis

Organization of F-actin, largely determines the cellular morphology and surface movement [256, 257]. Because of their close proximity, any mechanical damage to the membrane might also disrupt the F-actin. During cryopreservation, extracellular ice, mechanical stress and differential thermal contraction between cells and substrates occur that may cause depolymerization and accumulation of F-actin. Cytoskeleton integrity plays a major role in cell viability, cell proliferation and differentiation. Loss of function of cell membranes interferes with transport systems such as pH regulatory systems on the cell membrane. Disruption of organelle membranes affects transport systems such as mitochondrial transport systems that is essential for oxidative phosphorylation, the major energy-generating pathway [258-259] Therefore, cytoskeleton integrity of TECs was assessed by confocal microscopy and confocal images are shown in Figure 43. The investigation on the effect of cryopreservation on the organization of F-actin has shown no remarkable impact on F-actin of MSCs in TECs by cryopreservation using $T_{30}/E_{30}/A_{100}/I$. Whereas significant distortion of F-actin, ruptured membrane, low intensity of stress fiber and unclear boundary are observed with cryopreserved TECs using Me_2SO.

It is further observed that the noncryopreserved TECs has retained its cell morphology as represented by the existence of intact cell membrane. Immunofloroscence has shown better cell attachment in TECs cryopreserved in $T_{30}/E_{30}/A_{100}/I$ compared to the cell attachment using Me_2SO. However, cell attachment is lower than that observed with unfrozen TECs in all the cases.

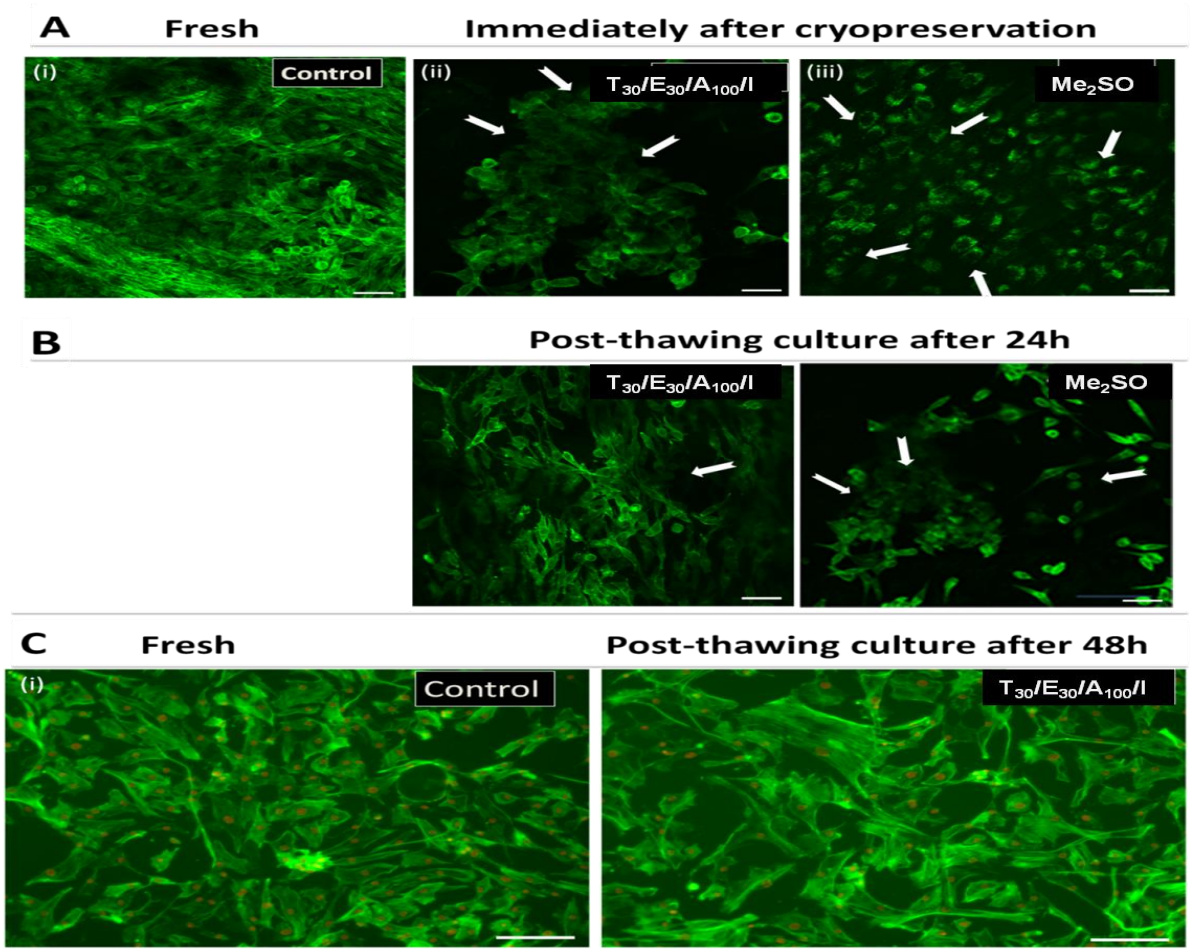


Figure 43: Comparative confocal images (F-actin) of cryopreserved and non cryopreserved silk nanofiber TECs. (A) (i) Shows noncryopreserved, (ii and iii) shows the TECs immediately after cryopreservation with scale bar 5 lm, (B) 24 h after cryopreservation with scale bar 5 mm and (C) close-up images of actin cytoskeleton 48 h after cryopreservation (ii) with scale bar 40 µm. In both the Me_2SO and $T_{30}/E_{30}/A_{100}/I$ groups, the cytoskeleton in the frozen/thawed regions is damaged, with shortened extensions and less number of cells compare to noncryopreserved cells. In the Me_2SO frozen group, the cells have condensed, distorted after freezing, and less damage is observed in $T_{30}/E_{30}/A_{100}/I$ groups.

5.3.5 Alkaline Phosphatase activity

The ALP activity of MSCs on the nanofibrous scaffolds up to 21 days culture in osteogenic differentiation media is shown in Figure 44. The study shows the increase in ALP production progressively on cryopreserved and non cryopreserved TECs indicating the osteoblastic differentiation ability of MSCs on scaffold. Additionally, a highly significant difference ($p < 0.05$) in ALP production is observed between Me_2SO and $T_{30}/E_{30}/C_{100}/I$. Overall, an increase in ALP activity from 8±0.9µg to 4000±67µg and 9±0.3µg to 3848±400 with cryopreserved TECs in Me_2SO and $T_{30}E_{30}C_{100}/I$ are achieved during 1 to 21 days of culture period.

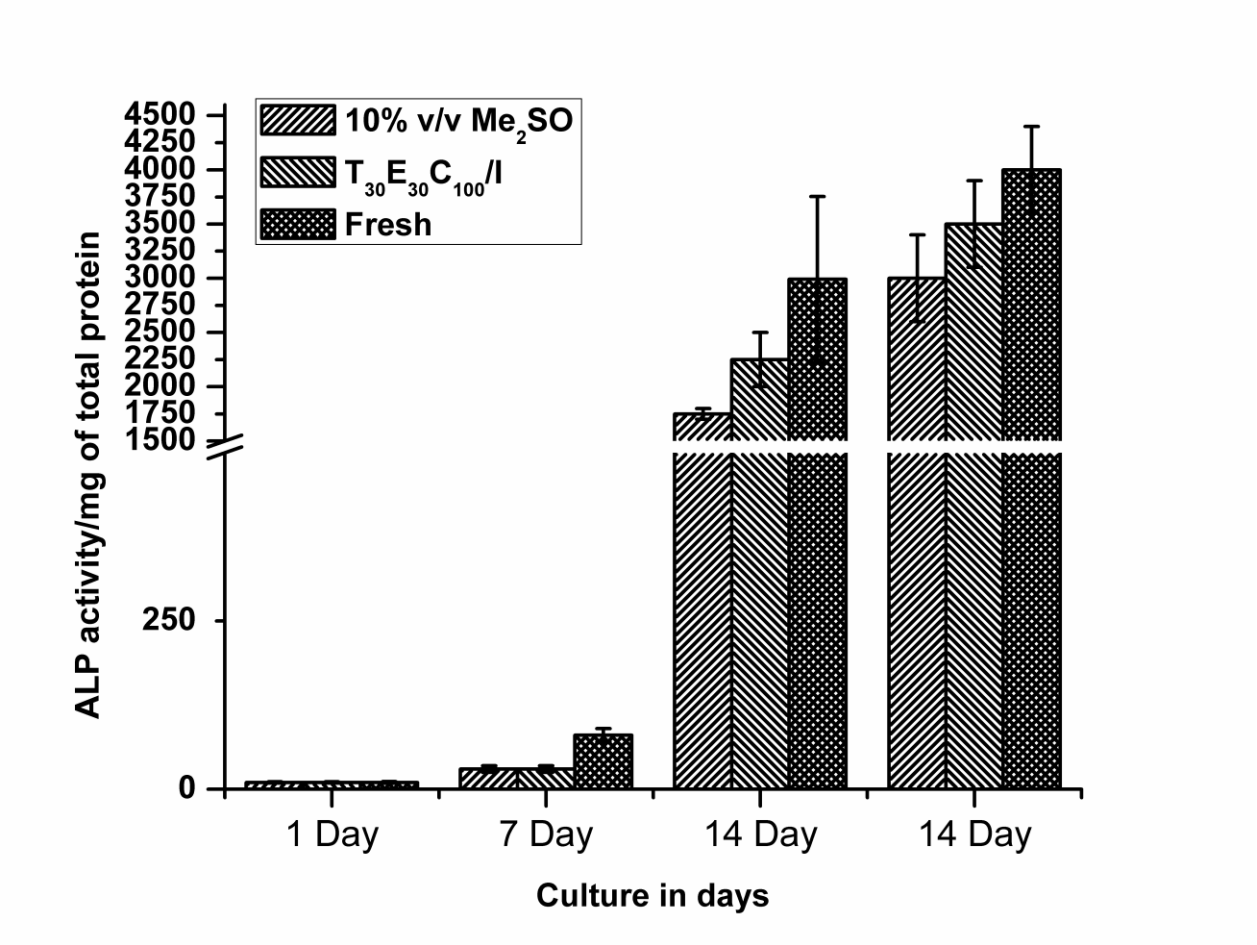


Figure 44: ALP activity was studied on day 1, 7 and 21 of post thaw MSCs cultured on silk nanofiber scaffolds in freezing solution of 10% Me_2SO and $T_{30}/E_{30}/A_{100}/I$ as evaluated by NBT substrate in osteogenic induction medium. Results shows significant difference ($p > 0.05$) in ALP activity after 7 and 14 days post thaw culture.

5.3.6 Effect of cryopreservation on Mechanical properties of TECs

In tissue engineering, scaffold must have desired mechanical properties which become critical when it is used for bone tissue regeneration in particular. During cryopreservation, the mechanical strength of scaffold may be altered due to the occurrence of thermal stress[75-76]. Therefore, mechanical test was performed on cryopreserved and non cryopreserved TECs to investigate the effect of cryopreservation on tensile strength of scaffold. Statistical analysis indicates that, tensile strength of the non-cryopreserved, TECs cryopreserved in Me_2SO and $T_{30}/E_{30}/C_{100}/I$ were 1.82 ±0.31Mpa, 1.35±0.19Mpa and 1.79 ±0.21Mpa respectively (Figure 45). Furthermore, no significant difference was observed between non-cryopreserved and cryopreserved TECs in $T_{30}/E_{30}/C_{100}/I$. It has been assessed that the combined CPA $T_{30}/E_{30}/C_{100}/I$ did not show any remarkable effect on the mechanical property of the scaffold. However, the tensile strength of the scaffold decreases significantly with Me_2SO preserved TECs. Similar result is also reported in another study [15]. Thus it is established the superiority of $T_{30}E_{30}C_{100}/I$ over Me_2SO as freezing solution.

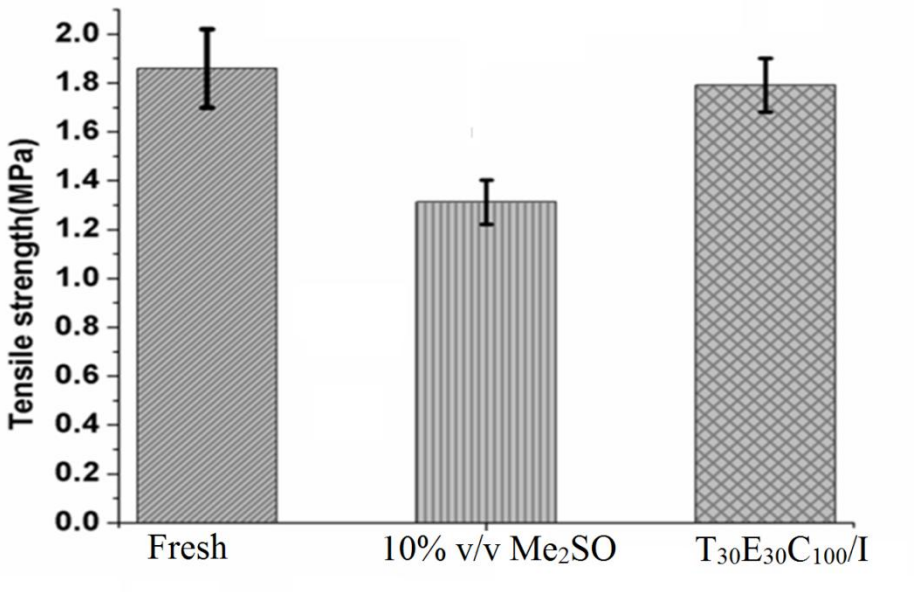


Figure 45: Ultimate tensile strength of TECs cryopreserved in $T_{30}/E_{30}/C_{100}/I$, Me_2SO and noncryopreserved TECs (Fresh)

5.3.7 Calcium deposition

The effect of cryopreservation on the osteogenic differentiation capacity of MSCs in TECs was investigated qualitatively by histological staining of calcium deposition on the scaffold. As shown in Figure 46 and 47, MSCs are found to attach and spread throughout the scaffolds. A network of cells and ECM with a relatively small amount of granular deposition of calcium is covered on the scaffold surface when TECs is cryopreserved in Me_2SO. Whereas, a higher deposition of calcium is observed with noncryopreserved and cryopreserved $T_{30}/E_{30}/C_{100}/I$ TECs indicating the higher degree of osteogenic activity of MSCs. SEM results indicated the MSCs remained adhered to the scaffold and mineralization structures intact (Figure 47). EDXA detected the presence of phosphorus and calcium confirming the presence of mineralized structures (Figure 47 B and D).

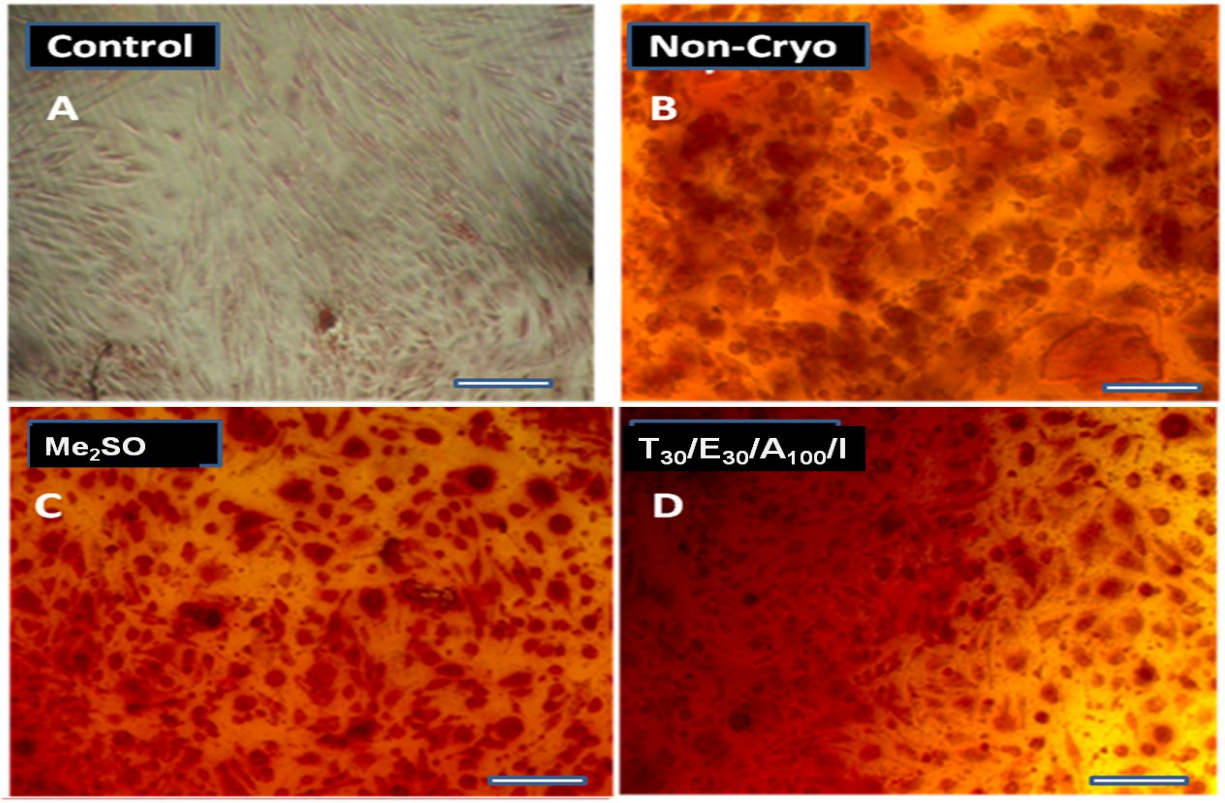


Figure 46: Optical images of alizarin red staining for calcium deposition of osteogenically induced MSCs in post thaw TECs (A) TECs without osteogenic media (B) TECs with osteogenic media without cryopreservation, (C) TECs cryopreserved in Me_2SO (D) TECs cryopreserved in $T_{30}/E_{30}/C_{100}/I$. Scale bars represent 50 mm

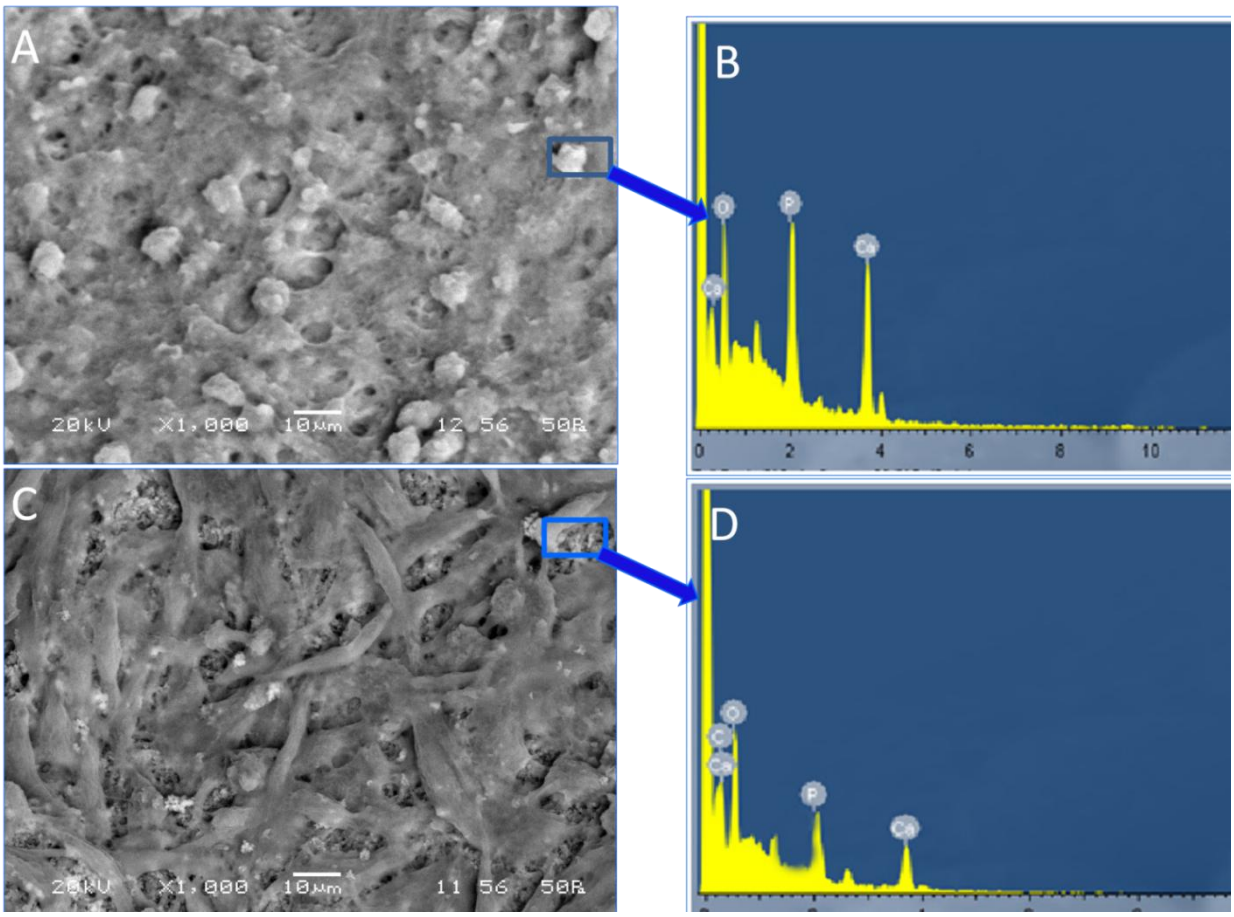


Figure 47: SEM and EDX analysis of post-thaw TECs cultured in osteogenic differentiation medium for 21 days -(A) TECs cryopreserved in $T_{30}/E_{30}/C_{100}/I$, (C) TECs cryopreserved in 10% Me_2SO, (B) and (D) depicts the EDX analysis of the elemental components of calcium phosphate salts, consisting of P, Ca and O elements in both groups scaffolds

5.3.8 Expression of osteogenic-specific genes

The quantitative osteogenic differentiation level of seeded MSCs on TECs was assessed by quantitative PCR of bone-specific genes. From the analysis of Figure 48, the osteogenic differentiation genes such as collagenase-1[Col 1], core binding protein [Cbfa 1], osteocalcin [OCN] and alkaline phosphate [APL] are observed to be increasingly expressed on $T_{30}/E_{30}/C_{100}/I$ cryopreserved group during 14 day of culture in comparison to Me_2SO cryopreserved groups.

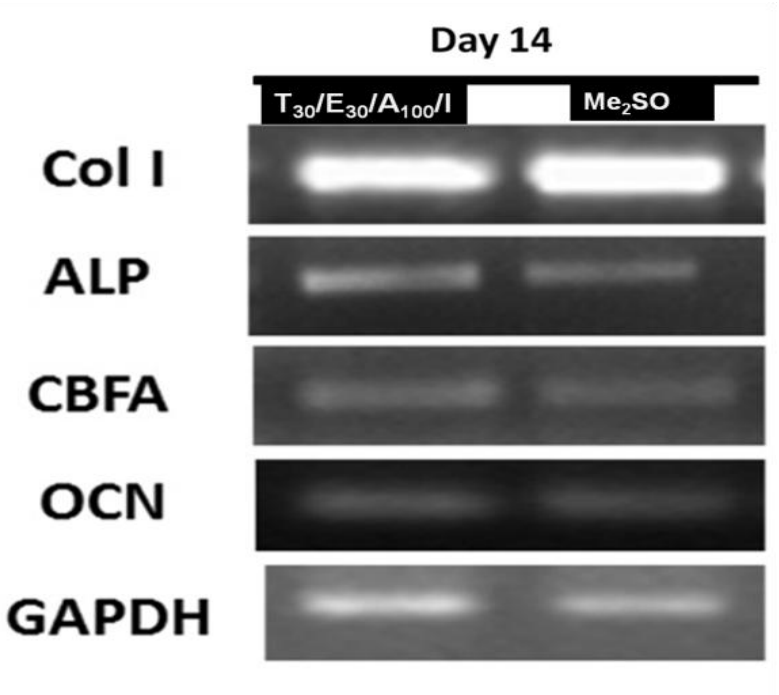


Figure 48: Osteogenic gene expression levels of post thaw MSCs in TECs cultured in osteogenic media

5.3.9 Optimization of controlled rate freezing parameters for preservation of TECs using $T_{30}/E_{30}/C_{100}$ freezing medium

In this section, it was further investigated the effect of key controlled rate freezing parameters on cryopreservation of TECs and establish the optimum cryopreservation condition using the most effective freezing medium $T_{30}/E_{30}/C_{100}/I$ in presence of apoptotic inhibitors.

5.3.9.1 Effect of cooling rate on MSCs viability in TECs

The effect of cooling rate (-0.5°C/min, -1.0°C/min, -2.5°C/min, -5°C/min and -10°C/min) on MSCs viability was studied and the experimental result is shown in Figure 49. As it is indicated the highest cell viability of 74% is achieved at cooling rate of -1.0°C/min and a decline in cell viability is observed with either higher or lower than -1.0°C/min cooling rate at constant seeding temperature of -7.5 °C

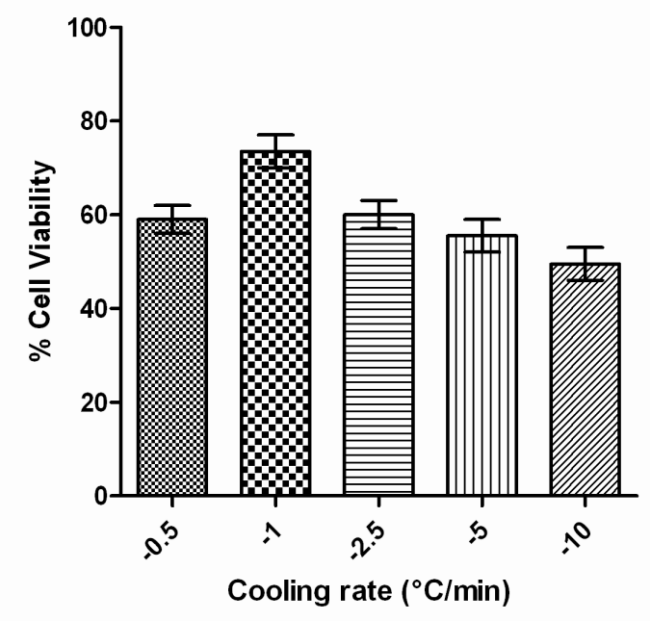


Figure 49: Effect of different cooling rate on MSCs seeded TECs

5.3.9.2 The effect of seeding temperature on cell viability

The effect of seeding temperature on cell viability was investigated by performing cryopreservation experiments at varying seeding temperature at -5 °C, -7.5 °C and -10°C. The optimal cell viability was recorded as 80 % at -7.5 °C seeding temperature. The rate of cooling temperature is maintained constant at1°C /min.

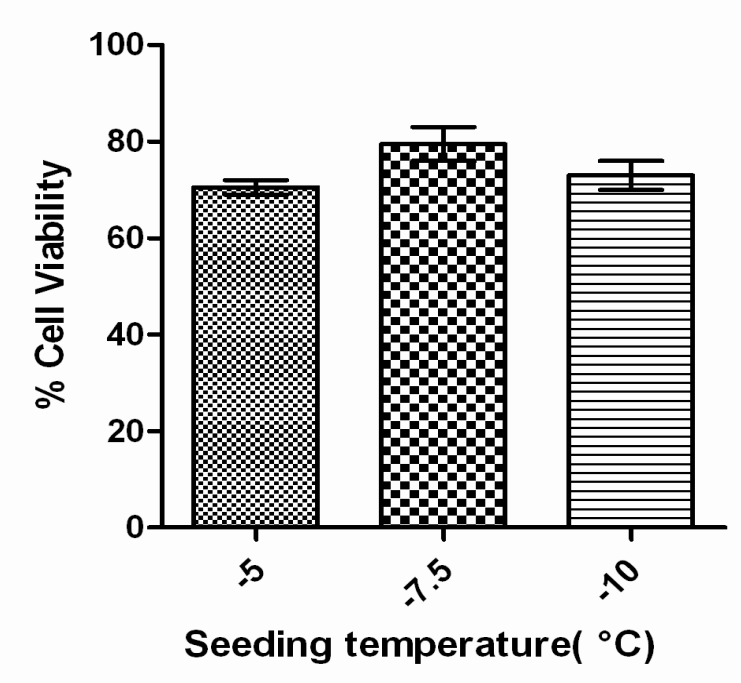


Figure 50: Effect of seeding temperature on MSCs seeded TECs

5.3.9.3 Cell Morphology

After cryopreservation of MSCs seeded scaffold, the cell population was observed to be decreased in comparison to fresh unfrozen MSCs, representing that cryopreserved induced stress resulted in cell detachment as shown in Figure 51. Furthermore, more number of cells is detached in 5°C/min and 10°C/min rate cryopreserved cells in comparison to the cells cryopreserved at 1°C/min. SEM result reveals that before cryopreservation, the adherent hMSCs were in spindle shape. But after cryopreservation at 5 and 10°C/min, the cells did not maintain their original shape, either in flatten shape or round shape. Moreover, the cell number becomes less after cryopreservation at the cooling rates of 5 and 10 C/min than the fresh MSCs.

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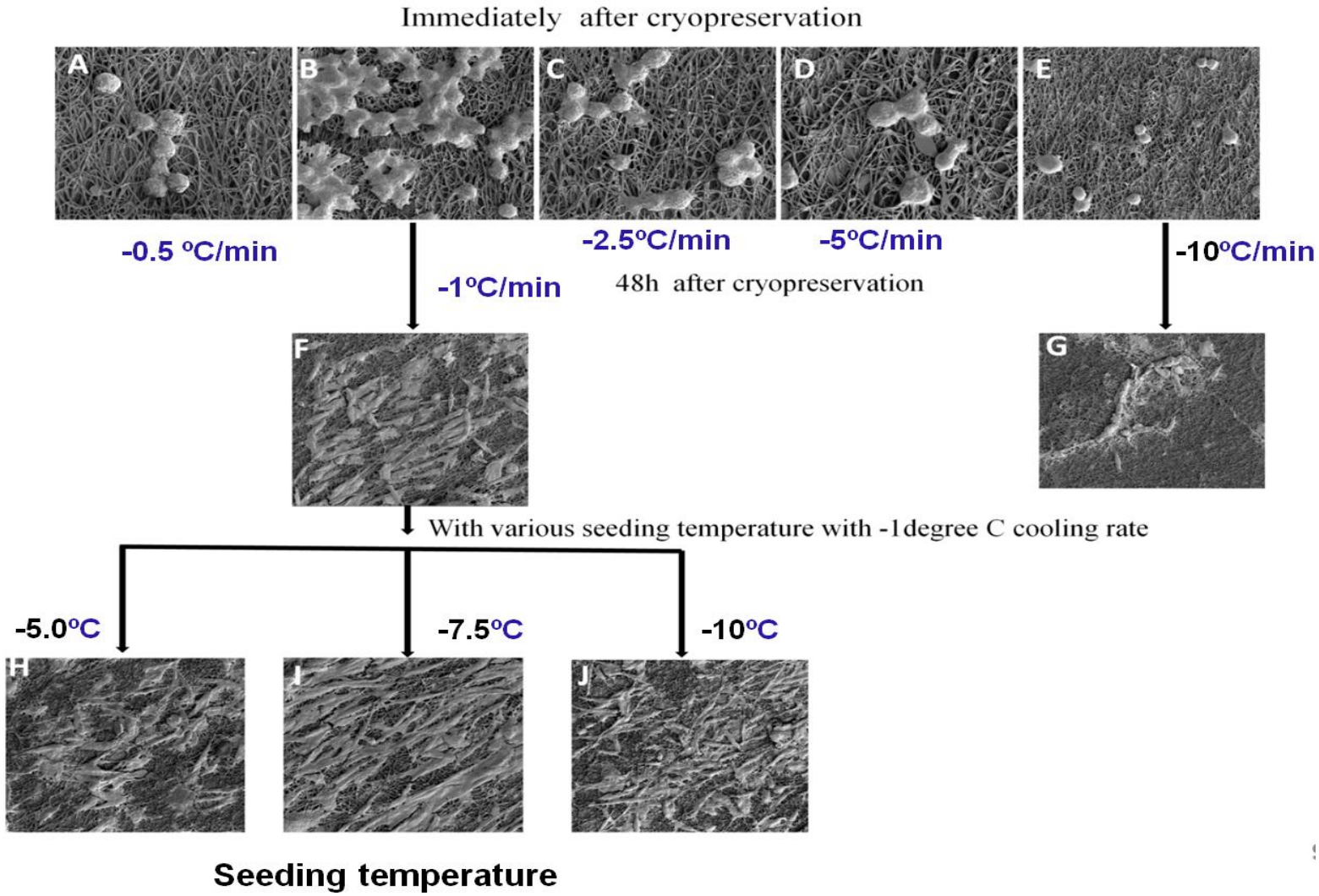

Figure 51: SEM images of TECs under different cooling rate and seeding temperature

Conclusion

From this study, it is established that $T_{30}/E_{30}/C_{100}/I$ is found to be an effective freezing medium in preserving TECs developed from hMSCs and nanofiberous SF scaffold as confirmed by the cell viability, proliferation and differention ability of hMSCs in TECs after and before cryopreservation. Furthermore, an enhanced cell vitality of of MSCs is obtained by optimizing the controlled rate freezing parameters. The optimum cryopreservation condition was established s cooling rate of 1^oC / min and nucleation temperature of -7.5^o C giving maximum cell viability of 80%.

CHAPTER V

Summary & Conclusion

SUMMARY & CONCLUSION

In recent years, mesenchymal stem cells (MSCs) and MSCs derived tissue engineered constructs (TECs) have been considered as important biological products that can be used for a variety of therapeutic and tissue engineering applications including bone, cartilage, tendon, muscles, blood vessel and other tissue regeneration. Therefore, recent research is directed towards the production of MSCs and MSCs seeded tissue engineered constructs (TECs) to repair defect and/or diseased tissues and organs. However, the successful clinical applications of these cells and tissue engineering products rely on the development of an appropriate cryopreservation technology that offers long time storage of such products with desired cell viability, metabolic activity and cellular differential ability. Me_2SO a traditional freezing solution is currently used for preservation of various cells and other biological species. However, freezing protocol using Me_2SO as cryoprotecting solution is reported to have several detrimental effects on the cryopreserved cells and hence harmful to patients.

In this context, a combination of a variety of natural osmolytes seems to be effective cryoprotective agents in preserving MSCs and other cells. Furthermore, it has been reported that the controlled rate freezing technology is superior to the uncontrolled freezing methods of preserving cells. However, no systematic research effort has so far been given to develop an efficient cryopreservation strategy for long term preservation of MSCs and TECs using natural osmolytes. Therefore, the present dissertation work has been undertaken to develop a cryopreservation strategy using a freezing medium consisting of natural cryoprotective agents that can be useful for the long term preservation of MSCs and MSCs seeded tissue engineered constructs.

The major outcome achieved through this research work is summarized as follows-

I. In the first phase of the dissertation work, a variety of natural cryoprotective agents in combination were investigated to see their ability towards cryopreservation with the aim of developing an effective freezing medium that can be useful for preserving MNCs and other cells like MSCs. MNCs were isolated from umbilical cord blood by Ficol hypaque technique and cultured. The extracellular cryoprotectants (10mM) namely trehalose, hydroxyl ethyl starch,

polyvinyl pyrrolidine and intracellular CPAs (5mM) like erythritol, taurine and ectoine were used to prepare different combinations of freezing medium following L9 (34) Taguchi orthogonal array. Catalase, coenzyme Q10 and n-acetyl cystine (100µg/m) were added as antioxidants. The cryopreservation experiments were conducted in a controlled rate freezing condition. The combination of extracellular, intracellular CPA and antioxidant has shown encouraging results towards the maintenance and survival of cell during and after freezing. Among the CPAs and antioxidants used under study, trehalose, ectoin and catalase have shown the best cryopreservation performance achieving high cell viability. The post thaw viability is also observed to be 10% higher than the cell viability obtained using conventional Me_2SO as CPA. Furthermore, SEM and phase contrast microscopy confirmed the normal cell morphology of the post-thaw cultured cells with retaining their membrane integrity.

Thus it has been demonstrated that the cryopreservation medium consisting of trehalose, ectoin and catalase is the most effective freezing solution that may be of further interest of research to design a standard cryopreservation protocol for MSCs

II. In this part of the thesis work, the most effective natural osmolytes such as trehalose and ectoin in presence of catalase antioxidant as obtained in the previous section were further evaluated for their effectiveness towards the long term preservation of much demanding MSCs for therapeutic and tissue engineering applications. To accomplish this task, MSCs were sorted from cultured MNCs by fluorescence activated cell sorter (FACS), cultured and characterised for their morphology, and immune-phenotype. MSCs were then subjected to controlled rate freezing using various freezing solutions using trehalose and ectoin as CPAs individually and in combination with varying ratios of trehalose and ectoin. The freezing solution containing the combination of CPAs has shown better post thaw cell viability than the individual CPAs as well as control (Me_2SO). Furthermore, among various composition of CPAs tested under study, the freezing medium having combination of 30mM trehalose, 30mM ectoin and 100µg catalase ($T_{30}/E_{30}/C_{100}/I$) exhibited maximum post thaw survival with retention of cytoskeleton, membrane potential and rate of proliferation. The cryopreserved MSCs have also shown the desired differentiation ability even after 12months of storage. It is thus concluded that $T_{30}/E_{30}/C_{100}$ freezing solution developed

in this study is effective in preserving UCB derived MSCs in long term basis and the maximum cell viability archived was 73%.

III. In this phase of research work, an attempt has been made to determine the cryopreservation induced apoptosis signalling pathways and evaluate the role of caspase-dependent and independent apoptotic pathways in MSCs. to accomplish the task MSCs were cryopreserved in a control rate freezer, using $T_{30}/E_{30}/C_{100}/I$ as cryoprotective agent either with or without pre-treatment with general caspase inhibitor z-VAD-fmk, or with the more selective caspase inhibitors such as z-IETD-fmk, z-LEHD-fmk, and z-DEVD-fmk. Furthermore, to evaluate the effect of calcium mediated pathway, the cryopreserved MSCs samples were tested with and without calpain inhibitor. FACS was used to measure the cell viability, mitochondrial membrane potential and cell cycle analysis. Processing of procaspases 3, 8, 9, µ calpain and Bid was determined by Western blotting. Cryopreservation of MSCs resulted in characteristic apoptosis within 24 h after post thaw. It has been established that the addition of calpain inhibitor and pan caspase inhibitor are used in CPA solution increases the cell viability to 80 %.

IV. In this phase of work, the cryopreservation condition was further optimised to increase the efficiency of the best obtained freezing solution ($T_{30}/E_{30}/C_{100}/I$) in presence of caspase and calpain inhibitors. The various parameters were investigated are prenucleation cooling rate, nucleation temperature, cold spike temperature, post nucleation holding temperature, post nucleation cooling rate, cell concentration and storage temperature. The result indicated that the cooling profile has a significant impact on cell viability. The optimum condition for the controlled rate freezing of MSCs was established as (i) Prenucleation cooling rate -1^{o}C / min (ii) Nucleation temperature -7.5^{o}C (iii) Cold spike -80^{o}C/min (iv) Post nucleation holding time of 5 min (v) Post nucleation cooling rate -1^{o}C/min. (vi) 3×10^{6}/ml cell concentration. The maximum cell viability obtained at optimum condition is 85%.

V. In this part of the thesis, research work was further extended to evaluate the potentiality of the most effective freezing solution obtained in the previous section towards the preservation of TECs

derived by seeding of MSCs on nanofibrous silk fibroin scaffold (TECs) by conducting cryopreservation experiments in a controlled rate freezer. The SF scaffolds used for TECs formation is made from eri and tasar SF blend. To the best of our knowledge, the present study is the first report on the cryopreservation of MSCs-seeded nanofibrous scaffold fabricated from silk fibroin which is considered as a promising biomaterial for tissue engineering applications. It has been observed from the experimental results that the viability and functionality of hMSCs on SF scaffolds were well maintained and cultured after cryopreservation. These are well supported by the assessment of cell viability (flowcytometry analysis & MTT assay), structural integrity (SEM and confocal images) and functionality in terms of osteogenic differentiation ability (Immunohistochemistry, RT-PCR analysis & ALP assay) of TECs. The newly formulated freezing solution of combined CPAs ($T_{30}/E_{30}/C_{100}/I$) further did not show any adverse impact on the mechanical property of the scaffold in cryopreserved TECs. Thus it is established the effectiveness of $T_{30}/E_{30}/C_{100}/I$ as freezing medium in preserving TECs developed from hMSCs and SF scaffold.

VI. In this phase of research work, an optimization study was carried out to maximise the recovery of MSCs on TECs cryopreserved in $T_{30}/E_{30}/C_{100}/I$ freezing medium. The effect of key controlled rate freezing parameters was investigated and optimum freezing condition was established as cooling rate of $-1^{o}C$/min and seeding temperature of $-7.5^{o}C$/min. The maximum MSCs viability of 80% was achieved at optimum condition.

Overall, in this dissertation work, a Me2SO free freezing solution consisting of a combination of natural osmolytes such as trehalose (extracellular CPA) and ectoin (intracellular CPA) with catalase as antioxidant has been established as an effective cryopreservation medium for long term preservation of MSCs and MSCs seeded on tissue engineered constructs. The efficiency of the freezing medium achieving high MSCs recovery can further be improved by the addition of apoptotic inhibitors. It is finally concluded that the development of a controlled rate freezing system using the freezing medium ($T_{30}/E_{30}/C_{100}/I$)established in this study may pave the way for long term preservation of MSCs and MSCs seeded tissue engineered products for clinical applications

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List of Publications

On PhD work

Patent

1. Patent applied: Niladri N Panda, Krishna pramanik, Amit Biswas, **Akalabya Bissoyi**. Electrospun silk fibroin bland nanofibrous scaffold for tissue engineering (TR-5 C.B.R NO: 4716) Date-27/3/2012 17:07:01

Journals

- **Bissoyi, A**., Nayak, B., Pramanik, K., & Sarangi, S. K. (2014). Targeting Cryopreservation-Induced Cell Death:A Review. *Biopreservation and Biobanking*, *12*(1), 23-34.

- **Bissoyi, A**., Pramanik, K., Panda, N. N., & Sarangi, S. K. (2014). Cryopreservation of hMSCs seeded silk nanofibers based tissue engineered constructs. *Cryobiology*.(68)332–342.

- **Bissoyi, A.**, & Pramanik, K. (2013). Effects of Non-Toxic Cryoprotective Agents on the Viability of Cord Blood Derived MNCs. *Cryoletters*, *34*(5), 453-465.

- **Bissoyi, A**., Pramanik, K., (2014). Role of apoptosis pathway in cryopreserved induced cell death in mesenchymal stem cells derived from umbilical Cord blood, *Biopreservation and Biobanking*, 12(4), 21-32.

- **Bissoyi, A.**, Pramanik, K. Isolation, characterization and preservation of MSCs derived from umbilical cord blood. Journal of Tissue Engineering and Regenerative Medicine Vol 6, Issue Supplement s1, Article first published online: 3 SEP 2012

Manuscript under preparation

- To be communicated: ***Akalabya Bissoyi****, K. Pramanik, Sribatsha Mohapatra. "Long term Cryopreservation of Umbilical Cord blood derived Mesenchymal Stem cell using natural osmolytes and antioxidant" Biopreservation and Biobanking*.

- To be communicated: **Akalabya Bissoyi**, K. Pramanik. Optimization of cryopreservation protocol for hMSCs using osmolytes in combination with antioxidant and apoptotic inhibitor.

Conference

1. **A. Bissoyi**, K. Pramanik‘ A Study of the apoptosis on cord blood derived MNC as novel method for selection of cryoprotectant solution’-2011 National conference on Tissue engineering: prospects & challenges & post conference workshop on Flowcytometer in tissue engineering (tepc-2011) JAN 22

2. **A. Bissoyi**, K. Pramanik ‘Molecular dynamic study of caspase-8 protein in cryopreserved condition’-2011 National conference on Tissue engineering: prospects & challenges & post conference workshop on Flowcytometer in tissue engineering (tepc-2011) JAN-22

3. **A. Bissoyi**, K. Pramanik ‘Effect of osmolytes on apoptotic protein in cryopreservation temperature by molecular dynamic simulation study’ . 2011 International Conference on Tissue Engineering & Regenerative Medicine

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