

OPTIMIZATION OF METHODS FOR IN-VITRO EXPANSION AND
CRYOPRESERVATION OF MAMMALIAN STEM CELLS

By

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ABSTRACT

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The recent advances in the field of Tissue Engineering have given rise to the need for a tremendous quantity of cells. The search for and use of an appropriate multipotent or pluripotent stem cells in tissue engineering is an emerging concept.

Mesenchymal stem cells hold a very great deal of promise because of their capacity to self-renew and to differentiate into various lineages. These cells are very low in number in the body but their in vitro expansion capacity is very unique. Taking into consideration the lack of uniform approach for expansion of these MSCs and storing these cells for future use, in this work focus is laid on identifying the optimal culture and storage conditions for MSCs. The data obtained from this study indicates

that the media and the quantity of the serum play a very important role in the expansion. Addition of HB-EGF has been shown to increase the proliferation.

The current viability rates, post recovery vary according to the cell type used. This gives rise to the need for analyzing various cells. In this thesis the experiments are based on two cell lines models. The viability rates obtained as the end result are used as a standard for optimizing the process for all mammalian cells. A pilot study involving rat mesenchymal stem cells was also performed to evaluate if the cryopreservation of stem cells is similar to the mammalian cell models. The results show that the viability rate of the cells post cryopreservation is high when there is 5% of DMSO in the freeze media and when the step-by-step slow cooling process is used. In conclusion, this thesis defines the optimal culture conditions for the expansion of MSCs in high numbers and storage of these cells for subsequent therapeutic approaches.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
ABSTRACT	iii
LIST OF ILLUSTRATIONS.....	viii
LIST OF TABLES.....	xi
Chapter	
1. INTRODUCTION	1
1.1 Tissue Engineering-A step towards realization of a dream	1
1.2 Stem cells and stem cell culture	2
1.2.1 Types of cells	2
1.2.2 Culture condition and growth media	5
1.3 Cryopreservation and Cryoinjuries	7
1.3.1 Cryoinjury during freezing of cells	9
1.3.2 Cryoinjury during storage	13
1.3.3 Cryoinjury associated with the thawing process	13
1.4 Cryoprotectants	14
1.4.1 The toxicity of cryoprotectants	16
1.4.2 Osmotic effects of cryoprotectants.....	17
1.5 Need for optimization of culture condition and cryopreservation	18

2. MATERIALS AND METHODS	20
2.1 Cells	20
2.1.1 B16F1 melanoma cells	20
2.1.2 3T3 fibroblast cells	21
2.1.3 Rat stem cells	22
2.2 Culture conditions	25
2.3 Cryopreservation and cell recovery	26
2.3.1 Cryopreservation techniques.....	27
2.3.2 Viability test-Trypan blue exclusion	32
3. CHARACTERIZATION OF OPTIMUM CULTURE CONDITIONS AND PROTOCOL FOR IN VITRO EXPANSION OF RAT MESENCHYMAL STEM CELL.....	36
3.1 Effect of growth media cell proliferation.....	36
3.2 Effect of serum	38
3.3 Effect of growth factors	42
3.3.1 Effect of EGF on cell proliferation.....	42
3.3.2 Effect of bFGF on cell proliferation.....	44
3.3.3 Effect of HB-EGF on cell proliferation.....	46
3.4 Effect of Trypsin	50
4. OPTIMIZATION OF FREEZING CONDITIONS TO INCREASE THE VIABILITY OF CRYOPRESERVED CELLS.....	52
4.1 Cell Lines	52
4.1.1 Effect of freeze media.....	52

4.1.2 Effect of cell density	57
4.1.3 Effect of cryopreservation process	61
4.2 Rat peritoneal stem cells	67
4.2.1 Effect of freeze media.....	67
4.2.2 Effect of cell density	68
4.2.3 Effect of cryopreservation process	69
5. CONCLUSION.....	71
6. FUTURE DIRECTIONS	72
REFERENCES	74
BIOGRAPHICAL INFORMATION	85

LIST OF ILLUSTRATIONS

Figure		Page
1.1	Schematic diagram of physical events in cells during freezing.....	10
1.2	Effect of cooling rate on cell survival.....	11
2.1	B16F1 melanoma cells at 10X and 20X magnification.....	21
2.2	3T3 fibroblasts at 10X and 20X magnification.....	22
2.3	Rat stem cells at 10X and 20X magnification.....	23
2.4	Experimental design for testing various culture conditions for Rat MSCs.....	26
2.5	Nalgene ‘Mr.Frosty’ cooler box.....	29
2.6	Cryo-matic CELLevator- Semi-programmable freezer.....	30
2.7	Experimental design for cryopreservation of cells.....	31
2.8	Trypan blue viability assay for recovered cells.....	34
2.9	Trypan blue stained cells in a hemacytometer at 20X magnification.....	35
3.1	Morphology and confluency of Rat stem cells in various media on the 5 th day of culture A. DMEM+20%FBS; B. MEM+20%FBS; C. DMEM F12.....	37
3.2	Growth rates of rat stem cells in a various media.....	38
3.3	Morphology and confluency of Rat stem cells in various serum on the 5 th day of culture in DMEM with A. 20%FBS; B. 20%BGS; C. 10%FBS+10%BGS.....	40
3.4	Growth rates of rat stem cells in a media in various serum.....	41

3.5	Morphology and confluency of Rat stem cells in a media containing various concentrations of EGF on the 7 th day of culture. A. Control B. 10 ng/ml; C. 30ng/ml; D. 100ng/ml	43
3.6	Growth rates of rat stem cells in a media containing various concentrations of EGF	44
3.7	Morphology and confluency of Rat stem cells in a media containing various concentrations of bFGF on the 7 th day of culture. A. Control; B. 2 ng/ml; C. 10ng/ml; D. 50ng/ml.....	45
3.8	Growth rates of rat stem cells in a media containing various concentrations of bFGF	46
3.9	Morphology and confluency of Rat stem cells in a media containing various concentrations of HB-EGF on the 7 th day of culture. A. Control; B. 5 ng/ml; C. 25ng/ml; D. 50ng/ml.....	47
3.10	Growth rates of rat stem cells in a media containing various concentrations of HB-EGF	48
3.11	Growth rates of rat stem cells in a media containing cocktail of growth factors.....	49
3.12	Effect of Trypsin on morphology and confluency of Rat stem cells on the 7 th day of culture A. Cell scraper; B. Trypsin	50
3.13	Growth rates of rat stem cells subcultured with various methods	51
4.1	Freezing the cells: In varying percentages of DMSO and using the step-by-step freezing process	53
4.2	Viability rates for B16F1 melanoma cells cryopreserved with varying percentages of DMSO and using the step-by-step freezing process.....	55
4.3	Viability rates for 3T3 fibroblast cryopreserved with varying percentages of DMSO and using the step-by-step freezing process. (n=2)	56
4.4	Freezing the cells: In varying cell densities using 10%DMSO and using the step-by-step freezing procedure.....	58

4.5	Viability rates for B16F1 melanoma cells cryopreserved with varying cell density in 10% DMSO and using the step-by-step freezing process. (n=2).....	59
4.6	Viability rates for 3T3 fibroblast cryopreserved with varying percentages of DMSO and using the step-by-step freezing process. (n=2).....	60
4.7	Freezing the cells: In 10% DMSO with 5 million cells/vial using the step-by-step freezing procedure.....	62
4.8	Freezing the cells: In 10% DMSO with 5 million cells/vial using Nalgene 'Mr.Frosty' cooler.....	63
4.9	Freezing the cells: In 10% DMSO with 5 million cells/vial using the CELLerator.....	64
4.10	Viability rates for B16F1 melanoma cells cryopreserved with various methods (n=2).....	65
4.11	Viability rates for 3T3 fibroblasts cryopreserved with various methods (n=2).....	66
4.12	Viability rates for Rat stem cells cryopreserved with varying percentages of DMSO(n=3).....	68
4.13	Viability rates for Rat stem cells cryopreserved with various methods (n=3).....	70

LIST OF TABLES

Table	Page
1.1 Potential Cellular Response due to the stress encountered while freezing.....	12
1.2 Current recovery rates	18
4.1 Composition of freeze media.....	54
4.2 Viability of B16F1 cells cryopreserved with varying percentages of DMSO	54
4.3 Viability of 3T3 fibroblast cryopreserved with varying percentages of DMSO	54
4.4 Viability of B16F1 melanoma cells cryopreserved with various cell densities	59
4.5 Viability of 3T3 fibroblast cryopreserved with various cell densities	59
4.6 Viability of B16F1 melanoma cells cryopreserved with various methods	65
4.7 Viability of 3T3 fibroblast cryopreserved with various methods	65
4.8 Viability of Rat stem cells cryopreserved with varying percentages of DMSO.....	67
4.9 Viability of Rat stem cells cryopreserved with various methods	69

CHAPTER 1

INTRODUCTION

1.1 Tissue Engineering-A step towards realization of a dream

The dream of imitating nature has enticed humanity for thousands of years. In the twentieth century, for the first time, scientific evidence finally provided rational hope that the dream might become a reality. The advancements in the field of Tissue Engineering provided a new ray of hope towards realizing the dream. Tissue Engineering has to imitate the nature's triad involving the cells, the extracellular matrix and the signaling systems. Currently a wide range of cells either from the person who needs the cell (autologous) or from other human sources (allogenic) or from different species (xenogenic) are used as the cell sources [1]. These cells are removed from their sources and grown in a microenvironment very similar to the human body and they are manipulated to achieve the desired behavior. These cells need to be expanded in culture to ensure that there are enough cells for transplant. The cells are grown onto 3 dimensional matrices designated as scaffolds. These scaffolds are made of biocompatible materials like Poly Lactic Acid or Poly Glycolic acid. The necessary signals are incorporated into the scaffold to draw cells into the scaffold. Since every Tissue Engineered product is a combination of various components there are some limitations.

1.2 Stem cells and stem cell culture

When selecting the cellular component of an engineered product, it is important to identify appropriate cells and to be able to isolate them from the primary source. In addition, expansion of these cells without permanently altering the phenotype and function during the expansion phase and without introduction of any adventitious and species-specific bacterial or viral agents poses significant challenges. The issues with contaminating viruses and bacterial agents are even more significant when xenogenic cells are used. Finally, when genetically modified cells are used in a tissue-engineered product, there are additional concerns such as cell transformation [2].

1.2.1 Types of cells

Based on the stage of the culture, cells are classified into primary cell culture or continuous cell lines. The stage of the culture following isolation of the cells, but before the first subculture is called the primary culture. This is obtained either by allowing the cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately adhere to the substrate [3]. The culture consists of mixed population of cell types. Frequently some of the cells may survive but will not proliferate and will therefore be lost in the increasing population of those which are able to multiply in the conditions supplied *in vitro*. The preparation of primary cultures is labor intensive and they can be maintained *in vitro* only for a limited period of time. During their relatively limited life span primary cells usually retain many of the differentiated characteristics of the cell *in vivo*.

A cell line comprises of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumor cells [4].

Stem cells: Apart from the cell lines and the primary cell cultures that are obtained from specialized organs, a relative new member to the cell type is the stem cell. Stem cells are specialized cells that can replicate themselves and can produce cells that take on more specialized functions. Stem cells have been gaining importance over the past few years because they are considered as blank cells that can be programmed into desired cell types. Obtaining reliable sources of stem cells has been one of the major challenges of tissue engineering. Since stem cells can be differentiated into a variety of specialized cells, there are intensive research efforts being conducted to study the use of stem cells in manufacturing tissue engineering products.

Stem cells can be obtained from several sources:

- Embryos - Spare embryos, special purpose embryos, Cloned embryos
- Aborted fetuses and Post-partum Umbilical cords and placenta [5,6].
- Adult tissue or organs: stem cells are obtained from the tissue or organs of living adults during surgery.
- Cadavers: isolation and survival of neural progenitor cells from human post-mortem tissues (up to 20 hours after death) has been reported and provides an additional source of human stem cells [7,8].

Embryonic stem cells are found to trigger immune reactions when transplanted into patients. Further the use of embryonic stem cell is still an ethical issue. Adult stem cell research has been gaining importance because of the fact that the cells can be obtained from the patient himself and can be differentiated into the desired cell type. Isolation of several adult stem cells including mesenchymal, hematopoietic, neural, and hepatic stem cells has opened a novel avenue for obtaining an unlimited supply of cells. These stem cells are normally extracted from the bone marrow. Newer sources like Umbilical cord blood, Placental stem cells and testicular stem cells have been identified which overcome the ethical issues related to embryonic stem cells.

Out of all the stem cells, Mesenchymal stem cells (MSCs) have generated a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self renew and differentiate into functional cell types that constitute the tissue in which they exist. These cells may be directly obtained from individual patients, thereby eliminating the complications associated with immune rejection of allogenic tissue. The quantity of the MSCs needed for a transplant far exceeds the quantity obtained from a patient's bone marrow. Hence the need to find the optimum conditions for the growth and proliferation of these MSCs in vitro.

1.2.2 Culture condition and growth media

The growth media for the cells used initially was natural media based on tissue extracts and body fluids. But as the demand for the amounts of medium increased, various chemically defined media were introduced. Eagle's Basal Medium and subsequently Eagle's Minimal Essential Medium (MEM) became widely adopted. Later on modifications of the above two like Dulbecco's modification DMEM and RPMI1640 (Roosevelt Park Memorial Institute media) were introduced. These media were mixed with various serum types like calf serum, horse serum or fetal bovine serum (FBS) which are good sources of growth factors(which in turn promote cell proliferation), and adhesion factors and antitrypsin activity, which promotes cell attachment. Animal serum is routinely added to culture media as a source of nutrients, despite technical disadvantages to its inclusion and its high cost. Technical disadvantages to using serum include the undefined nature of serum, batch-to-batch variability in composition, and the risk of contamination. There are increasing concerns about animal suffering inflicted during serum collection that add an ethical imperative to move away from the use of serum wherever possible. Hence in this study the effectiveness of a serum-free media DMEM-F12 is compared with the DMEM and MEM media.

Several strategies have been employed to expand, enhance and maintain the multilineage potential of MSCs, such as culturing cells with specific growth factors, enriching cells prior to initial plating, and/or culturing cells in a non-contact suspension culture configuration. Under normal conditions, these MSCs can be expanded typically to 40 Passages until their growth rate is significantly reduced. In addition to the growth

factors in the serum various additives like bFGF (basic Fibroblast growth factor), EGF (Epidermal Growth Factor) and HB-EGF (Heparin Bound Epidermal Growth Factor) are available.

The fibroblast growth factors (FGFs) are a complex family of mostly mitogenic proteins that share varying degrees of amino acid homology and bind to receptors that contain overlapping recognition sites. bFGF preferentially selects for the survival of a particular subset of MSCs with a higher self-renewal potential [9]. However bFGF affects MSC multilineage differentiation capacity, favoring differentiation toward the osteogenic lineage and limiting neurogenic differentiation [10].

EGF has been found to enhance the proliferation capacity of MSCs as well as their differentiation into the osteogenic and hepatocytic lineages [11,12]. HBEGF has been found to have a positive effect on the cell proliferation and it does not affect the stem cells' capacity to self renew [13]. Heparin-binding epidermal growth factor HB-EGF is a member of the epidermal growth factor (EGF) family of growth factors that stimulate growth and differentiation [14].

The need for cultured cells for further use has given rise to the need to find optimum conditions for storing. The cells obtained cannot be maintained in culture for very long duration of time. In 1940, pioneer biologist Basil J. Luyet known as the 'Father of cryobiology', [15] published a work titled "Life and Death at Low Temperatures" in which he described his experiments involving freezing living cells. Many organisms were damaged irrevocably by physical and chemical changes which were triggered by the freezing process, but in some cases he managed to restore normal

function when organisms were rewarmed after freezing. Luyet observed that a high proportion of frog sperm treated with sucrose, would survive freezing to -192°C , four degrees above liquid nitrogen temperature [16]. But the foundations for this study were laid before the 20th century.

1.3 Cryopreservation and Cryoinjuries

The process of preserving cells at very low sub-zero temperatures for long term usage is called cryopreservation. Sub-Zero temperatures are essential to stop any biological activity that might lead to the cell death. Cryopreservation is a pivotal process in cellular engineering for creating a continuous source of generated functional cell lines and for the convenience of various medical treatments that involve cell culture [17]. Cryopreservation offers the potential to maximize the use and availability of biological materials that have a limited supply. It allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years [18], but probably much longer. Important progress in cryobiology, the science of cryopreservation was achieved in the second half of the previous century. Much progress resulted from empirical studies. In later years, progress was also strongly stimulated by the development of fundamental theoretical cryobiology. In so-called ‘slow cooling’ methods that are commonly used, the cells are cooled at a range of cooling rates that are fast enough to prevent ‘slow cooling damage’ but are slow enough to allow sufficient dehydration of the cells to prevent intracellular ice formation (IIF) [19]. The dehydrated cells in the ‘unfrozen fraction’ that remains between the masses of ice will ultimately reach a desirable stable glassy state. The cells can stay in this state

for long periods of time. The thawing process while recovery should be rapid to ensure minimum cell damage [1].

The main advantages of cryopreservation are

1. The genotype of the cell is maintained
2. Senescence is prevented
3. Transformation is prevented
4. Risk of a microbial contamination and cell death due to equipment failure are reduced
5. Reduced risk of cross contamination with other cell lines.
6. Reduction in risk of genetic drift and morphological changes
7. Reduction in the cost involved in cell culturing and maintaining.
8. Convenient for distribution

The main aim of cryopreservation is to prevent the need to have numerous cell lines in culture at the same time. Compared to other methods of storing cells and tissue like refrigeration, chemical preservation, continuous in vitro culturing, freezing to cryogenic temperatures has the benefits of affording long shelf lives with assured genetic stability, virtually no risk of microbial contamination during storage and improved cost effectiveness. Thus, cryopreservation is currently the most viable approach to meet the requirements of production, distribution and end-use of tissue-engineered products.

1.3.1 Cryoinjury during freezing of cells

The cryopreservation process causes significant changes in the thermal, chemical and physical environment in the tissue, with added risks of biological damage [1]. While freezing cell suspensions, the cooling rate is very vital. If the rate of cooling is sufficiently slow to allow the intracellular solution to equilibrate with its external environment by expressing water through the cell membrane, the cell will dehydrate extensively with decreasing temperature. On the other hand, if the cooling is fast compared to the rate of water efflux, low temperatures are reached before significant dehydration can occur. In this latter case, the cell remains largely undeformed, but there is a very high probability of intracellular ice formation (IIF) in the cell. Therefore the best freezing protocol involves intermediate cooling rates, wherein the cells are partially dehydrated, and thus near equilibrium. If the cooling rate is not rapid ice nucleates first in the extracellular space between the cells. This will create an osmotic gradient between the intracellular isotonic solution and the freeze-concentrated extracellular solution, across the cell membrane. If the cooling rate is low the intracellular water moves out of the cell into the extracellular ice phases which cause shrinking of the cell. Both the above mentioned conditions lead to lysis of the cell [20].

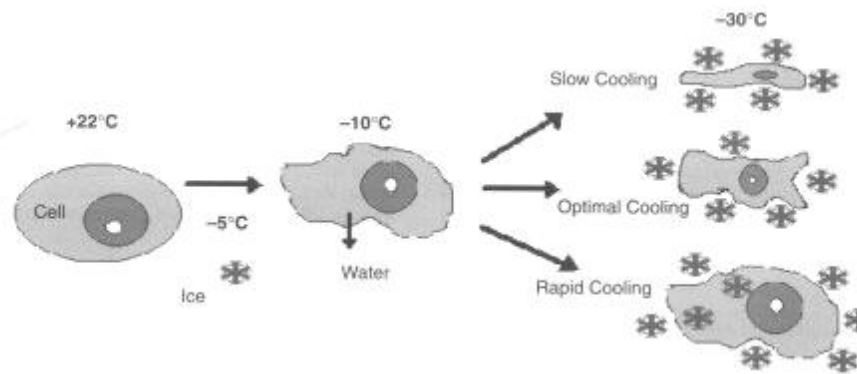


Fig 1.1 Schematic diagram of physical events in cells during freezing [20]

1.3.1.1 Two-factor Hypothesis

Based on experimental observations of the cooling rate dependence of cell survival, Mazur *et al* proposed a two-factor hypothesis of cell freezing injury, which posits that two distinct mechanisms are responsible for cell damage during cryopreservation.

1. At slow cooling rates, the cryoinjury occurs due to the solution effects (i.e., the solute/electrolyte concentration, severe cell dehydration, and the reduction of unfrozen fraction in the extracellular space); and [21]

2. At high cooling rates, cryoinjury occurs due to the lethal Intracellular Ice Formation. The optimal cooling rate for cell survival should be low enough to avoid Intracellular Ice Formation but high enough to minimize the solution effects [22,23].

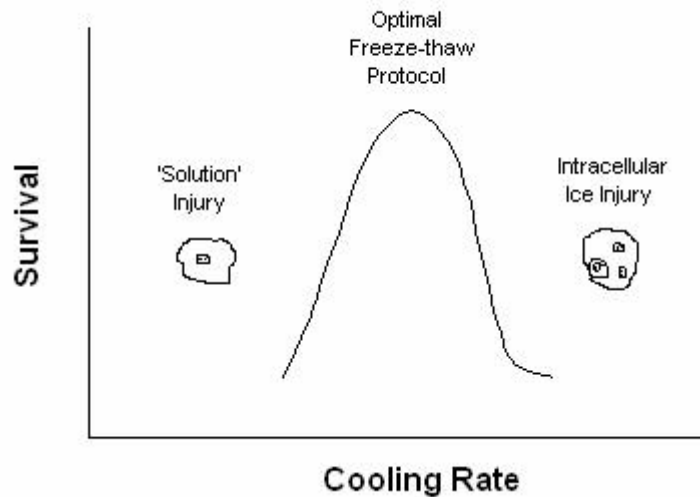


Fig 1.2 Effect of cooling rate on cell survival

Figure 1.2 shows that there is an optimum cooling rate at which the two mechanisms of damage are balanced, and the probability of cell survival reaches a maximum. Optimal values for all the freezing processes that are used can be explained in terms of a balance between solution effects and intracellular ice formation. Although the experimental evidence supports the universality of the two-factor hypothesis as a qualitative explanation of cell damage during freezing, the optimum cryopreservation method is known to differ from cell type to cell type. This is a result not only of variable sensitivity to freezing injury, but also of variations in cell biophysical properties such as water permeability of the plasma membrane and the activity of ice nucleating catalytic sites in the cell, which affect the rate of dehydration, and the probability of Intracellular Ice Formation, respectively [24,25].

In addition to these two injury mechanisms, other mechanisms like freezing induced immunologic injury [26] and apoptosis [27,28] could also be responsible for cellular damage.

Cells rarely come into direct contact with ice crystals; rather they become concentrated into the unfrozen fraction, where they are exposed to a number of physical stresses. It is the cellular response to these stresses, which determines survival [29].

Table 1.1 Potential Cellular Response due to the stress encountered while freezing

Stress encountered	Potential cellular response
Reduction in temperature	<ul style="list-style-type: none"> • Membrane lipid phase changes. • Depolymerization of the cytoskeleton
Increase in solute concentration	Osmotic shrinkage
Increase in ionic concentration	Direct effects on membranes, including solubilisation of membrane proteins
Dehydration	Destabilization of the lipid bilayers
Precipitation of salts and eutectic Formation	Under dispute
Gas bubble formation	Mechanical damage
Solution becomes extremely Viscous	Diffusion processes, including osmosis may become limited
Changes in pH	Denaturation of proteins
Cells are packed closely	Membrane damage

Cells are exposed to all these stresses which are changing during solidification. However, it is the osmotic response of cells that is the primary determinant of viability. The hypertonic conditions the cells encounter lead to an osmotic loss of water, the extent of which is dependent on the rate of cooling. The tolerance of cells to freezing at slow cooling rates depends on their ability to withstand osmotic stress [30].

Resistance to water diffusion increases as the cooling rate increases and cell contents may supercool before all the freezable water has been removed. Under these conditions, cell shrinkage and membrane damage will be minimized. This happens when the cooling rate is optimum [31].

1.3.2 Cryoinjury during storage

Usually, the cryopreserved samples are stored at or below -70°C . It has been observed however that at -70°C the samples have good viability for 2-3 months. At less than -120°C , chemical reactions cannot occur in human-relevant times. At -196°C , no thermally driven reactions can occur in less than geologically relevant times. The reactions that can occur are the slow accumulation of direct damage from ionizing radiation, but this accumulation would probably become significant only after centuries of storage [27].

1.3.3 Cryoinjury associated with the thawing process

A cell that has survived cooling to low subzero temperatures is still challenged during warming and thawing, which can exert effects on survival comparable with those of cooling. These effects depend on whether the prior rate of cooling has induced intracellular freezing or cell dehydration. In the former case, rapid thawing can rescue

many cells, possibly because it can prevent the growth of small intracellular ice crystals into harmful large ice crystals (i.e., so-called recrystallization). Even when cells are cooled slowly enough to preclude intracellular freezing, the response to warming rate is often highly dependent on the freezing conditions and cell type. The warming methods include thawing in ambient air, in water baths with temperatures ranging between 5°C and 37°C, in coat pockets, and thawing the samples in the investigator's hands. There is little effect of warming rate when a fast cooling rate was used, probably because the cells had been killed by the Intracellular Ice Formation during the fast cooling process before the warming process started [26,27].

Cells will be exposed to osmotic shock as the suspension is thawed, resulting in a progressive decrease in the extracellular solute concentrations as the ice melts. The optimum thawing rate would be one in which this osmotic shock is prevented.

1.4 Cryoprotectants

Cryoprotectants as described by Computer Retrieval of Information on Scientific Projects are substances that provide protection against the harmful effects of low or freezing temperatures. A cryoprotectant can make water harden like glass, with no crystal formation [32]. Cryoprotectants also prevent the build-up of salts as water in cells crystallizes during freezing. High concentrations of salts and perhaps the ice crystals themselves can mortally wound cells either during freezing or thawing.

In 1949 Polge, Smith, Parkes in England made the serendipitous discovery of the protective action of glycerol which marked a milestone in the field of cryobiology. While working in the area of reproductive biology, Audrey Smith and Chris Polge were

attempting to cryopreserve spermatozoa from roosters. The media commonly used at that time for handling sperm cells was a saline solution supplemented with skim milk powder, egg yolk, levulose, and other compounds [33]. The researchers were modifying the composition of the cryopreservation solution, and testing the motility of sperm after cryopreservation. Normal results were about 5% motility. Glycerol was used as an agent to immobilize the sperm for microscopic examination. During the routine set of experiments the scientists noticed that the motility rate was 50% which was unusual. They repeated the experiment several times with the same remarkable result. The next day, while demonstrating the remarkable development to their colleagues with a new batch of cryopreservation medium, the post-thaw was back the normal 5%. Analyses of the earlier cryoprotectant revealed that the solution had a high Glycerol concentration. It was later discovered that the labels containing the glycerol and the cryopreservation media had been interchanged and that the cryopreservation solution was, in fact, the Glycerol solution used to immobilize the sperm for morphological assessment [34].

Cryoprotectants have different chemical compositions and share high water solubility associated with toxicity directly related to their concentration and temperature. They are water soluble and form hydrogen bonds with water [35]. Cryoprotectants are categorized as penetrating or non-penetrating. The former ones exert their protective effects through colligative action. Penetrating cryoprotectants penetrate the cell membranes and subsequently stabilize the intracellular proteins, reduce the temperature at which cells undergo lethal intracellular ice formation and moderate the impact of concentrated intra and extra- cellular electrolytes [36]. Thus the

potential toxic effect of freeze-induced solute concentration is reduced. The protective action of non-penetrating cryoprotectants is largely by osmotic dehydration [37]. The commonly used cryoprotective agents are Glycerol, Ethylene Glycol, DMSO (Dimethyl Sulfoxide) and 1,2 propandiol. DMSO has been shown to be a better cryoprotectant than Glycerol [38]. Their molecular weight is relatively low (<100). This makes it easy for them to penetrate into the cell through the cell membrane. Large molecules like sucrose, proteins and lipoproteins as a result of their size or polarity remain in the extracellular solution and act as non-penetrating cryoprotectants [35,37]. The non-penetrating cryoprotectants like Hydroxyethylstarch work due to their ability to reduce the amount of crystallizing water during freezing [39,40].

1.4.1 The toxicity of cryoprotectants

Cryoprotectants can suppress most cryoinjuries but, when used at higher concentrations, most of them become toxic to biological material [41]. Cryoprotectants are of necessity tolerated in high concentrations, but at a sufficiently high concentration any compound will be toxic. In practice it is found that the maximum concentration that can be achieved without impairment of viability is dependent on the temperature and rate of addition and removal; temperature dependence is due partly to chemical toxicity (which is reduced by reduction in temperature) and partly to osmotic effects (which are increased by reduction in temperature). To reduce the chemical toxicity cryoprotectants are frequently added at 0°C to -4°C rather than at room temperature. This process takes advantage of the positive temperature coefficient of chemical toxicity [33]. According

to Freshney the cryoprotectants can be effective when used at concentrations between 5 and 15%. At these concentrations the toxicity is considerably low [3].

1.4.2 Osmotic effects of cryoprotectants

The most effective cryoprotectants penetrate cell membranes but they do so more slowly than water, which means that some osmotic imbalance is inevitable during the addition or removal of these compounds [33]. The addition of a cryoprotectant to a sperm suspension was hypothesized to affect (lower) the hydraulic conductivity. Studies of human RBC have shown that approximately 90% of the RBC membrane water flux is carried by protein channels and that 10% passes through the lipid bilayer [42,43]. Given these two routes of water movement, a nonionic solute could affect the membrane permeability in at least three ways. First, the solute could modify the bilayer, causing a change in bilayer permeability [44]. Second, a modification of the bilayer could indirectly affect membrane transporter protein activity [45]. Third, the solute could interfere directly with the function of the water-transporting proteins (either primary water channels specific for water transport like CHIP 28, or secondary water channels that are presumably for some other molecule, like glucose, but that also facilitate water transport) [46]. This movement of water affects the osmotic balance of the cell.

Table 1.2 Current recovery rates

Cell Type	Cryopreservation Method	Viability
3T3	Hydrogel encapsulated	50-60% [47]
3T3	Trehalose loading after poration by Staphylococcus aureus α -hemolysin	80% [48]

1.5 Need for optimization of culture condition and cryopreservation

The MSCs have gained wide interest because of their capacity to differentiate and self renew. However the number of cells obtained from the source is not enough for transplant. Therefore the need to expand them in vitro arises. The aim of this project is to arrive at the best possible method and condition for culture and cryopreservation of mammalian stem cells with cell lines as models. The rat peritoneal mesenchymal stem cells are cultured in media containing serum and additive growth factors and the effect of various factors are analyzed. The best cryopreservation technique is arrived by testing various mammalian cells with different cell densities in various percentage compositions and frozen using a variety of techniques. The best cell density, percentage composition of the freeze media and the best technique for freezing are selected from the results obtained. DMSO and glycerol are commonly used for the cryopreservation of cells as well as tissues and organs. However, because of their toxicities to the cells, optimum amounts were required for the protection and less damage of to the cells. Based on the studies done by Jeong Hwa Son *et al* it was found that DMSO is a more effective cryoprotectant than glycerol [49]. This forms the basis of the investigations conducted.

The mechanism of action of DMSO as a cryoprotectant is at present poorly understood. It is considered that there are atleast three locations where DMSO may act to avoid freezing injury: on the exterior solution, on the membrane itself and on the interior solution [50]. DMSO protects the cells against freezing by intracellular dehydration. Dehydration is induced by the replacement of intracellular water with the cryoprotectant itself. This is achieved by penetrating the cell membrane. Hence DMSO belongs to the category of penetrating cryoprotectants [51]. The specific mode of action of the penetrating cryoprotectant is considered to be related to the following effects:

1. They lower the equilibrium freezing point
2. They lower the homogenous nucleation temperature.
3. They enhance undercooling and raise the recrystallization temperature.
4. They act as a solvent, keeping potentially harmful salts in solution during freezing [52].

CHAPTER 2

MATERIALS AND METHODS

2.1 Cells

Two cell lines and a primary cell were analyzed. The B16F1 melanoma and 3T3 fibroblast cell lines were obtained from ATCC (American Type Culture Collection).

2.1.1 B16F1 melanoma cells

The cell line was created by successive B16 melanoma tumor lines which were selected for their ability to form tumor nodules. B16 melanoma variant lines, which resisted lysis by syngeneic lymphocytes, were selected in vitro by repeated exposure of the tumor cells to purified cytotoxic lymphocytes [53]. This was accomplished by injecting tumor cells intra venously into syngeneic C57BL/6 mice and 2 to 3 weeks later collecting the secondary tumor growths and placing them into tissue culture. These tumor cells were then injected i.v. into new syngeneic mice and the process was repeated several times. With each successive tumor line the number of experimental tumor nodules was significantly increased.

The B16 lines were found to be stable in their metastatic properties even after many subculturings in vitro. These results suggest that an increased initial arrest of highly metastatic cells in a capillary bed may be a major factor in their increased survival and stability. Studies have demonstrated that tumor cells that succeed in forming tumor colonies also have increased invasive properties into normal tissues

when implanted subcutaneously [54]. Cells that detached more readily were spindlier in culture than parent cells, covered less surface area per cell, and formed fewer pulmonary tumors when injected iv into C57BL/6J mice. Cells that detached less readily were flatter in culture and formed more pulmonary tumors when injected iv than the parent cells. After detachment with EDTA, the cells reattached to culture dishes at the same rate [55]. The normal subculture ratio for this adherent cell line is 1:10 with a media change every 2 to 3 days.

The B16F1 used for this experiment have the ATCC number CTRL-6323 and are obtained from *Mus musculus* (mouse). These adherent cells are obtained from the skin of a mouse with melanoma.

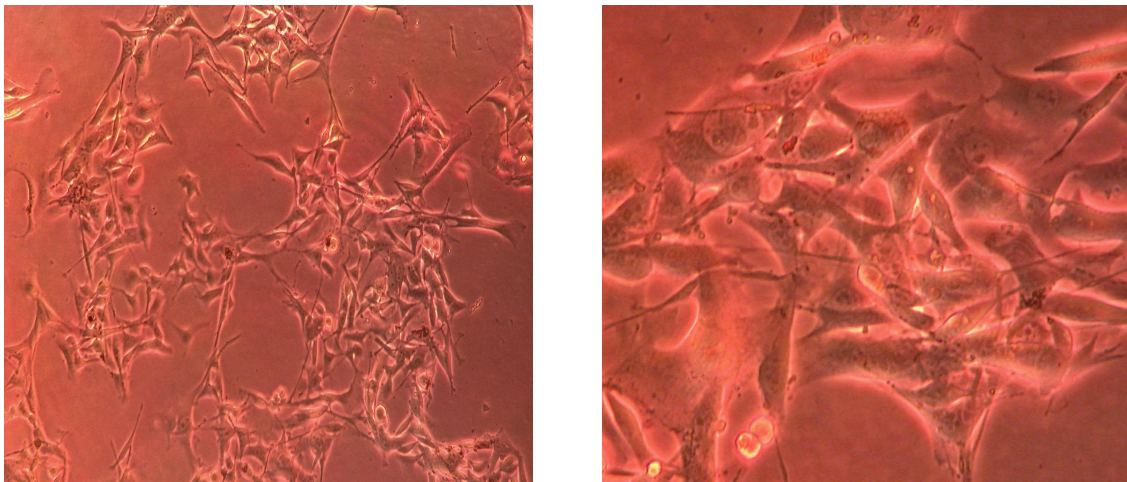


Fig 2.1 B16F1 melanoma cells at 10X and 20X magnification

2.1.2 3T3 fibroblast cells

The BALB/3T3 clone A31 is one of several adherent cell lines developed by S.A. Aaronson and G.T. Todaro in 1968 from disaggregated 14- to 17-day-old BALB/C mouse embryos. These cells have been found to grow well with the interaction with the

growth factors in serum [56]. The cells are extremely sensitive to contact inhibition of cell division, grow at a high dilution and exhibit a low saturation density. The normal subculture ratio is 1:4 or 1:10 with media change for atleast twice a week.

The 3T3 cell line is obtained by the continuous culturing of mouse embryo cells. After the initial decline in growth, after three months in culture these embryo cells developed into established lines which have a constant or a potential growth rate. The cells of line 3T3 differed in appearance from the other lines. In sparse culture, they also looked fibroblastic but grew considerably flatter, appeared finely granular, and were more difficult to trypsinize [57]. The 3T3 fibroblast cell line used for this experiment has the ATCC number CTRL 163 obtained from BALB/C mouse.

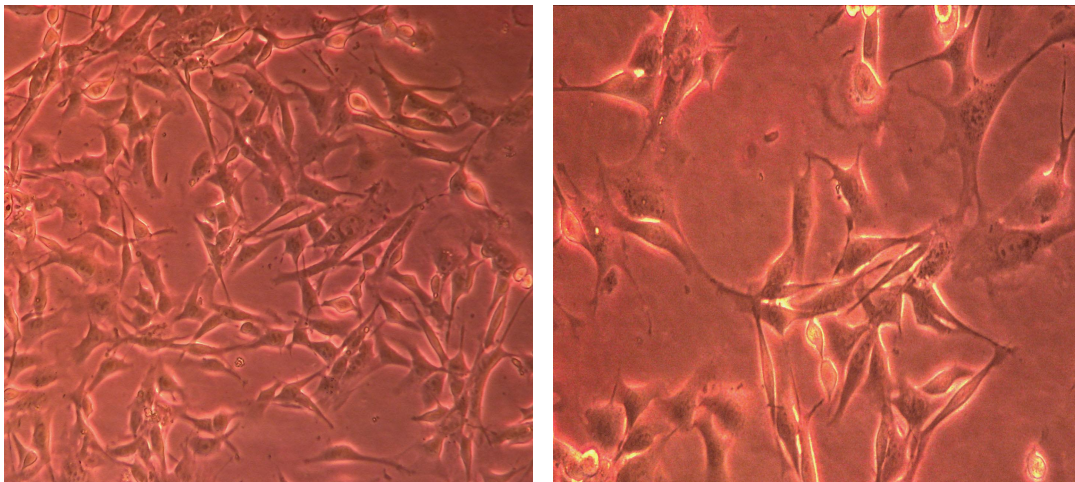


Fig 2.2 3T3 fibroblasts at 10X and 20X magnification

2.1.3 Rat stem cells

Previous studies conducted in the lab have shown that implanting a polymer particle into a rat recruits stem cells that resemble the mesenchymal stem cell morphology. The rat stem cells were obtained from the peritoneum of a Sprague

Dawley rat which was injected with PLLA particles. The peritoneum was washed with DMEM and the solution was cultured with 20% FBS. The cells were allowed to attach for 5-6 days and then the media was changed. The cells that adhered belong to the mesenchymal type while the floating ones are a mixture of hematopoietic stem cells and RBCs. The cells were observed and media was changed twice in a week with a subculture done at 70-80% confluence. The cells that were used in the experiment were in their second passage.

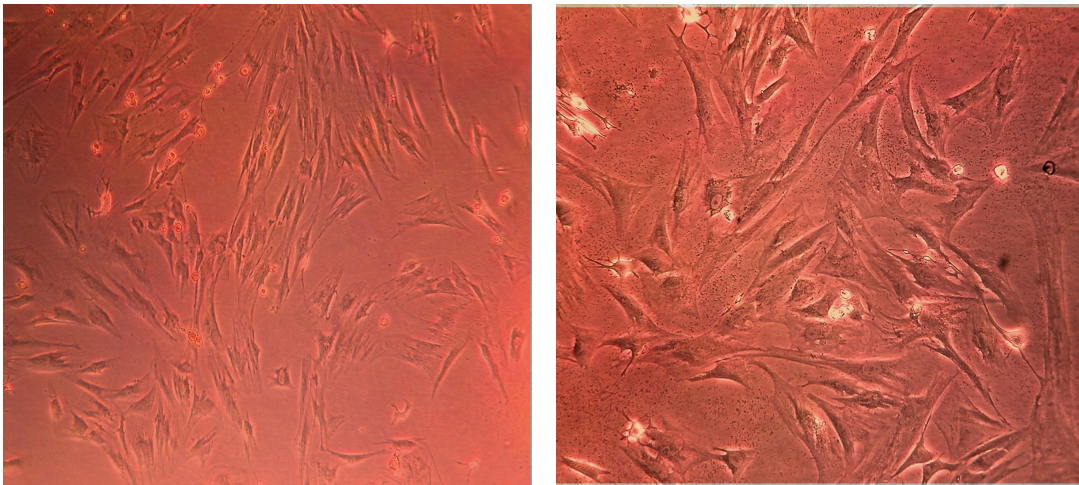


Fig 2.3 Rat stem cells at 10X and 20X magnification

For both the cell lines the serum used is calf serum because fetal bovine serum causes transformation and loss of contact inhibition. The primary stem cell culture needs fetal bovine serum because there are more proteins and growth factors in it than calf serum.

2.1.3.1 Culture In-Vitro

The cells that are used for the experiment are grown in a medium with 80% DMEM (Dulbecco's Modified Eagles Medium, Sigma Aldrich, St.Louis, MO) and 20%

Serum (Atlanta Biologicals, Lawrenceville, GA). The same brands of media and serum are used throughout the entire culture. DMEM is rich with essential amino acids, vitamins and salts needed for the cell growth. Serum contains growth factors, which promote cell proliferation and adhesion factors and anti-trypsin activity, which promote cell attachment. Serum is also a source of minerals, protein, lipids and hormones [3]. DMEM and serum that are used for the experiments are filtered using a cellulose acetate filter (of 0.22µm pore size). The cells are grown in a 25 cm² flask (Corning, Corning, NY) under constant supervision with media changes every alternate day. The flasks are maintained in a sterile environment inside an incubator which is maintained at 37⁰C with 5% Carbon dioxide supply and 95% humidity. Humidity is supplied by a tray filled with distilled water. The cells are allowed to grow till a confluency of 70-80% is attained. This is considered as the late log phase in the growth curve of the cell. If the cells exceed the mentioned confluency they are subcultured.

Subculture is done by using 0.05% trypsin (Sigma Aldrich, St.Louis, MO) and 0.025% EDTA (Sigma Aldrich, St.Louis, MO). 1ml of trypsin is added into the 25cm² flask, shaken gently and kept inside the incubator for 1-2 minutes. The flask is then observed under the microscope to check for detached cells. Detached cells appear to be round and they float around while the adhered ones are flat and have projections. The trypsinized cell solution is neutralized with 1.5ml of serum and flushed gently to ensure that all the cells are detached. 5ml of DMEM is added and the entire suspension is flushed, with the tip of the pipette resting on the bottom corner of the flask, taking care not to create any foam. The flask is observed under the microscope to ensure that there

are no clumps in the suspension. Another method of subculture is to use a cell scraper (Greiner Bio-One Inc Longwood, FL). The cells are gently scraped and complete media is added and the suspension is flushed well and then transferred into the well plate.

2.1.3.2 Quantification

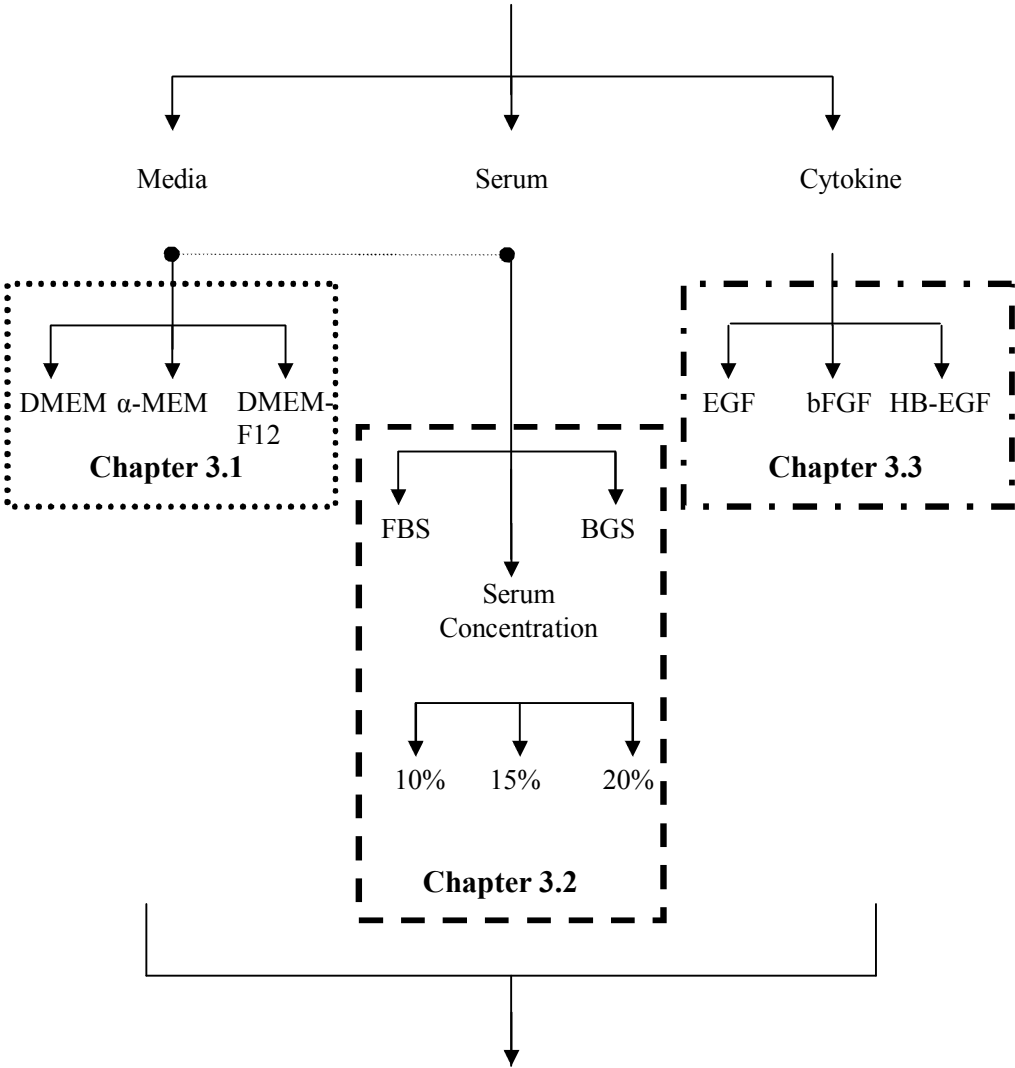
The cell solution is dropped onto a hemacytometer and covered with a cover slip and the cell count is taken. The total number of cells for the entire flask is calculated and the value is noted.

2.2 Culture conditions

The cells are grown in DMEM, α -MEM (Sigma Aldrich, St.Louis, MO) and DMEM F12 (Sigma Aldrich, St.Louis, MO) to analyze the effect of the various media on growth. These media were supplemented with 20% FBS. For analyzing the effects of serum on growth DMEM mixed with 20% FBS, 20% BGS and 10% FBS + 10% BGS. For evaluating the effect of various growth factors on the cell proliferation in vitro, the cells are plated in a well plate with a cell density of 3000 cells/well. They were supplemented with varying concentrations of following growth factors (Sigma Aldrich, St.Louis, MO).

- EGF (10 ng/ml, 30ng/ml, 100ng/ml),
- bFGF (2 ng/ml, 10ng/ml, 50ng/ml),
- HB-EGF (5 ng/ml, 25 ng/ml, 50 ng/ml)

Current Culture conditions for In-Vitro expansion of Mesenchymal Stem Cells



Optimum Culture conditions for In-Vitro expansion of Mesenchymal Stem Cells

Fig 2.4 Experimental design for testing various culture conditions for Rat MSCs

2.3 Cryopreservation and cell recovery

The cell solution is centrifuged (Beckman, Model TJ-6 Centrifuge) at 1100 RPM for 10 minutes. The cells are resuspended at approximately $0.2 \times 10^6 - 5 \times 10^6$

cells/ml of various freeze media containing the cryoprotectant (DMSO). DMSO used for this experiment is laboratory grade and supplied by Sigma Aldrich, St.Louis, MO and its melting point is 18⁰C. The cell solution is then dispensed into pre-labeled cryovials, and the vials are sealed. The cryovials used are Mikro-Schraubrohre 2ml vials made of Polypropylene. The cryovials are then frozen by a slow cooling process which involves cooling the cells at the rate of 1⁰C/min. The cryovials are placed on canes and transferred into the canisters inside the liquid Nitrogen Dewar which provides a very low temperature of -196⁰C.

2.3.1 Cryopreservation techniques

2.3.1.1 Step-by-step freezing

The cells are trypsinized and resuspended in a freeze media containing 10% DMSO maintained at 4⁰C. This solution is aliquoted into cryovials and is refrigerated at 4⁰C for 30 minutes. The vials are then refrigerated at -20⁰C for 30 minutes. They are frozen in a -70⁰C freezer for 24 hours. The vials are then placed on vial holders which are Aluminium canes maintained at -70⁰C. These vials are transferred into the canisters in the liquid Nitrogen tank. This transfer is done quickly, as the vials will reheat at about 10⁰C/min, and the cells will deteriorate rapidly if the temperature rises above -50⁰C. When the vials are safely located in the freezer, entries are made to ensure the location of these vials.

2.3.1.2 Nalgene 'Mr. Frosty' cooler

The cells are trypsinized and resuspended in a freeze media containing 10% DMSO maintained at 4⁰C. This solution is aliquoted into cryovials and the vials are

transferred into the Nalgene 'Mr. Frosty' cooler which is a plastic holder with fluid-filled base. The specific heat of the coolant in the base insulates the container and gives a cooling rate of about $1^{\circ}\text{C}/\text{min}$ in the vials. Typical cooling rates for homemade freezing systems lead to uncontrolled cooling that averages $1^{\circ}\text{C}/\text{min}$ but the cells actually experience more rapid rates of cooling during some parts of the cooling cycle. Home made freezing systems are also non-repeatable.

Mr. Frosty has a high density polyethylene vial holder inside a polycarbonate vessel. The closure is a high density polyethylene lid. Mr. Frosty requires only 100% isopropyl alcohol and a mechanical freezer. The freezing container is stored at room temperature when not in use. When the vials are ready to be cryopreserved 250ml of isopropyl alcohol is poured into the container and the vial holder is placed in position. The foam insert provides thermal insulation. The vials are then transferred into the holder and allowed to cool in a -70°C freezer for 24 hours. The container can hold upto 18 vials.

The major advantages of this method are that

1. Holder prevents the vials from contact with the alcohol-so no contamination by wicking.
2. Labels are not removed.



Fig 2.5 Nalgene 'Mr. Frosty' cooler box

2.3.1.3 Cryo-matic CELlevator

The cells are trypsinized and resuspended in a freeze media containing 10% DMSO maintained at 4⁰C. This solution is aliquoted into cryovials and the vials are transferred onto the sample tray of the cryo-matic CELlevator is an inexpensive semi programmable freezer. This is used in conjunction with liquid nitrogen tank that has a 2.3 inch opening at the neck. The instrument simplifies the cryopreservation of cells by freezing them automatically at one degree per minute over a four hour period. Once the cells reach the designated temperature of -186⁰C, the instrument stops and alerts the user that the run is complete by illuminating the red stop light.

The main body of the CELlevator encloses the motor and the electronic board which operates the instrument. The control buttons are located on the top of the instrument. One rocker switch turns the power on and off and the other controls the motor causing the sample tray to be lowered, when the switch is in the down position,

and raised, when the switch is in the up position. The bottom of the instrument has a threaded rod which extends from the unit through a piece of Styrofoam, with the sample tray attached to it at the bottom. The sample tray is designed to accommodate and freeze six samples at one time.

The vials are placed in the sample tray and the instrument is placed in such a way that the Styrofoam on the cryomatic aligns with the opening of the opening of the tank. The instrument is turned on and the other rocker switch is put in the down position and the instrument is allowed to run. This will turn on the motor and initiate the very slow decent of the sample tray. The light indicator turns red when the run is completed and the motor is automatically turned off. This generally takes 4 hours. The rocker switch is put in the up position. The light turns up when the sample tray is brought back to its initial position (fully up). At this time the cryomatic CELlevator is removed from the tank and the cryovials are removed from the sample tray and arranged on cane and then placed in canisters inside the liquid Nitrogen.



Fig 2.6 Cryo-matic CELlevator- Semi-programmable freezer

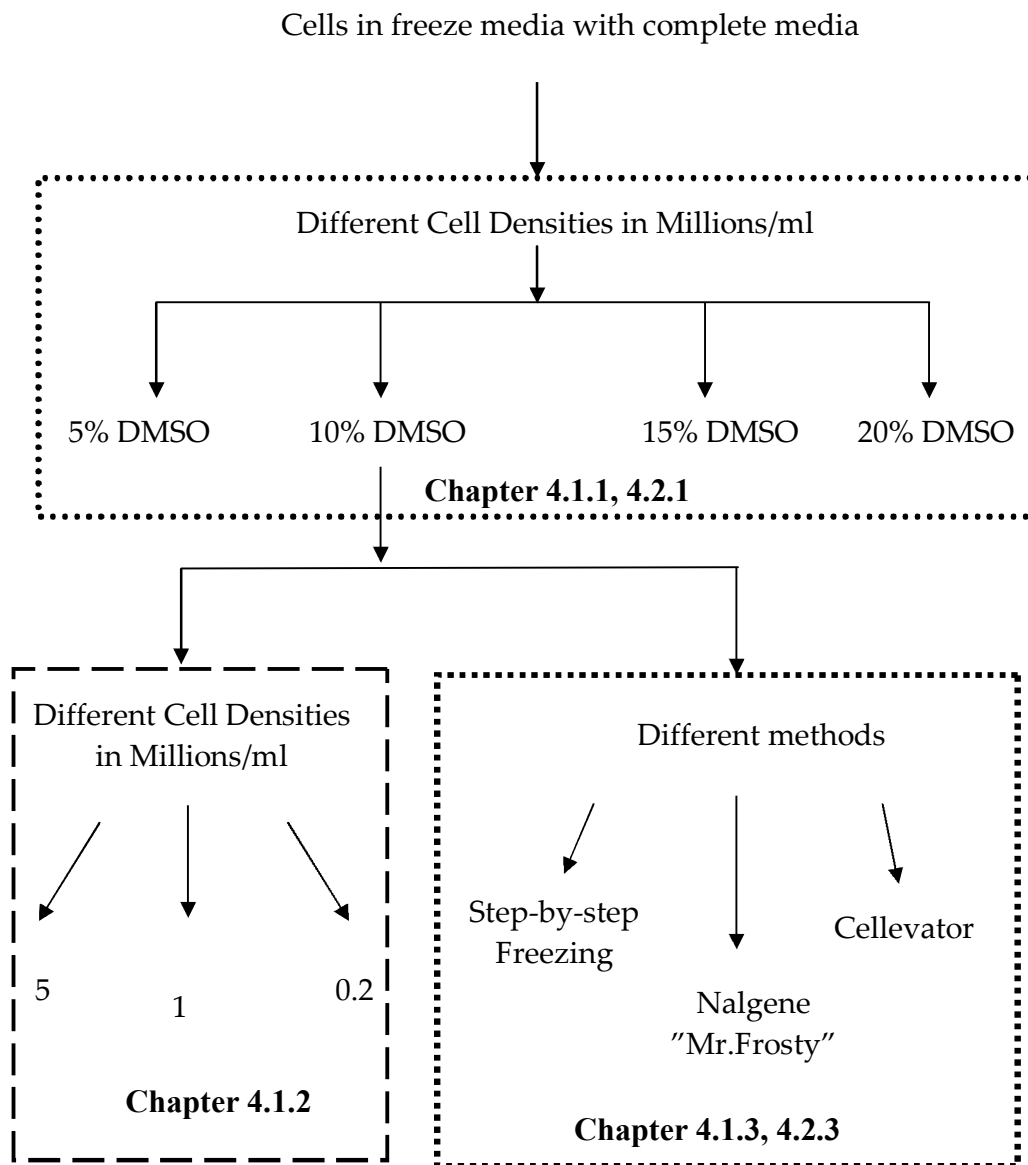


Fig 2.7 Experimental design for cryopreservation of cells

The vials are retrieved from liquid nitrogen and plunged immediately into a waterbath maintained at 37°C. The rapid thawing process is allowed to continue for two minutes. When the vials are thawed, the labels checked to confirm the identity of the contents. The vials are swabbed with 70% ethanol and taken inside a biological

hood. The contents are transferred into a 50ml plastic tube and centrifuged at 1100 RPM for 10 minutes with DMEM added to it. This washing step plays a very important role in reducing the toxicity of the cell solution. Adverse reactions like cardiovascular complications and neurologic problems along with nausea and chills have been observed in patients, due to infusion of DMSO from a transplanted organ [58,59]. The supernatant is discarded and the cells are resuspended in DMEM for cell culture and viability tests (Trypan blue exclusion).

2.3.2 Viability test-Trypan blue exclusion

Viability assays are used to measure the proportion of viable cells following a potentially traumatic procedure like freezing and thawing. Most viability tests rely on a breakdown in membrane integrity that is determined by the uptake of a dye to which the cell is normally impermeable [18].

For mammalian cells, the number or percentage of viable cells can be determined by staining with trypan blue. Viable cells exclude the dye, whereas dead cells take up the dye. After staining with trypan blue, cells should be counted almost immediately since viable cells also can take up the dye after a few minutes of exposure to the dye. Also trypan blue has a great affinity for proteins. To avoid interface serum is not added in the resuspending media [60].

To perform the test, transfer 0.1ml of the cell solution into an eppendorf tube and add an equal volume of 0.4% Trypan blue (Sigma Aldrich) to the cell suspension and mix well. After about 5 minutes, transfer a small volume of the cell suspension to one of the chambers of a hemacytometer so as to completely fill the chamber, and then

count the stained and unstained cells in the four corner squares using a microscope.

Determine the percent cell viability by the following formula [61]:

$$\text{Viability} = \frac{\text{Number of stained cells} \times 100\%}{\text{Total cell number (stained plus unstained)}}$$

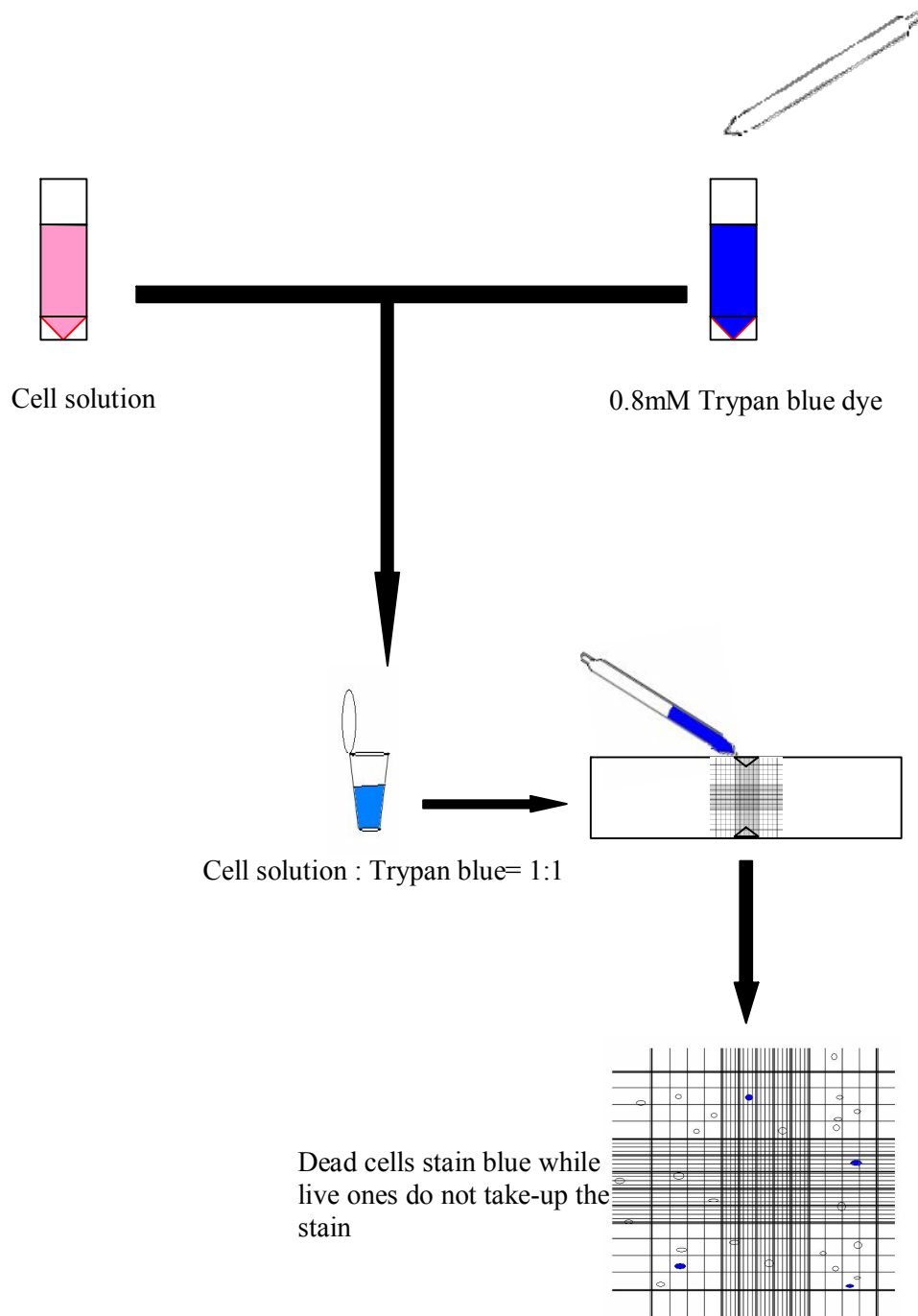


Fig 2.8 Trypan blue viability assay for recovered cells

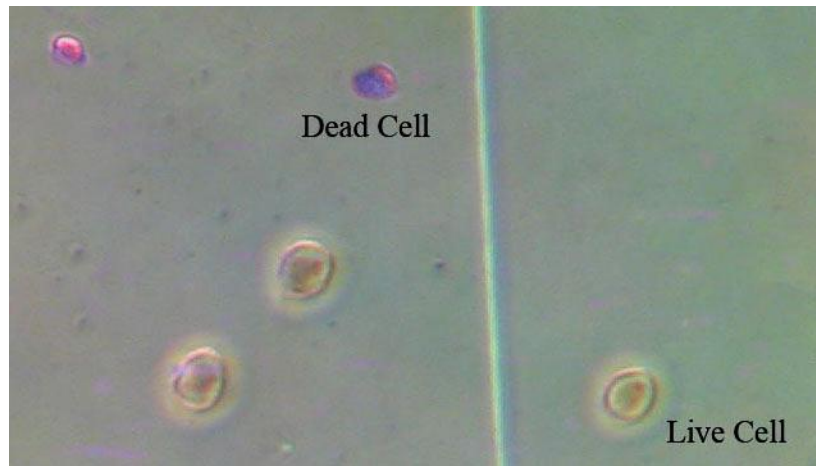


Fig 2.9 Trypan blue stained cells in a hemacytometer at 20X magnification

CHAPTER 3

CHARACTERIZATION OF OPTIMUM CULTURE CONDITIONS AND CULTURE PROTOCOL FOR IN VITRO EXPANSION OF RAT MESENCHYMAL STEM CELL

3.1 Effect of growth media on cell proliferation

Previous studies have indicated that the growth media plays a very important role in the proliferation of cells. The culture of the rat MSCs in DMEM, α MEM and serum-free DMEM-F12 media were tested. The former ones were supplemented with varying percentages of FBS.

The cells were seeded with a density of 500 cells/well. The 10%, 15% and 20% of FBS was added to each well. The cell growth was observed for a period of 7 days and a bar chart was plotted. No significant difference observed in the effect of 15% and 20% FBS on cell growth and morphology in culture (Figures 3.1, 3.2). Not much of significant difference was observed between DMEM and α -MEM on cell proliferation and morphology. Serum free media showed relatively fewer cells when compared to the other media used in combination with Serum. This states that the serum-free media does not support the growth of the MSCs in vitro (Figure 3.1.C). The above experiment negates the replacement of serum rich media with serum free media. Amongst the various basal media tested, DMEM was selected for the subsequent studies. Contradictory to the studies conducted earlier, which states that the serum-free media is as good as the serum rich media is negated from the above experiments. Previous

studies have shown that in the case of Bone marrow mesenchymal stem cells, α -MEM induced a better growth than DMEM [10,62]. But our results confirmed that the peritoneal stem cells possess different culture condition requirements than the bone marrow stem cells because DMEM evoked a better response.

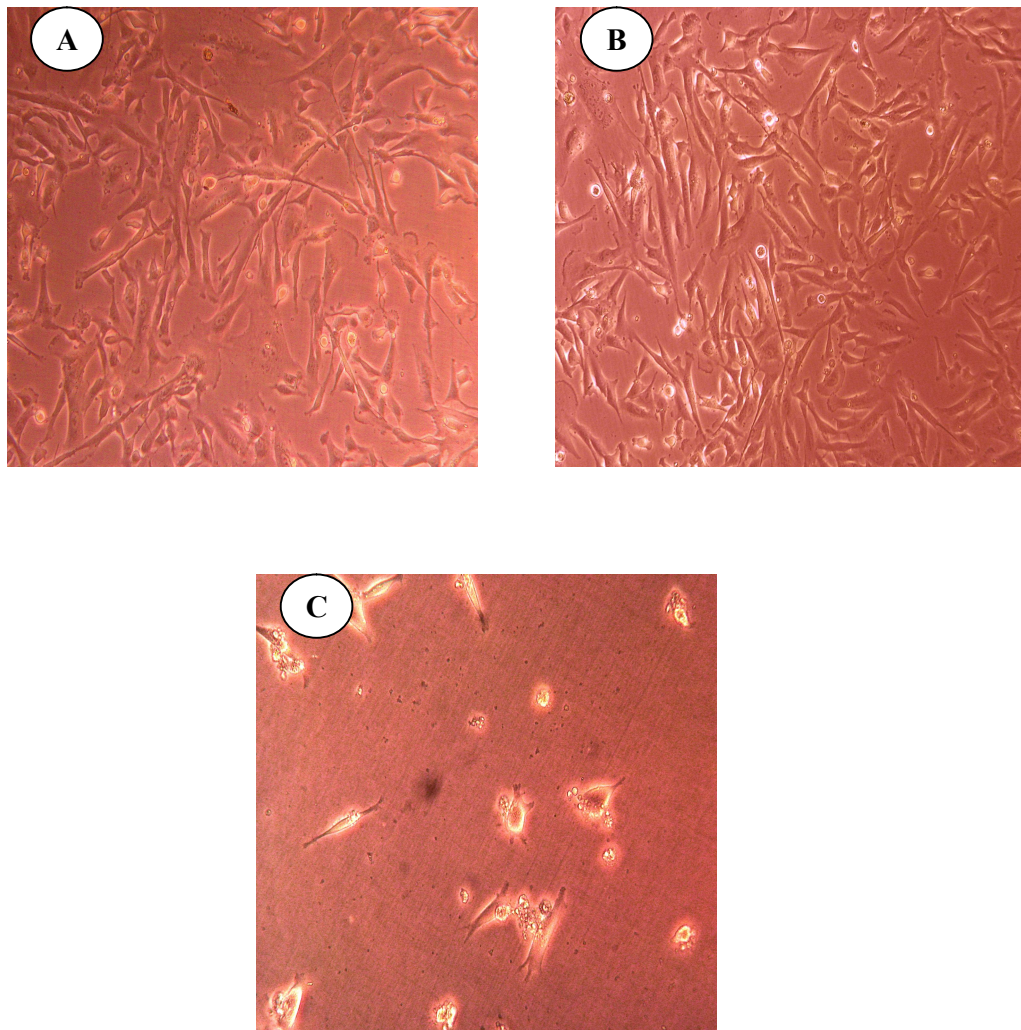


Fig 3.1 Morphology and confluency of Rat stem cells in various media on the 5th day of culture A. DMEM+20%FBS; B. MEM+20%FBS; C. DMEM F12

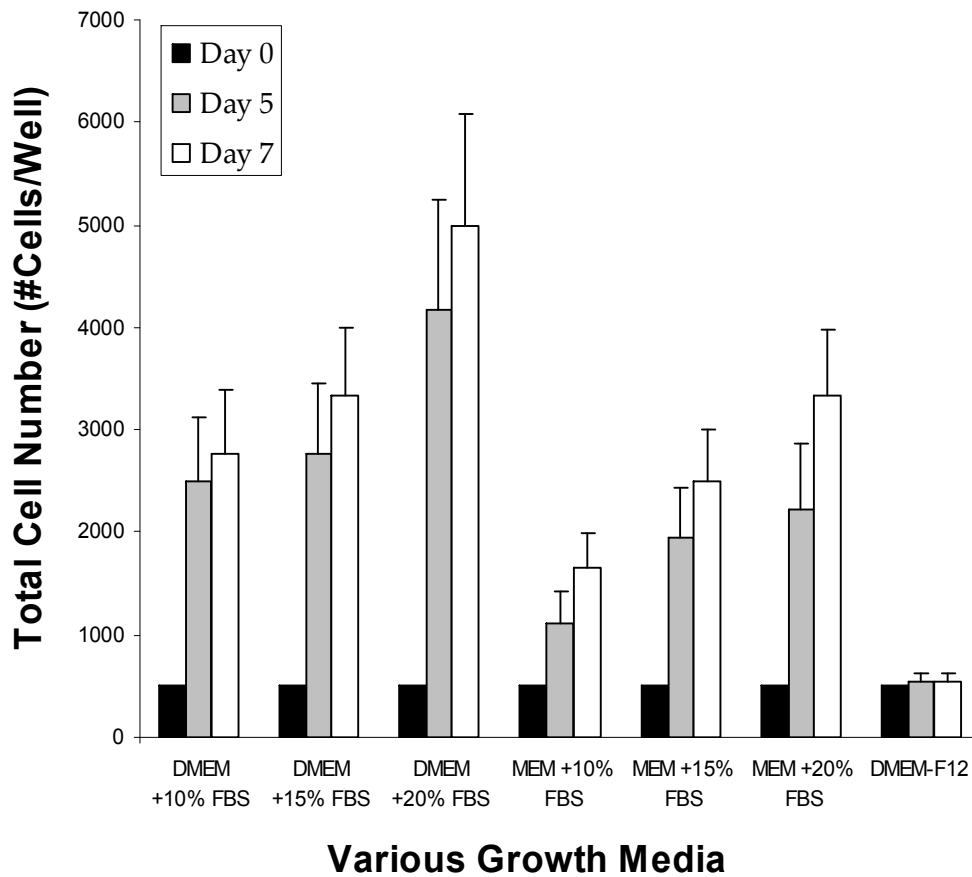


Fig 3.2 Growth rates of rat stem cells in various media

3.2 Effect of serum

The general approach to the culture of MSCs involves isolating the mononucleated cells containing MSCs and seeding these cells on tissue culture plates at a standard plating density in a medium containing fetal bovine serum (FBS). All current protocols for in vitro culture of hMSC include fetal bovine serum (FBS) as nutritional supplement [10,58].

The effect of various types of serum on the proliferation of cells was analyzed by culturing the cells in DMEM supplemented with 20%FBS, 20%BGS and a 20% mixture of the both in a 1:1 ratio. The cells were observed for a week and the growth curve was obtained. The cells were seeded with a density of 500 cells/well. The cells were quantified by counting under a microscope and a bar chart was plotted (Figures 3.3, 3.4).

Results show that FBS is a relatively better choice of serum over BGS. However the risk of transmission of prion diseases and zoonoses from the use of FBS is considered to be small. A greater risk associated with the use of hMSC expanded in FBS seems to be the immunogenicity of the xenogeneic FBS proteins [62,63]. This risk is reduced by using heat inactivated serum. All the above experiments were conducted in heat inactivated fetal bovine serum. BGS has been found to contain lesser bovine serum proteins than FBS [60]. The growth of the stem cells in BGS did not differ significantly from that of the cells in FBS and therefore it can be used as a serum supplement. However BGS caused change in morphology by Day 5 in culture, leading to possible differentiation.

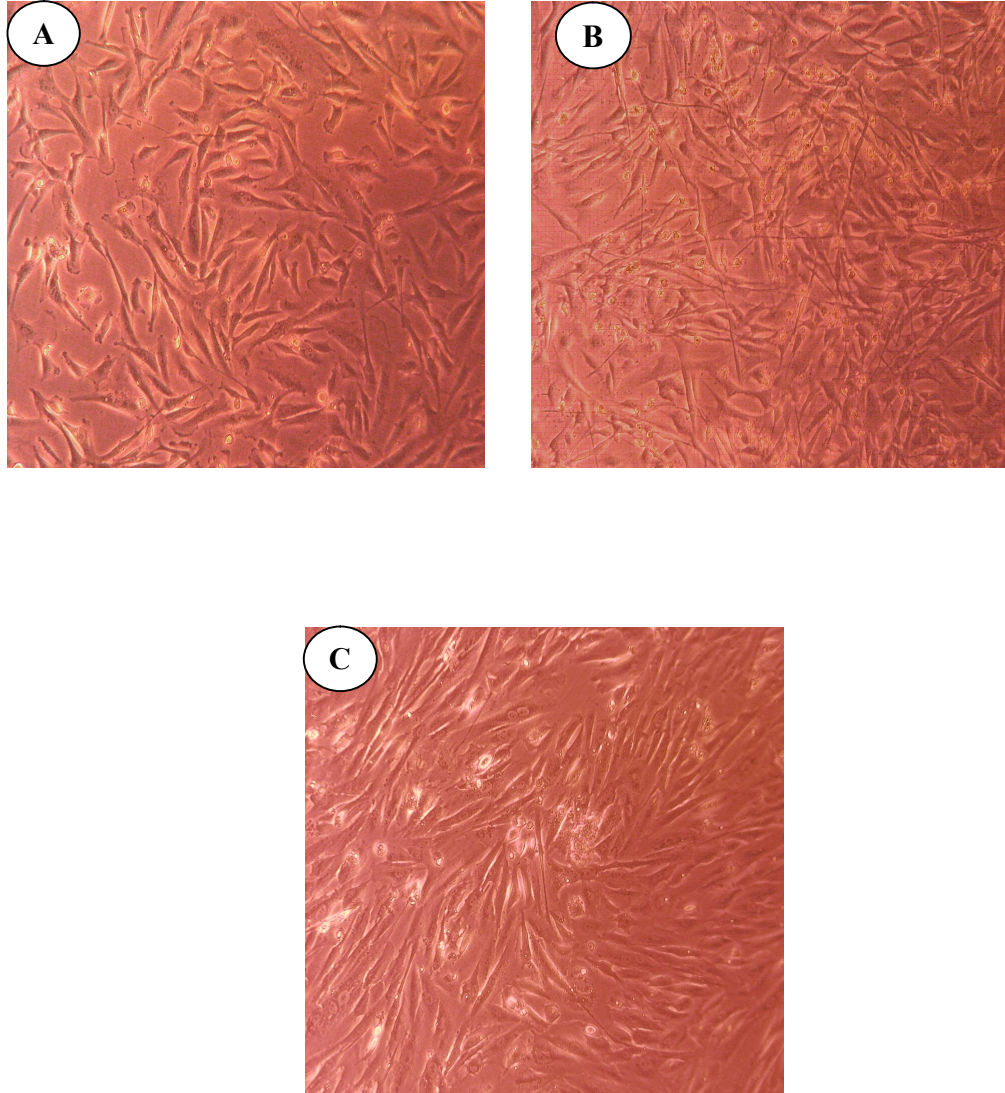


Fig 3.3 Morphology and confluency of Rat stem cells in various serum on the 5th day of culture in DMEM with A. 20%FBS; B. 20%BGS; C. 10%FBS+10%BGS.

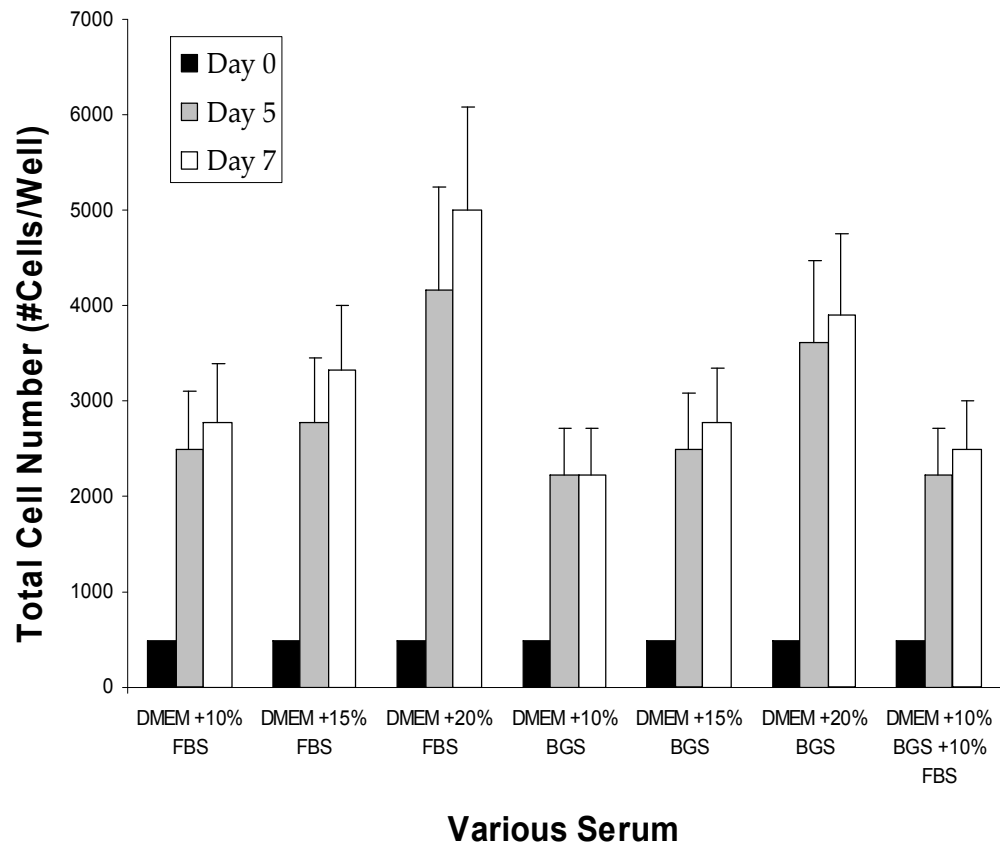


Fig 3.4 Growth rates of rat stem cells in various serum containing media

3.3 Effect of growth factors

Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types; while others are specific to a particular cell-type.

3.3.1 Effect of EGF on cell proliferation

The rat stem cells were seeded in a well plate with a cell density of 3000 cells/well. The media used was DMEM and 20% FBS. To this medium 10, 30 and 100 ng/ml of EGF was added and the growth pattern was observed for a period of two weeks. Cells were counted on the 7th and 14th day under the microscope and the growth pattern was obtained.

From the bar chart (Figure 3.6) it is proved that EGF promotes cell proliferation in culture. Higher concentration of EGF shows higher growth and proliferation in culture. There was no morphological difference between the cells cultured in the cytokine and the control sample (Figure 3.5).

It was seen that EGF induces a dose dependent increase in growth rate in bone marrow mesenchymal stem cells [11,12]. The results obtained prove that, this holds true even for peritoneal mesenchymal stem cells. However there was no significant difference between the 30ng/ml and 100ng/ml concentrations which can be explained by the saturation of the receptors available.

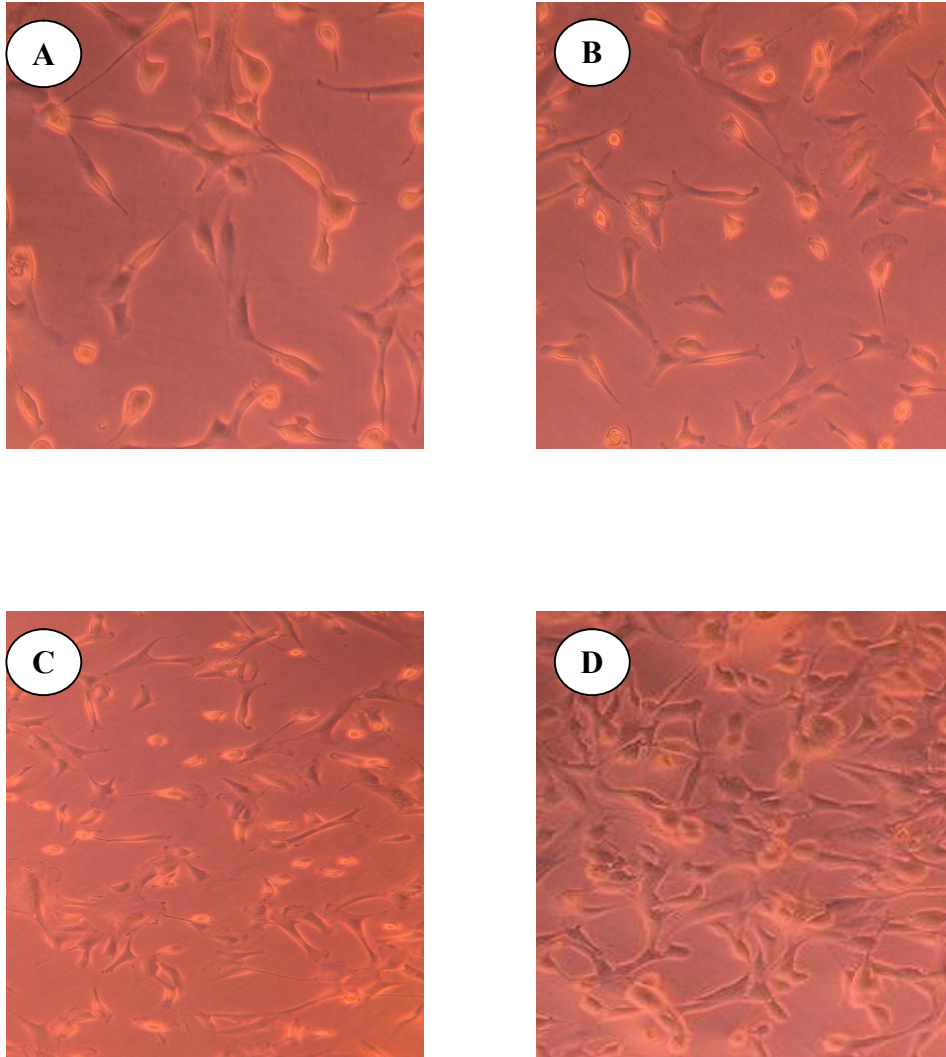


Fig 3.5 Morphology and confluency of Rat stem cells in a media containing various concentrations of EGF on the 7th day of culture. A. Control B. 10 ng/ml; C. 30ng/ml; D. 100ng/ml

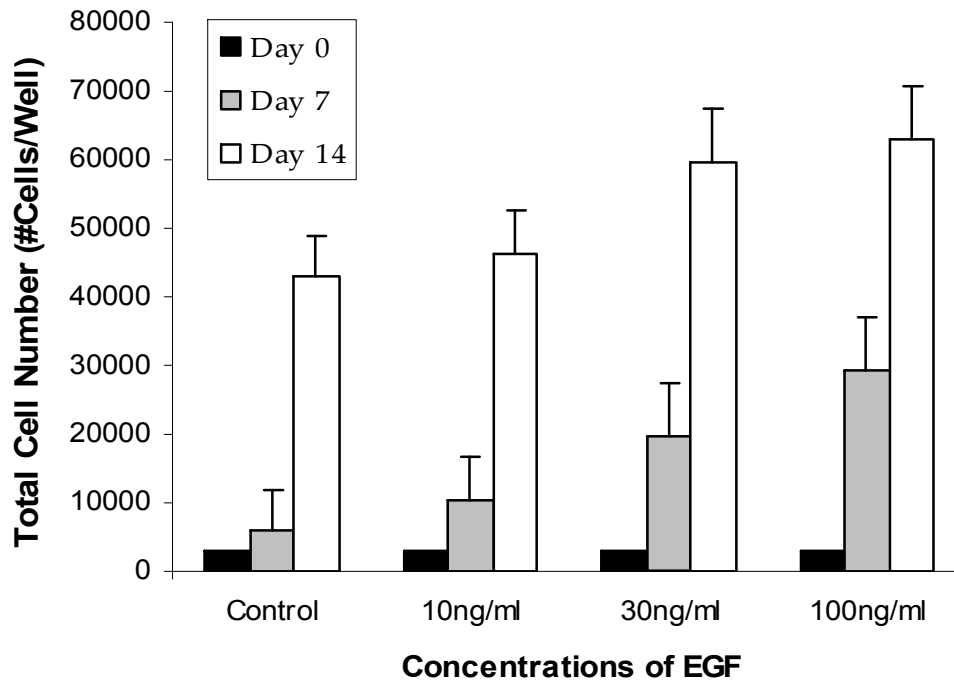


Fig 3.6 Growth rates of rat stem cells in a media containing various concentrations of EGF

3.3.2 Effect of bFGF on cell proliferation

The rat stem cells were seeded in a well plate with a cell density of 3000 cells/well. The media used was DMEM and 20% FBS. To this medium 2, 10 and 50 ng/ml bFGF was added and the growth pattern was observed for a period of two weeks. Cells were counted on the 7th and 14th day under the microscope and the growth pattern was obtained. In this experimental series the cell growth was maximum for the well containing 2 ng/ml of bFGF (Figures 3.7, 3.8). The well plates with increased concentration of bFGF had lesser MSCs because some of the cells underwent differentiation into the osteogenic lineage. This effect was not seen on replication. This is contradictory to the growth pattern observed in the bone marrow mesenchymal stem

cells [9,10], which showed a dose dependent increase in growth. This could be because certain FGFs have an inhibitory effect on cell growth [64].

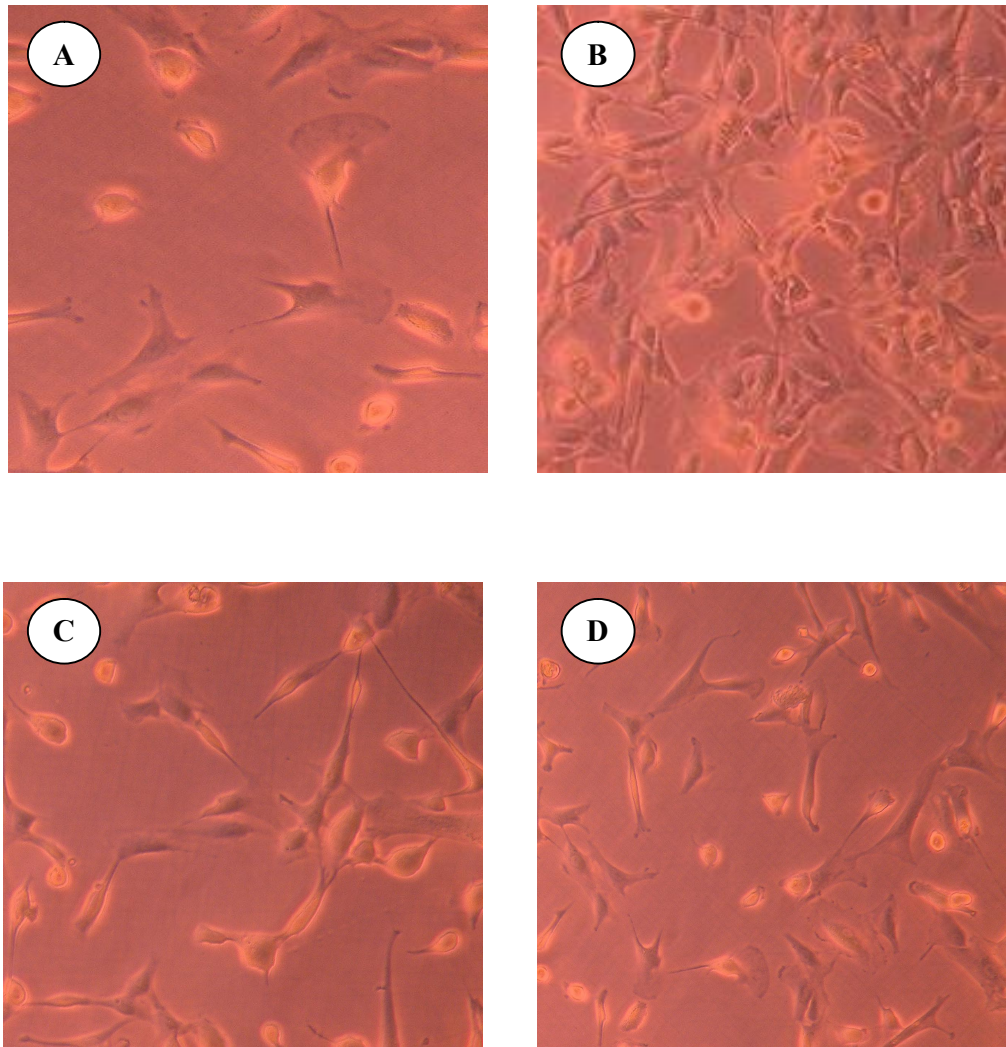


Fig 3.7 Morphology and confluency of Rat stem cells in a media containing various concentrations of bFGF on the 7th day of culture. A. Control; B. 2 ng/ml; C. 10ng/ml; D. 50ng/ml

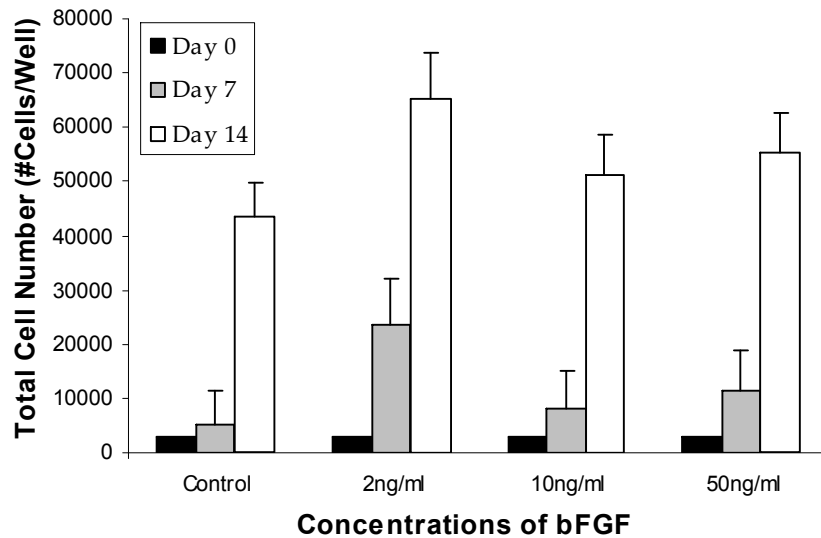


Fig 3.8 Growth rates of rat stem cells in a media bFGF

3.3.3 Effect of HB-EGF on cell proliferation

The rat stem cells were seeded in a well plate with a cell density of 3000 cells/well. The media used was DMEM and 20% FBS. To this medium 5, 25 and 50 ng/ml HB-EGF was added and the growth pattern was observed for a period of two weeks. Cells were counted on the 7th and 14th day under the microscope and the growth pattern was obtained.

HB-EGF permitted faster cell proliferation without any characteristic change in cell morphology (Figure 3.9). No significant difference in effect of 25 ng/ml and 50ng/ml of HB-EGF on the cells (Figures 3.9, 3.10). This proves that HB-EGF is involved in regulating proliferation [65].

The growth pattern observed in the peritoneal stem cells upon culture with HB-EGF evoked a similar response as to what was observed in the bone marrow mesenchymal stem cells [13,14]. There was a dose dependent increase in the growth rate which is a characteristic of the growth factors belonging to the EGF family [11,12].

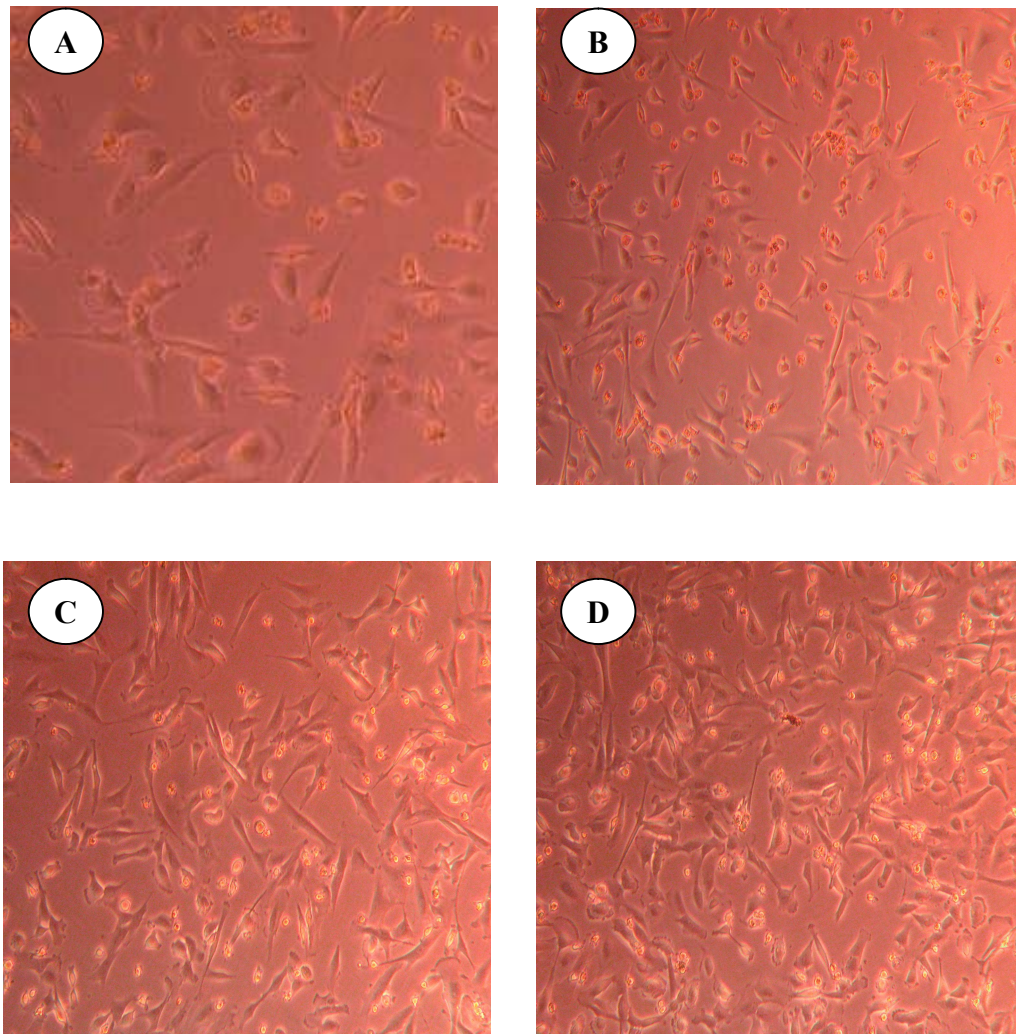


Fig 3.9 Morphology and confluency of Rat stem cells in a media containing various concentrations of HB-FGF on the 7th day of culture. A. Control; B. 5 ng/ml; C. 25ng/ml; D. 50ng/ml

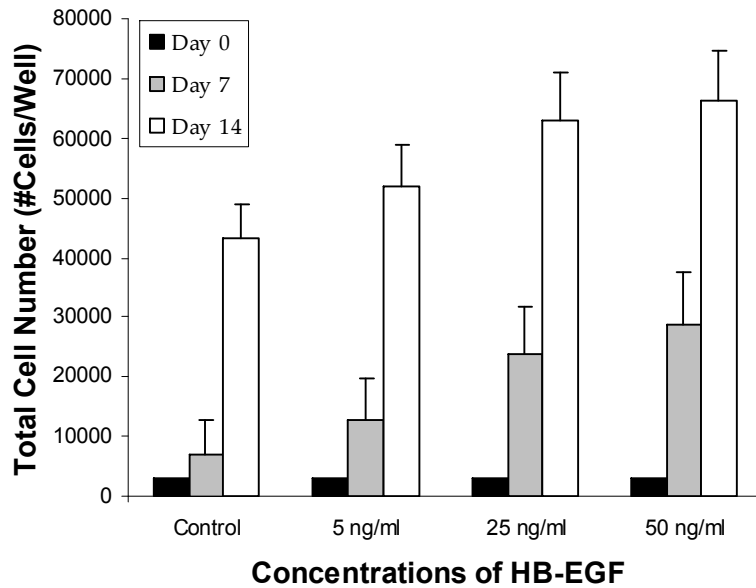


Fig 3.10 Growth rates of rat stem cells in a media containing various concentrations of HB-EGF

To compare the effect of these growth factors on the proliferation rate of the MSCs an experiment was designed using these growth factors as single entities as well as concoctions. Each well was seeded with a cell density of 500 cells/ml and the growth media was supplemented with 10ng/ml bFGF, 25ng/ml of HB-EGF, 25ng/ml HB-EGF +10ng/ml of bFGF, 25ng/ml EGF and 25ng/ml of EGF+10ng/ml of bFGF. The growth in these wells were compared to a control sample which had only DMEM and 20%FBS.

It is found that HB-EGF had a more positive effect on the proliferation of cells than any of the other growth factors. However HB-EGF does not allow the MSCs to differentiate into specific lineages. At the selected optimum seeding density, HBEGF

stimulates the highest cell growth when compared with EGF & bFGF and in combination with bFGF.

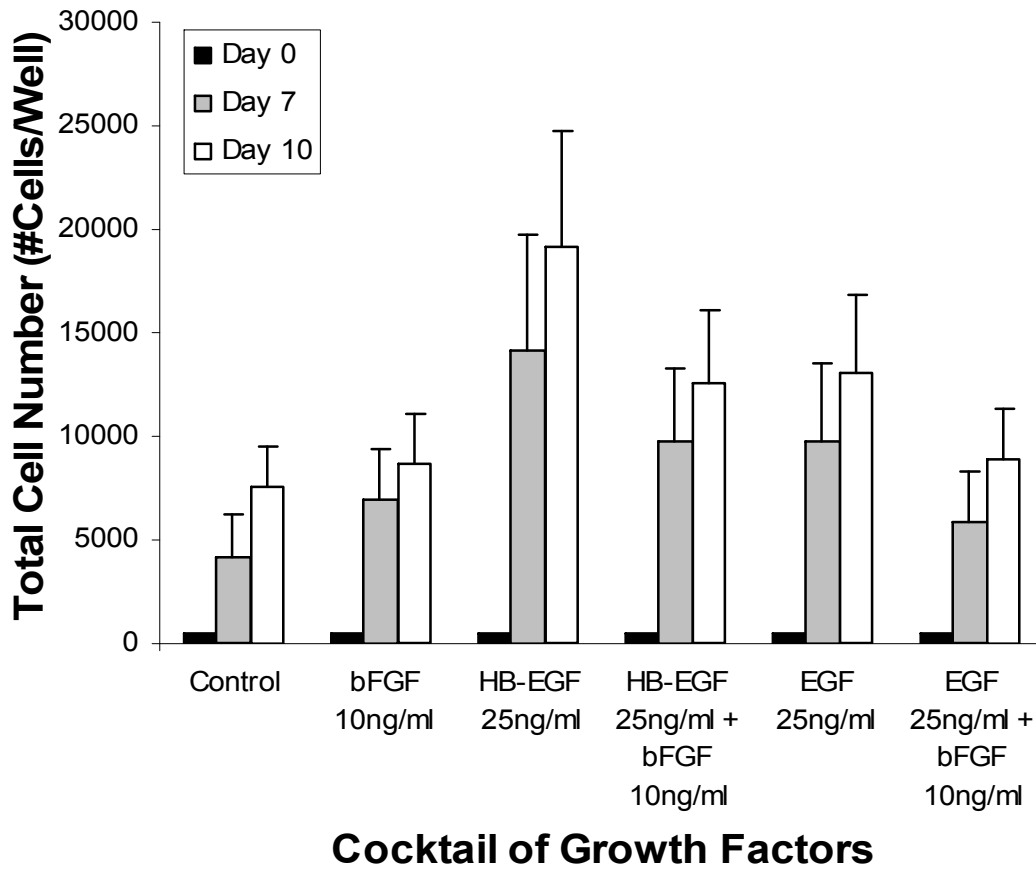


Fig 3.11 Growth rates of rat stem cells in a media containing cocktail of growth factors

3.4 Effect of Trypsin

The cells were seeded on a well plate with density of 500 cells/well. The growth media was DMEM+20%FBS. The cells were subcultured and counted on the 7th and the 10th day. The subculture was done using a cell scraper and this growth was compared with the cells that were subcultured with Trypsin and a growth analysis was conducted.

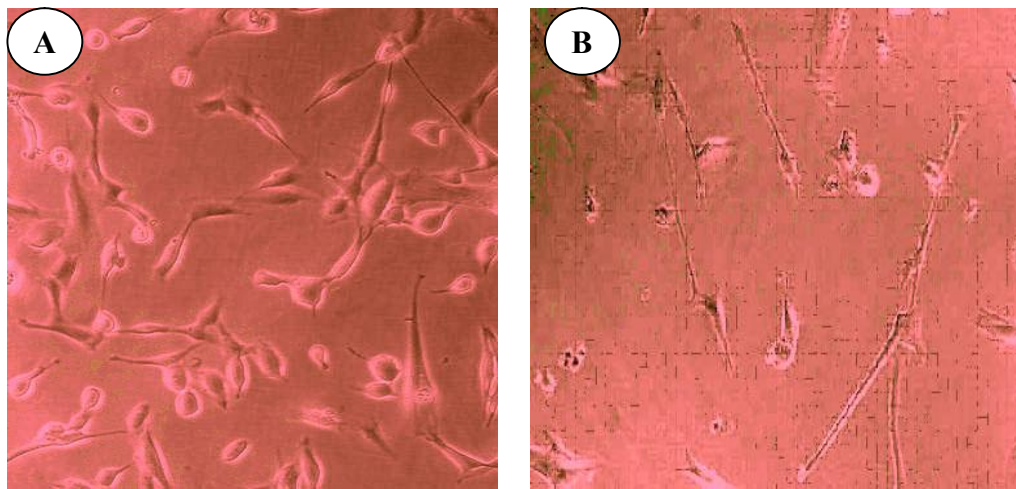


Fig 3.12 Effect of Trypsin on morphology and confluency of Rat stem cells on the 7th day of culture A. Cell scraper; B. Trypsin.

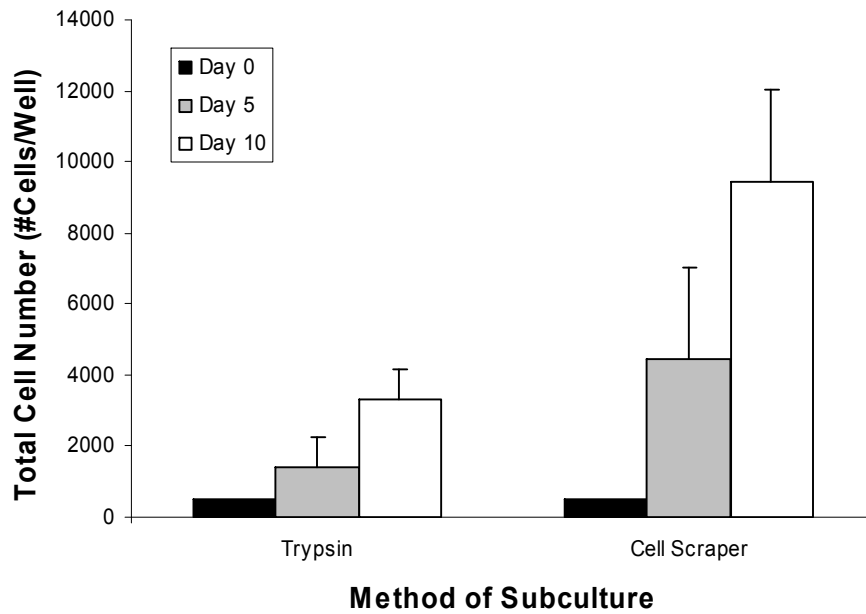


Fig 3.13 Growth rates of rat stem cells subcultured with various methods.

It was observed that the cells that were subcultured with a cell scraper grew faster than the ones subcultured with Trypsin (Figures 3.12, 3.13). This is attributed to the fact that Trypsin affects the adherence capacity of the cells.

CHAPTER 4

OPTIMIZATION OF FREEZING CONDITIONS TO INCREASE THE VIABILITY OF CRYOPRESERVED CELLS

4.1 Cell Lines

4.1.1 Effect of freeze media

The amount of DMSO plays a very critical role in maintaining the cells in the sub-zero temperatures. The freeze media is generally composed of DMEM, 20% calf serum and 5%-20% DMSO. DMSO in the medium has protective effects but it also increases the pH. This increase will further reduce the cell viability if the pH exceeds 8.0. This pH is balanced by the calf serum. In this experiment the freeze media containing 5%,10%,15% and 20% of DMSO in complete media are tested. The cells in the freeze media are aliquoted into cryovials with a cell density of 5 million cells/1 ml and then frozen using step by step process wherein the vials are frozen at 4⁰C for 30 minutes, at-20⁰C for 30 minutes and at -70⁰C for 24 hours before transferring into the liquid Nitrogen storage tank. The experimental design is given below.

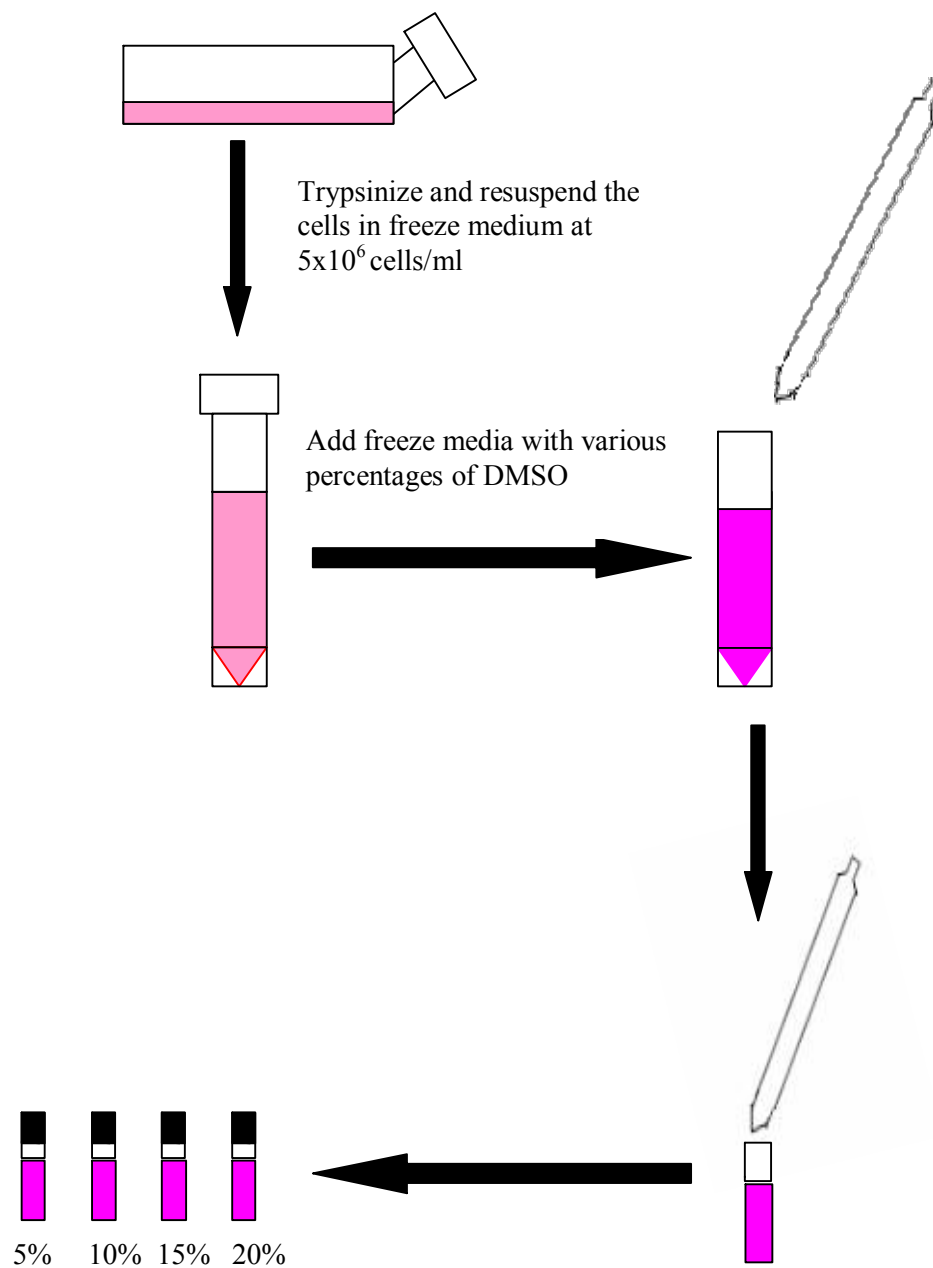


Fig 4.1 Freezing the cells: In varying percentages of DMSO and using the step-by-step freezing process.

Table 4.1 Composition of freeze media

Percentage of DMSO	Percentage of Complete Media
5%	95%
10%	90%
15%	85%
20%	80%

Table 4.2 Viability of B16F1 cells cryopreserved with varying percentages of DMSO

Percentage of DMSO in freeze media	Percentage viability post-thaw
5%	88.93% ± 0.59
10%	85.73% ± 1.08
15%	74.03% ± 1.38
20%	59.58% ± 2.65

Table 4.3 Viability of 3T3 fibroblast cryopreserved with varying percentages of DMSO

Percentage of DMSO in freeze media	Percentage viability post-thaw
5%	84.11% ± 5.51
10%	86.29% ± 4.59
15%	76.34% ± 2.20
20%	48.98% ± 9.31

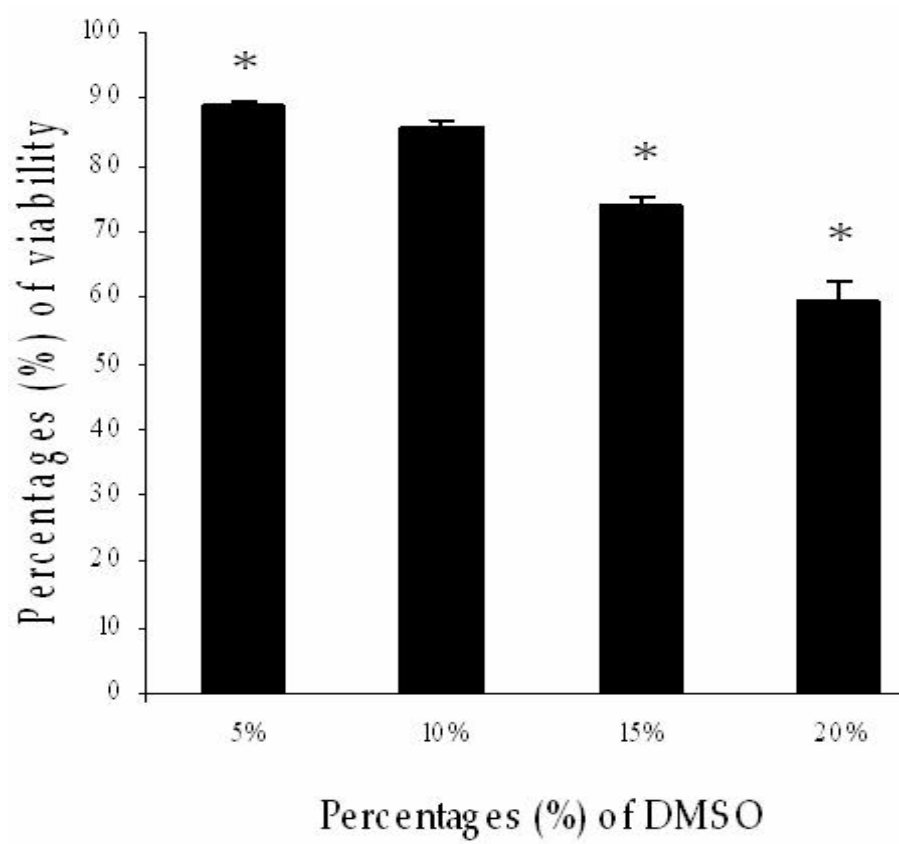


Fig 4.2 Viability rates for B16F1 melanoma cells cryopreserved with varying percentages of DMSO and using the step-by-step freezing process. (n=2)

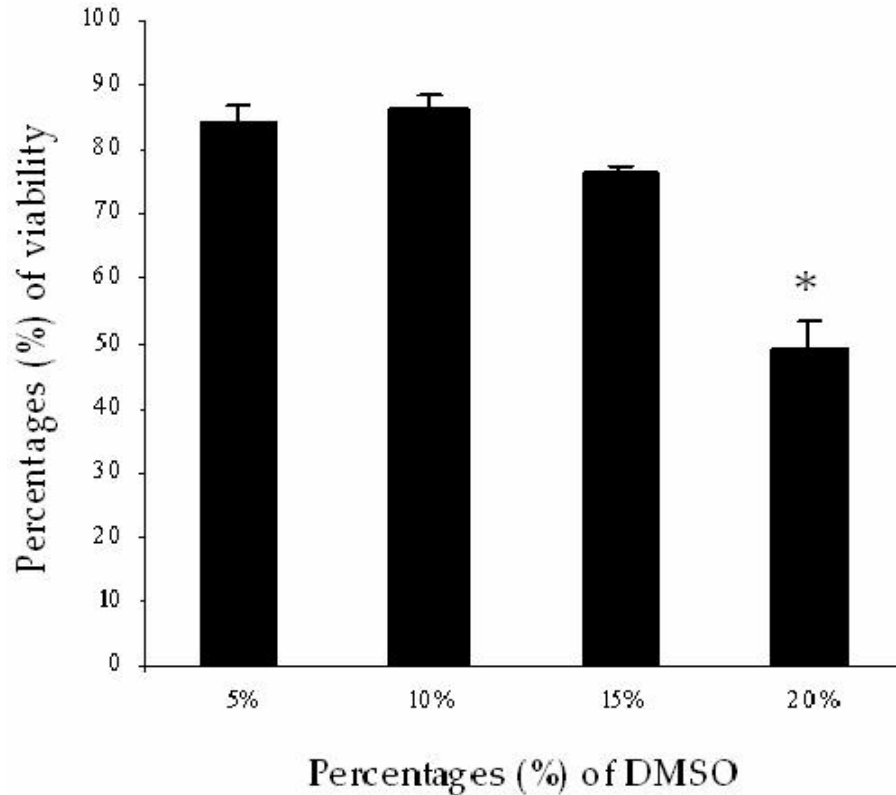


Fig 4.3 Viability rates for 3T3 fibroblast cryopreserved with varying percentages of DMSO and using the step-by-step freezing process. (n=2)

The effect of the various percentages of DMSO in the freeze media on the viability of the B16F1 melanoma cells are shown in the Figure (4.2) . Two vials of each sample are analyzed. A students t-test is conducted on the results obtained to understand the significance of the effect of composition. The control in the above experiment is the 10% DMSO. It is found that the viability of cells cryopreserved using 5% DMSO is significantly ($p=0.002$) higher than that obtained using 10% by a value of 3.2 %. Both 15% and 20% showed significantly ($p=0.0001$ each) lower recover rates than the 10%.

The effect of the various percentages of DMSO in the freeze media on the viability of the 3T3 fibroblast are shown in the (Figure 4.3). Two samples for each percentage are analyzed. A student's t-test is conducted on the results obtained to understand the significance of the effect of composition of freeze media on the post thaw viability of 3T3 fibroblasts. The control in this experiment is the 10% DMSO. It is found that the viability of cells cryopreserved using 5% and 15% of DMSO is lower than that obtained using 10% by a value of 2.8 % and 9.95% respectively. But these values did not hold significance ($p=0.71$ for 5% and 0.06% for 15%). 20% showed significantly ($p=0.04$) lower recover rate than the 10%.

4.1.2 Effect of cell density

The previous study using varying percentages of cryoprotectant has shown that the viability is affected by the composition of the freeze medium. The cell density that was used for the experiment was 5 million cells/ml in each vial. The results obtained indicate that the viability is comparable for both 5% and 10%.

Based on these observations the following experiment was designed to test if the viability is affected by the cell density. The capacity of a penetrating cryoprotectant to prevent any cryoinjury depends on the capacity and amount of cryoprotectant inside the cell. One factor that determines this penetrating capacity is cell density or the number of cells in 1ml of the freeze media. The optimum cell density that should be cryopreserved in each vial may vary according to the cell type.

For this experiment three different cell densities are analyzed. The cells are trypsinized and removed from the flask in the same procedure as described before and

they are resuspended in 1 ml of freeze media containing 10% DMSO and 90% complete media at three different cell densities of 5 million, 1 million and 0.2 million. The experimental design is given in figure 4.4.

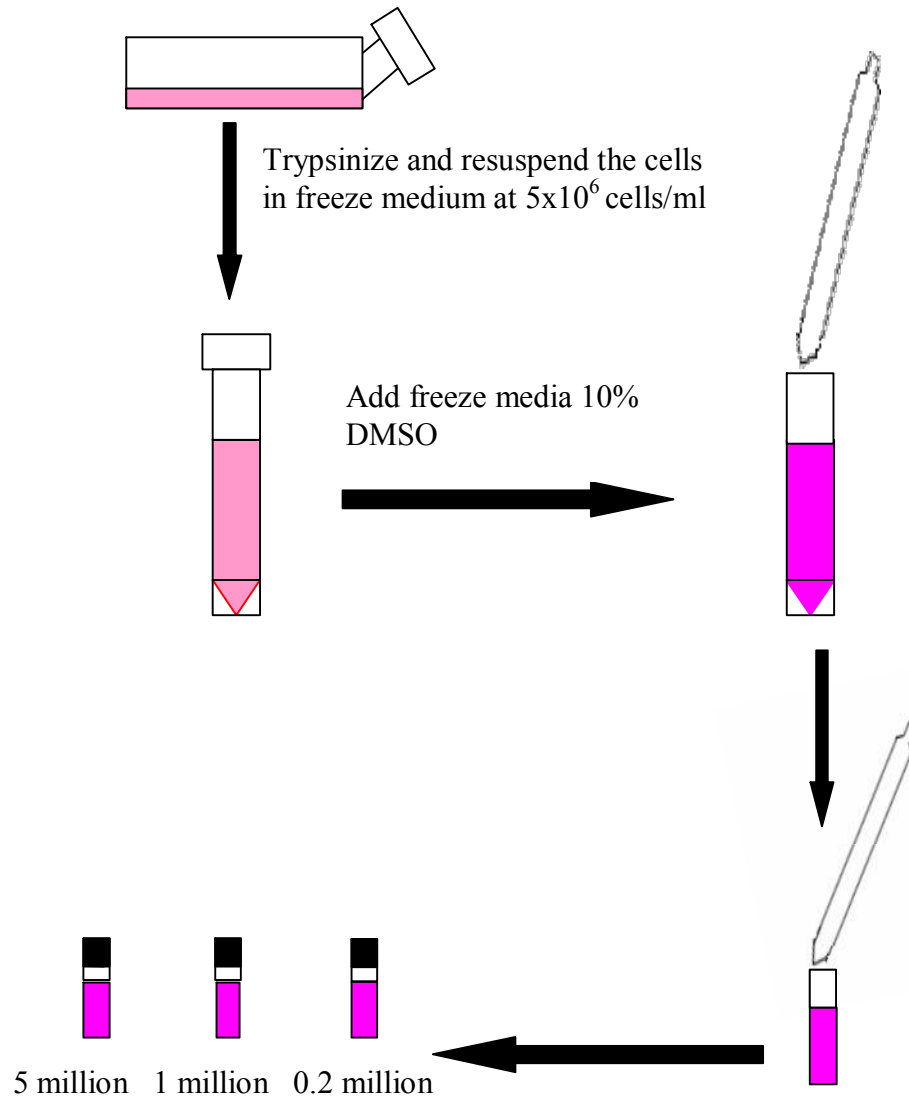


Fig 4.4 Freezing the cells: In varying cell densities using 10%DMSO and using the step-by-step freezing procedure.

Table 4.4 Viability of B16F1 melanoma cells cryopreserved with various cell densities

Various cell densities	Percentage viability post-thaw
5 Million	86.93% ± 3.28
1 Million	85.01% ± 3.98
0.2 Million	86.42% ± 5.27

Table 4.5 Viability of 3T3 fibroblast cryopreserved with various cell densities

Various cell densities	Percentage viability post-thaw
5 Million	90.51% ± 0.21
1 Million	80.74% ± 10.78
0.2 Million	77.95% ± 8.21

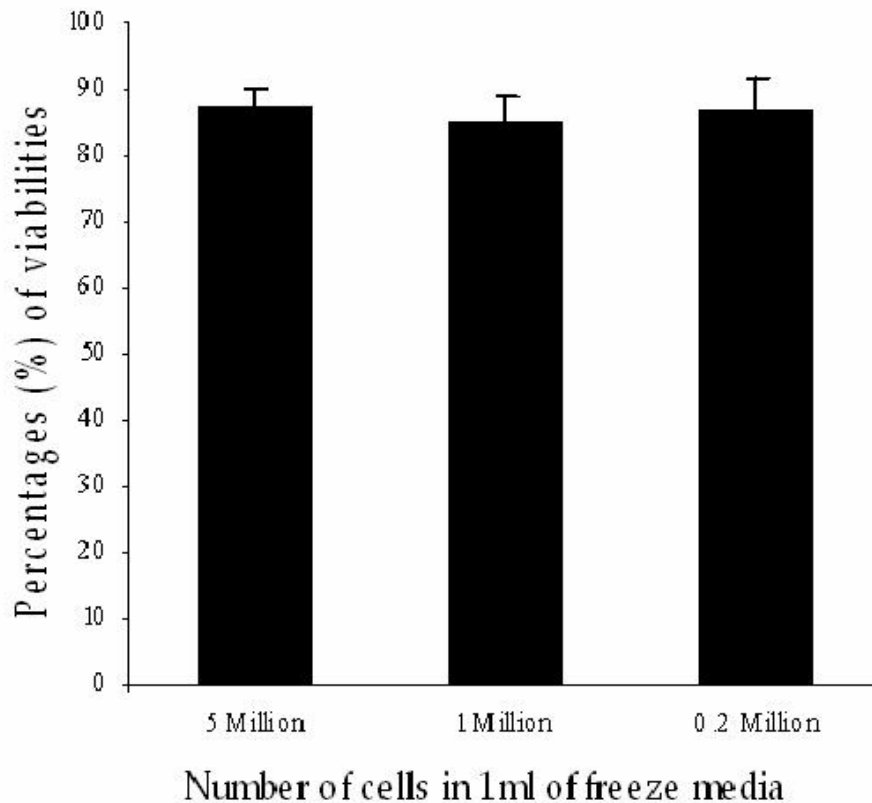


Fig 4.5 Viability rates for B16F1 melanoma cells cryopreserved with varying cell density in 10% of DMSO and using the step by step freezing process. (n=2)

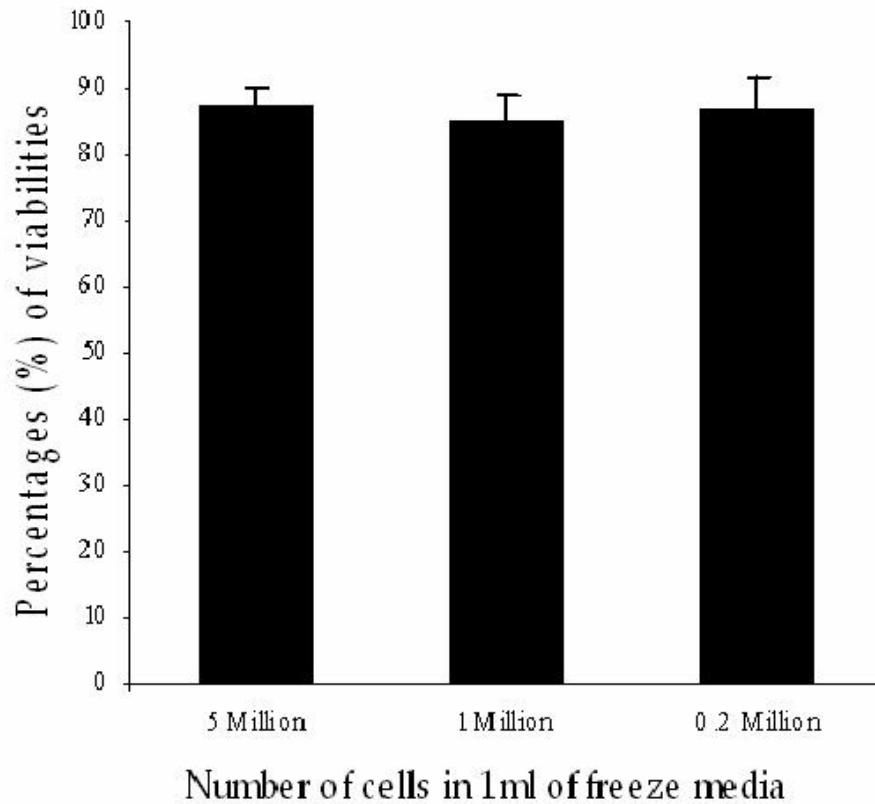


Fig 4.6 Viability rates for 3T3 fibroblast cryopreserved with varying cell density in 10% of DMSO and using the step-by-step freezing process. (n=2)

The effect of the various cell densities while cryopreserving B16F1 melanoma cells is shown in Table 4.5. Two samples for each cell densities are analyzed. A students t-test is conducted on the results obtained to understand the significance (Figure 4.4). The control in this experiment is 5 million cells/ml. It is found that the viability of cells remain the same irrespective of the cell density.

The effect of the various cell densities while cryopreserving 3T3 fibroblast are shown in the Table 4.5. Two samples for each cell densities are analyzed. A students t-test is conducted on the results obtained to understand the significance (Figure 4.6). The

control in this experiment is the 5 million cells/ml. It is found that the difference in viability of cells is insignificant ($p=0.32$ for 1 million and $p=0.16$ for 0.2 million).

4.1.3 Effect of cryopreservation process

The method of freezing employed while cryopreserving plays a very important role in viability of the cell upon recovery. The rate of cooling is essentially a way of controlling water loss from the cells embedded in an ice-hypertonic solution matrix, and has been shown to vary widely in successful cryopreservation procedures [53]. The commonly used freezing techniques involve cooling the vials at the rate of 0.1°C to $10^{\circ}\text{C}/\text{min}$. Most cultured cells survive best if they are cooled at $1^{\circ}\text{C}/\text{min}$. Programmable freezers can also be used, but they are relatively expensive.

Previous studies have shown that the cell viability is affected by the composition of freeze medium and the cell density in the cryovial. These studies were conducted using a regular step-by-step freezing manual method (Figure 4.7). However the following tests were designed to evaluate if this manual freezing method is comparable to standard industrial methods like Nalgene cooler box (Figure 4.8) or a semi-programmable freezer, CELLerator (Figure 4.9).

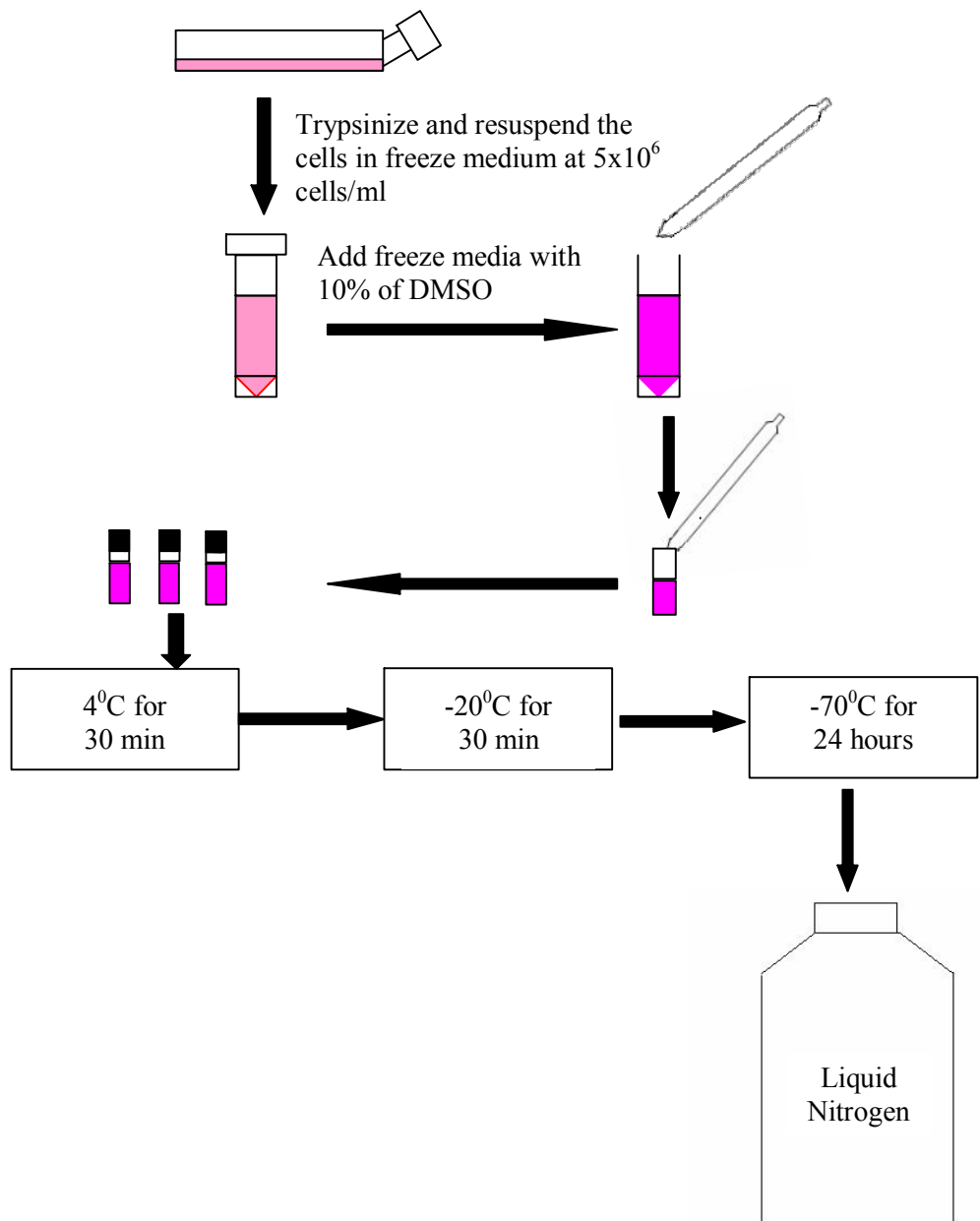
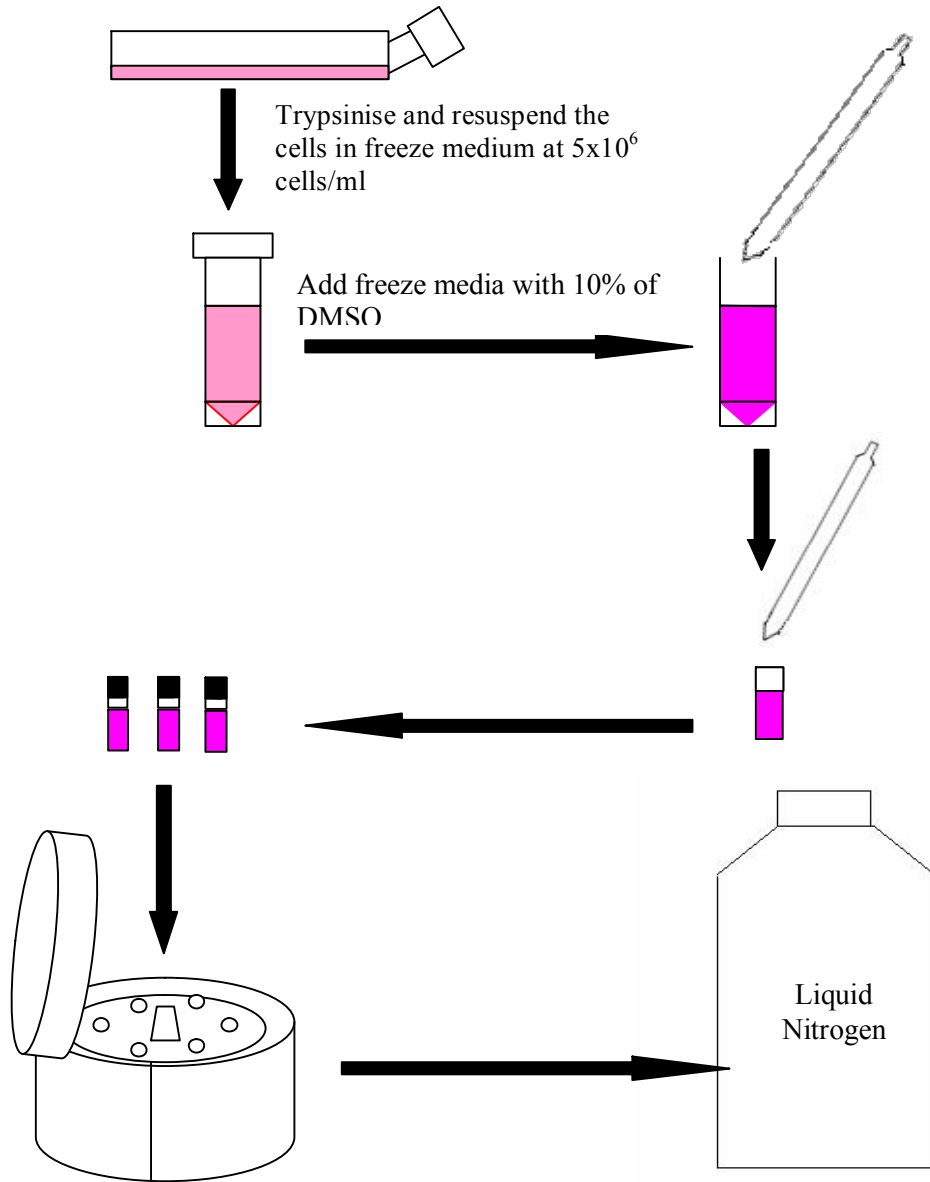


Fig 4.7 Freezing the cells: In 10% DMSO with 5 million cells/vial using the step-by-step freezing procedure



Vials in Nalgene 'Mr. Frosty' for 24 hours

Fig 4.8 Freezing the cells: In 10% DMSO with 5 million cells/vial using Nalgene 'Mr. Frosty' cooler.

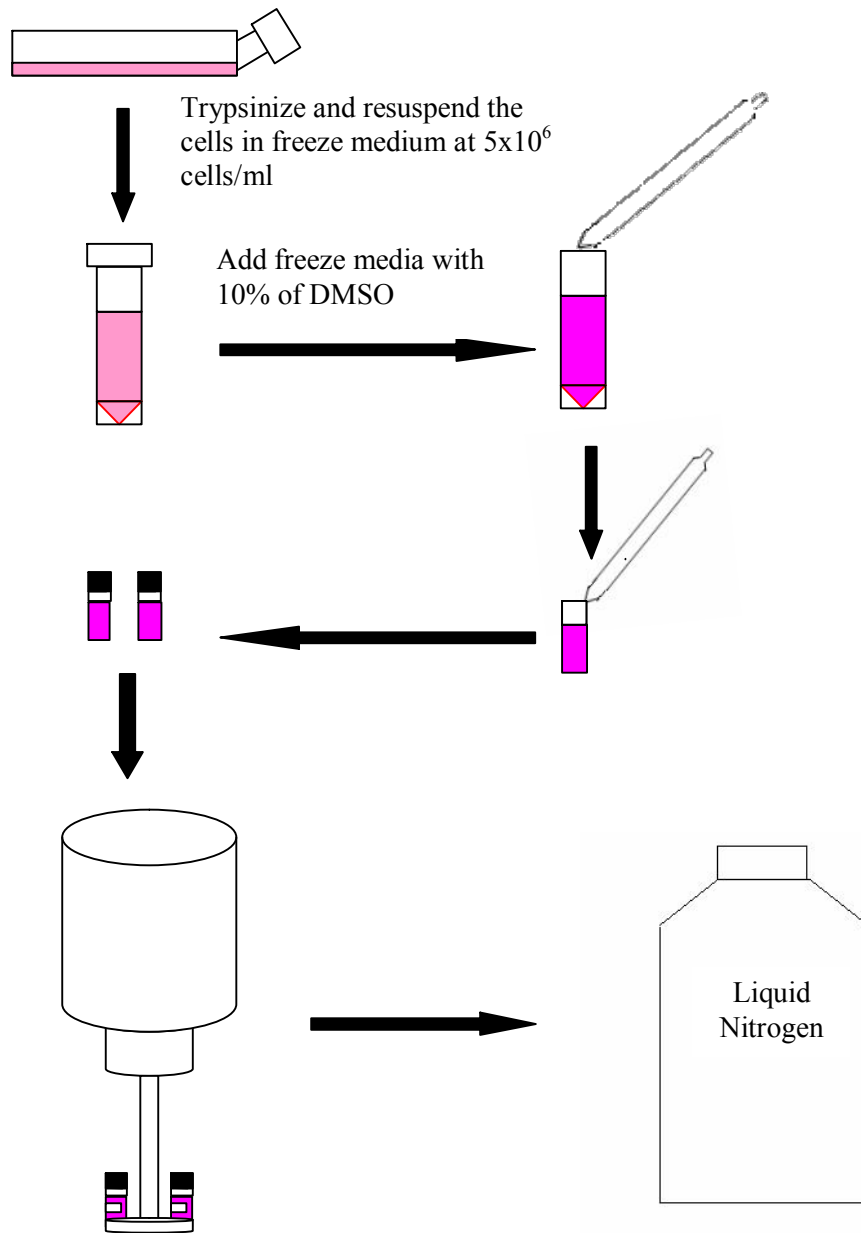


Fig 4.9 Freezing the cells: In 10% DMSO with 5 million cells/vial using the CELlevator

Table 4.6 Viability of B16F1 melanoma cells cryopreserved with various methods

Various methods	Percentage viability post-thaw
Step-by-step freezing	87.45 % ± 8.09
Nalgene	68.37 % ± 1.07
CELLevator	74.41 % ± 0.84

Table 4.7 Viability of 3T3 fibroblast cryopreserved with various methods

Various methods	Percentage viability post-thaw
Step-by-step freezing	91.16 % ± 2.91
Nalgene	89.97 % ± 0.09
CELLevator	92.40 % ± 1.38

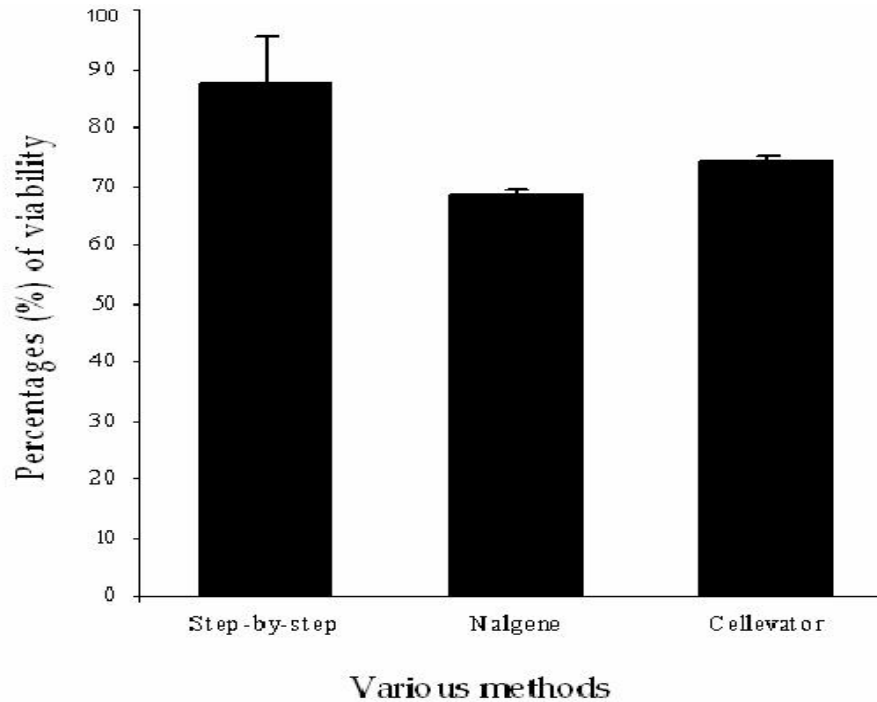


Fig 4.10 Viability rates for B16F1 melanoma cells cryopreserved with various methods in 10% DMSO at 5 million cells/ml. (n=2)

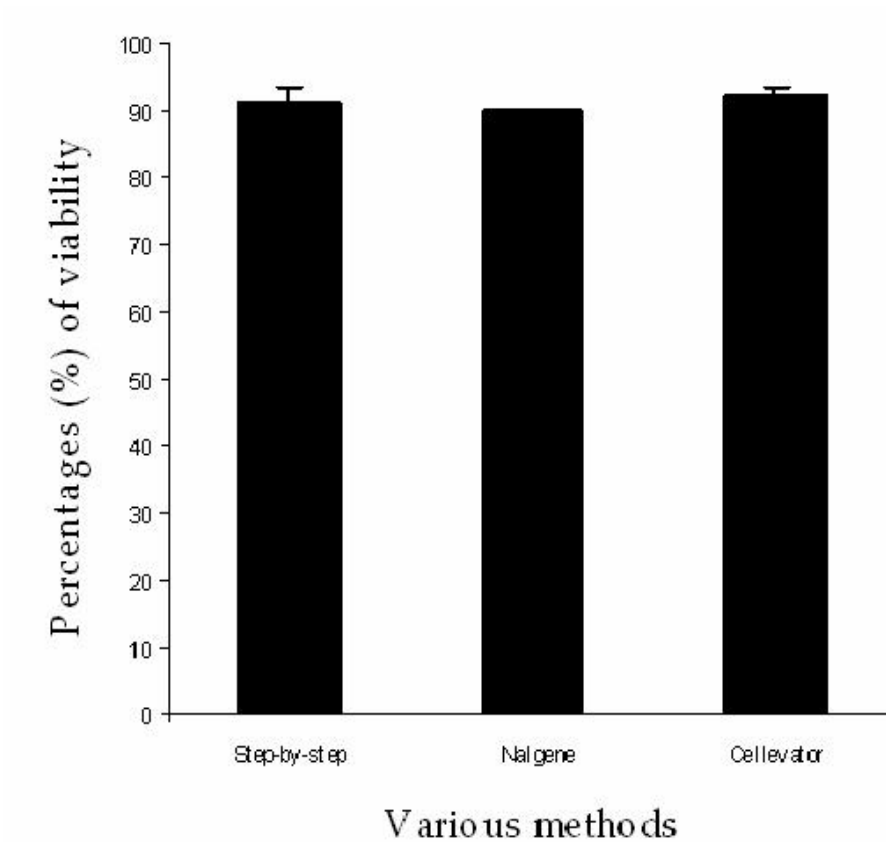


Fig 4.11 Viability rates for 3T3 fibroblast cryopreserved with various methods in 10% DMSO at 5 million cells/ml. (n=2)

The effect of the various methods of cryopreserving B16F1 melanoma cells are shown in Table 4.6. Two samples for each method are analyzed. A students t-test is conducted on the results obtained to understand the significance (Figure 4.10). The control in this experiment is the step-by-step method. It is found that the step-by-step method has better viability than the other two but the difference in viability of cells is insignificant ($p=0.08$ for Nalgene and $p=0.15$ for CELlevator).

The effect of the various methods of cryopreserving 3T3 fibroblast is shown in Table 4.7. Two samples for each method are analyzed. Students t-test is conducted on

the results obtained to understand the significance (Figure 4.11). The control in this experiment is the step-by-step method. It is found that cells cryopreserved using CELLevator has a better recovery rate than the control though not significant ($p=0.13$) while cells cryopreserved using Nalgene had lesser viability than the control, but even here it is insignificant ($p=0.6$).

4.2 Rat peritoneal stem cell

4.2.1 Effect of freeze media

From the results obtained from the cell lines, the experiments were designed for stem cells derived from the peritoneum of rats. The rat stem cells were cryopreserved with a cell density of 0.2 million cells in each cryovial in 1ml of freeze medium containing various percentages of DMSO.

Table 4.8 Viability of Rat stem cells cryopreserved with varying percentages of DMSO

Percentage of DMSO in freeze media	Percentage viability post-thaw
5%	$86.57\% \pm 2.89$
10%	$82.83\% \pm 16.69$
15%	$76.56\% \pm 18.28$
20%	$54.76\% \pm 4.12$

The effect of the various percentages of DMSO in the freeze media on the viability on Rat stem cells are shown in Table 4.8. Three samples for each percentage are analyzed. Students t-test is conducted on the results obtained to understand the significance. The control in this experiment is the 10% DMSO. It is found that the viability of cells cryopreserved using 5% of DMSO is higher than that obtained using

10% by a value of 3.74 % but is not very significant ($p=0.7$). Viabilities at 15% and 20% were lower than that at 10% by values of 22.27% and 28.07% (Figure 4.12). The former did not hold any significance ($p=0.19$) while the latter is significant ($p=0.047$).

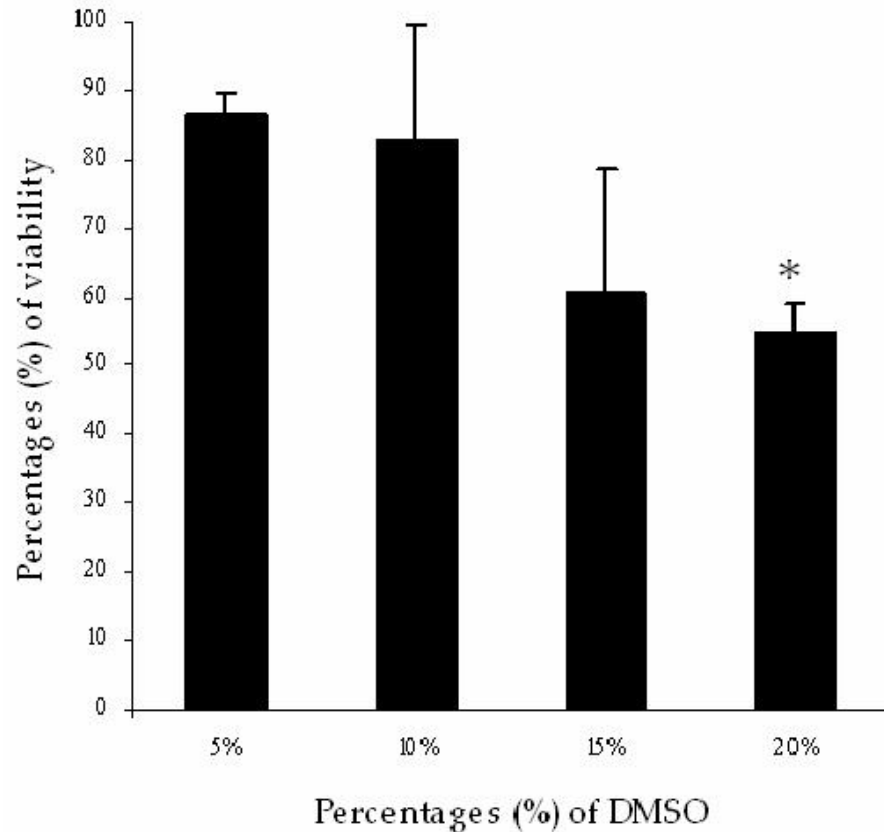


Fig 4.12 Viability rates for Rat stem cells cryopreserved with varying percentages of DMSO and using the step by step freezing process. (n=3)

4.2.2 Effect of cell density

The various cell densities were not tested because the tests in the cell lines revealed that there was no effect of cell densities on the viability.

4.2.3 Effect of cryopreservation process

The various methods are tested as described in the experiments for cell lines. A cell density of 0.2 million cells in each cryovial in 1ml of freeze medium containing 10% DMSO.

Table 4.9 Viability of Rat stem cells cryopreserved with various methods

Various methods	Percentage viability post-thaw
Step-by-step freezing	84.59 % \pm 2.59
Nalgene	68.37 % \pm 8.67
CELLerator	74.41 % \pm 4.81

The effect of the various methods of cryopreserving Rat stem cells are shown in the table above. Three samples for each method are analyzed. Students t-test is conducted on the results obtained to understand the significance (Figure 4.13). The control in this experiment is the step-by-step method. It is found that the difference in viability of cells is insignificant ($p=0.066$ for Nalgene and $p=0.097$ for CELLerator).

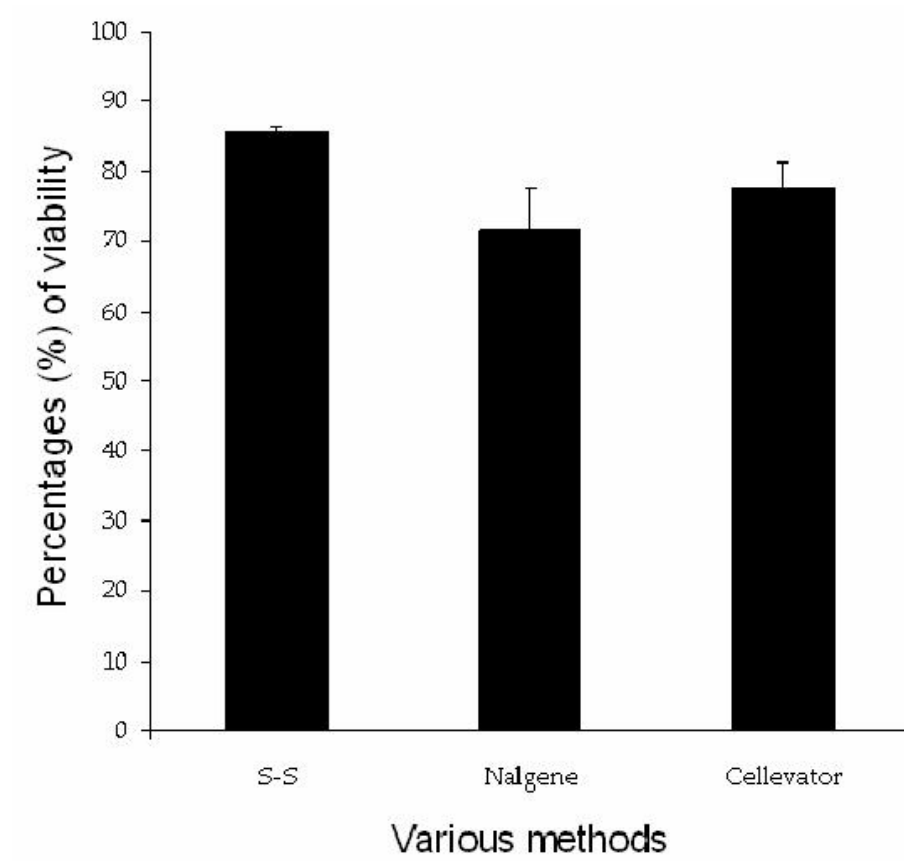


Fig 4.13 Viability rates for Rat stem cells cryopreserved with various methods in 10% DMSO at 5 million cells/ml. (n=3)

CHAPTER 5

CONCLUSION

Optimum Culture Conditions

The optimum culture media for the rat stem cells is DMEM supplemented with 20%FBS.The culturing of the MSCs with a media supplemented with HB-EGF increases the proliferation of the cells in vitro.Cells subcultured with a cell scraper tend to grow better and longer than the ones subcultured using Trypsin.

Optimum Cryopreservation Conditions

The optimum percentage of DMSO for effective cryopreservation is 5% in a Freeze media containing 80%DMEM and 20% The viability of the cells was found to be independent of the cell density in the cryopreservation media. This hold true for both the cell lines and the rat stem cell culture.The viability of the cells was also found to be independent of the method of cryopreservation. The step-by-step procedure which involves cooling at two different temperatures (-4°C and -20°C) for 30 minutes each was found to be better than the semi-programmable freezer used.

By combining the optimized conditions for culturing and cryopreservation the potentials of MSCs can be fully utilized.

CHAPTER 6

FUTURE DIRECTIONS

The future of the regenerative medicine is in the field of stem cells. Umbilical cord blood progenitor cells are gaining huge importance given the ethical problems with embryonic stem cells. Further these cells have been demonstrated to possess significant advantages over bone marrow in terms of proliferative capacity and immunogenic reactivity. But the most important limitation is the low number of hematopoietic stem cells obtained [66]. Therefore, the use of UCB for allogenic transplantation in adults has been hindered by the concern that a single CB may not contain sufficient numbers of stem/progenitor cells to reconstitute heavier patients in a timely manner [67]. Ex vivo expansion of stem/progenitor cells may circumvent this problem [68]. Because cryopreservation remains the method of choice for long-term preservation of stem/progenitor cells, UCB cryopreservation has become an important issue in banking and transplantation [69]. Newer sources of stem cells are being discovered. The conditions obtained can be further optimized by analyzing other growth factors that promote cell proliferation.

The viability of the rat peritoneal stem cells can be further improved by incorporating a combination of penetrating and non-penetrating cryoprotectant as it has been shown that a cocktail of cryoprotectants are more effective than a single one. A combination of DMSO and Dextran is harmful to mature cells and cells with large size

such as granulocytes, but suitable for lymphocytes and monocytes that are smaller in size. Therefore the size of the cell also plays an important role in determining the viability [70,71]. The damage to the cell could occur immediately before pre freezing or post thawing or post washing. The time duration for this wash also plays an important factor. So the analyses should include the viability steps at all the steps above [72,73,74]. Investigations into the time used for freezing using the step-by-step process can be used to further optimize the viability rate post recovery of the stem cells.

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Born of Dr.V.P.Balachandran and Dr.Jaya Balachandran, Harikrishnan Balachandran completed his Bachelor's in Electronics and Communication Engineering in the year 2003 at Maharaja Engineering College. He started his Master of Science in Biomedical Engineering at University of Texas at Arlington in Spring 2004. He started working in Dr. Liping Tang's lab in Summer 2005 wherein he began his work on his thesis 'Optimization of methods for in-vitro expansion and cryopreservation of mammalian cells'. The thesis was completed in Spring 2006.