AGEING OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS IN RELATION TO NUTRIENT STARVATION

MSc Thesis
2015

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

For the partial fulfillment of the requirement for degree in Master of Science in Biotechnology

Aarati Poudel
Exam Roll no: BT101-069
T.U. Regd. No.: 5-2-37-753-2008

AGEING OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS IN RELATION TO NUTRIENT STARVATION

M. Sc. Thesis
2015

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

<u>**Submitted by**</u>

Aarati Poudel

Roll No: BT 101/069

TU Regd no: 5-2-37-753-2008

Supervisor

Dr. Tilak R. Shrestha

Associate Professor

Central Department of Biotechnology

Tribhuvan University, Kathmandu, Nepal

Acknowledgement

I am deeply indebted to my Supervisor **Dr. Tilak R. Shrestha**, Central Department of Biotechnology, Tribhuwan University for facilitating and taking major initiating step for me to work at CCMB Hyderabad, his regular guidance, time to time critical evaluation of my work and impetus. The advent of this study and idea came from his preconception of research plan to produce a trained human resource in stem cell biology from CCMB and contribute for future research plan at CDBT, TU. I am feeling honored for the opportunity to work under the guidance of Dr. **Shashi Singh**, Senior principal scientist, CCMB. I am extremely grateful to her for providing me this golden opportunity of working with her and for valuable guidance, timely suggestion, words of encouragement, constructive remarks and dynamic supervision throughout this study.

My sincere admiration goes to Head of the Department Dr. Rajani Malla for providing every kind of facilities to undertake present study. I would like to thank Prof. Dr. Tribikram Bhattarai, Prof. Dr. Krishna Das Manandhar, Prof. Dr. Ganga Kharel, Mr. Bal Hari Poudel, Mrs. Jarina Joshi and all teaching and non teaching staffs of Central Department of Biotechnology for their support and encouragement.

My heartly thanks go to Mrs. Udayachandrika for providing guidance and valuable time while doing real time PCR. I am grateful to Ms. Snehal Raut for her constant support during FACS. I would also like to thank Rohit Rekulapelly and Sai Santhosh for their constant support, encouragement and help in clearing my doubts whenever asked. I express my sincere love and thanks to Radha aunty for her love and affection towards me. I also thank Rajesh Anna for providing me a helping hand always, at every step of my project.

I am immensely indebted to my friends Gomati, Santosh, Rajindra, Mitesh and all classmates for their constant encouragement. I would also like to thank Ujjwal Pradhan for technical and paper works. At last but not least, I wish to thank my husband, parents, brother, sister and relatives for their infinite encouragement, support and unwavering trust without which there is nothing in my ability to succeed.

ABBREVIATIONS

µL	Microlitre
AT	Adipose Tissue
BM	Bone Marrow
bp	Base Pair
BSA	Bovine Serum Albumin
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CFU	Colony Forming Units
DAPI	4', 6-diamidino-2-phenylindole
DCFDA	2’7’- dichlorofluorescein diacetate
DMF	N,N Dimethylformamide
DMSO	Dimethyl Sulhpoxide
DNA	Deoxy-Ribonucleic Acid
E2F	Elongation Factor-2
EDTA	Ethylenediaminetetraacetate
FACS	Fluorescence Assisted Cell Sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GAG	Glycosaminoglycan
GvHD	Graft Versus Host Disease
HRAS	Harvey Rat Sarcoma Viral Oncogene homologue
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cells Transplantation
hTERC	Telomerase RNA component
hTERT	Telomerase reverse transcriptase
hTR	human Telomerase RNA
IMDM	Iscove’s Modified Dulbecco’s Medium
iPSCs	Induced Pleuripotent Stem Cells
kDa	Kilo Dalton
LPL	Lipoprotein Lipase
mg	Miligram

mL	Mililitre
mM	Milli-Molar
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cell
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NF1	Neurofibromin 1
NTC	No Template Control
PBS	Phosphate Buffer Saline
pm	Pico-Molar
PPARγ2	Peroxisome Proliferation Activated Receptor γ2
PTEN	Phosphate and Tensin homologue
Rb	Retinoblastoma protein
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotation Per Minute
rRNA	Ribosomal RNA
RT-PCR	Real Time Polymerase Chain Reaction
SA-β-GAL	Senescence Associated Beta-Galactosidase
TGFβ	Transforming Growth Factor β
TRF	Terminal Restriction Fragment
UC	Umbilical Cord
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

TABLE OF CONTENTS

Appendix

LIST OF TABLES AND FIGURES

A] TABLES

B] FIGURES

Abstract

Human Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiation into several mesenchymal lineages. These cells have been isolated from different sources such as bone marrow, placenta, umbilical cord, adipose tissue, dental pulp, synovial membrane, peripheral blood, periodontal ligament and endometrium. Ability of Mesenchymal stem cells to self renew and differentiate into multiple lineages shows the great therapeutic potential of Mesenchymal stem cell but aging of MSCs in vitro limits this application. Many studies have shown that starvation in animal models causes prolongation of life. In this study, we tried to study the starvation effect of serum and glutamine on Mesenchymal stem cells in cellular and molecular level. Human bone marrow cells were isolated from fetal marrow aspirates after informed consent from hospital and were cultured in Iscove's modified dulbeccos media (IMDM) with six different concentrations of serum (0.5%, 2.5%, 5%, 10 % and 20%) and glutamine (0, 0.25mM, 0.5mM, 1mM, 2mM and 4mM). Comparative analysis was carried out for these cells in relation to aging markers such as morphology, expression of gene (senescense associate marker p21 and proliferation marker Ki67) by real time PCR and their products by immunostatining, senescence associated beta gal activity (SA-*B*-GAL), reactive oxygen species (ROS) estimation in both early (1 to 3 passage for glutamine and 1 to 2 passage for serum) and late passage cells (6 to 9 passage for glutamine and 4 to 5 for serum). The glutamine starvation was found to be effective than serum starvation as shown by cell count, passage number (two passage number for serum starvation Vs nine passage number for glutamine starvation) population doubling and morphological observations. Immunostatining results and real time PCR suggested that p21 was highly expressed in MSCs grown in lower concentrations of serum (0.5%, 2.5%, 5% and 10%) than MSCs grown in lower glutamine concentrations (0, 0.25mM, 0.5mM and 1 mM) implying . Flow cytometric analysis also reported accumulation of reactive oxygen species in MSCs cultured at serum limitation conditions. Thus, suggesting early ageing of serum starved Mesenchymal stem cells than glutamine starved Mesenchymal stem cells. The overall findings recommend that glutamine starvation seems to delay ageing and prolong longevity of bone marrow derived Mesenchymal stem cells and demand detail study of underlying mechanism.

Key words: Bone Marrow, Mesenchymal stem cell, Aging, p21, Ki67, Immunostatining, Flow cytometry

CHAPTER 1: INTRODUCTION

1. Introduction

1.1 Background

Stem cells are a class of undifferentiated cells that have the potential to differentiate into specialized cell types in the body. One of the main characteristics of stem cells is their remarkable potential to self-renew through cell division while maintaining the potential to develop into other types of cells. When a stem cell divides, each daughter cell either remains a stem cell or differentiates to become another type of cell such as a muscle cell, blood cells, or a nerve cell having a more specific function (Morrison *et al*, 2001). In some organs, such as the gut and bone marrow, stem cells divides on a regular basis to repair and replace worn out or injured tissues while in other organs such as pancreas and heart, these cells divide only under special conditions. Stem cells come from different sources such as blood, bone marrow, umbilical cord blood and adipose tissue. One of the elementary properties of stem cells is that these cells do not perform specialized functions except giving rise to specialized cells such as muscle cells, nerve cells and blood cells (Anderson *et al.,* 2001).

Human embryonic and adult stem cells are being used for cell based regenerative therapies each having their advantages and disadvantages. Human embryonic and adult stem cell differs in the number and type of cell that they can differentiate into. Different studies show that embryonic stem cells are pleuripotent and hence can give rise to all the differentiated cell types of the body while differentiation potential of adult stem cell is restricted to a specific set of cell types (Morrison *et al*, 2001; Wagers *et al.,* 2002). Besides these facts, it is also known that isolation of adult stem cells from adult tissue is quiet challenging and their expansion in culture medium have not yet been worked out. Embryonic stem cells can be grown relatively easily in culture (Bruno and Smith, 2010).

Furthermore, scientists believe that tissue derived from embryonic and adult stem cell would differ in the likelihood of causing transplant rejection (Alberio *et al.*, 2006). It is believed that transplantation of adult stem cell causes less rejection because these cells are taken from the patient itself and expanded in culture and reintroduced into the patient’s body (Madrigal *et al*., 2014). Thus, using adult stem cell for transplantation can avoid any type of side effects that can be caused by immune-suppressants. Likewise, an inner cell of blastocyst has the potential to give rise to the entire body of the organism including specialized cells and organs (Bruno and Smith, 2010).

Scientists have developed another type of cell called induced pleuripotent stem cell by genetically reprogramming adult cells. The adult cells are forced to express genes and factors that make the cells demonstrate the properties of embryonic stem cell. Human induced Pleuripotent Stem Cells (iPSCs) were discovered nearly a year after the discovery of mouse iPSCs. Human iPSCs demonstrate unique characteristics of pluripotent stem cells, being capable of expressing stem cell markers, forming cells characteristics of all three germ layer (Ralston and Rossant, 2010). Stem cells are advantageous for living organisms for many reasons. The regenerative abilities of stem cells make them potential source for being used as regenerative or reparative medicine. Various research have been carried out using stem cells to find out their essential properties that makes them different from other specialized cell types (Ralston and Rossant, 2010). Laboratory studies of stem cells are also being carried out to discover new drugs and find ways to study and correct birth defects (Ikebe and Suzuki, 2014).

Mesenchymal stem cells were first reported by Friedstein and his colleagues (1966) in bone marrow. Later other different sources of MSCs were found, including adipose tissue (AT), synovial membranes, bone, skin, pancreas, blood, fetal liver, lung, and umbilical cord blood (Eirin *et al*, 2012; Fukuchi *et al.,* 2004; Huang *et al.,* 2009; Hermida Gomez *et al.,* 2011; Tondreau *et al.,* 2005; Park *et al.,* 2011; Schwab *et al.,* 2008; Bakash *et al.,* 2005). In addition, it was found that these cell could differentiate into multiple cell types such as adipocytes, osteoblasts, and chondrocytes both in vitro and in vivo *(*Anker *et al.,* 2003; Pittenger *et al.,* 1998; Mackey *et al.,* 1998; Beshad *et al.,* 2011). In general, hMSCs are positive for CD63, CD105, CD166, CD54,CD55, CD13, CD44, CD73, and CD90 and negative for hematopoietic surface markers including CD34, CD45, CD14, CD11b, CD19, CD79α, CD31, CD133 (Caplan *et al*., 1994; Pittenger *et al.,* 1999).

1.2 Justification of the study

Expansion of MSCs in vitro depends on various conditions such as age and condition of the donor, harvesting technique, culture conditions and many more. These conditions also affect on the number of MSCs that can be expanded in vitro. Generally 10-20 ml of Bone marrow aspirate can yield hundreds of millions of cells (Sekiya *et al.,* 2002). MSCs are mainly expanded in vitro in the presence of FCS/FBS (Bruder *et al.,* 1997; Sekiya *et al.,* 2002). I*n vitro* expansion of MSCs has a major impact on the morphology, differentiation potential, length of telomere, expression of different genes and proteins and other major parameters used to define MSC (Dominici *et al.,* 2006).

Ability of Mesenchymal stem cells to self renew and differentiate into multiple lineages shows the great therapeutic potential of Mesenchymal stem cells (Krampera *et al.,* 2006). Administration of Mesenchymal stem cells represents a promising cell-based therapy for the treatment of range of diseases. A number of clinical trials have been done and still many others are ongoing. These days MSC have been used extensively in the clinic because these cells are relatively easy to isolate from many different sources and possess multilineage differentiation potential. There are many examples of success of clinical trials done using MSCs. A rapid hematopoietic recovery was noted as a result of autologous transplantation of MSCs in breast cancer patients (Koc *et al.,* 2000). Le Blanc (2004) reported that the infusion of BM derived MSCs isolated from mother can cure a child suffering from grade IV acute graft versus host disease (GvHD) of the liver and gut after allogenic hematopoietic stem cell transplantation (HSCT). Recent research on MSC has shown that life span of MSCs cultured in vitro can be extended by increasing the expression of telomerase i.e. the rate limiting enzyme. It is believed that the immunomodulatory properties of MSCs make it a potential candidate in systemic transplantation for generalized disease and local implantation for local tissue defects (Rahaman *et al.,* 2005). In addition, MSCs are believed to be useful in gene therapy and tissue engineering protocols.

Human mesenchymal stem cells (MSCs) are defined as plastic adherent cells with the potential to differentiate into multiple mesenchymal tissues (Dominici *et al.,* 2006). MSCs derived from bone marrow can proliferate in vitro and differentiate into lineages typical of bone, adipose tissue, cartilage and muscle (Caplan, 2007). Therefore, human bone marrow derived MSCs are interesting for tissue engineering strategies aiming to repair these tissues. However, MSCs are rare within the bone marrow aspirate, and need to be expanded *in vitro* for some passages in order to obtain clinical relevant numbers of cells. Although MSCs are considered as stem cells based on self renewing capacity, differentiation capability and functional reconstitution following clonal transfer (Sacchetti *et al*., 2007), they have a definitive life span and cease to proliferate after certain number of cell divisions. This state is referred as replicative senescence. It is characterized by cell enlargement and a certain metabolic activity, but the cells are completely refractory to mitogenic stimuli. This was first described in 1960s by Leonard Hayflick, and the number of population doublings (PD) inherent in a cell before the onset of replicative senescence is called the Hayflick limit (Hayflick, 1965). Since adult stem cells are responsible for cell renewal and maintenance of tissue homeostasis, it has been argued that cellular senescence could be related to the ageing process of the whole organism. In vitro, it has been shown that replicative senescence starts as soon as human bone marrow derived MSCs are placed in culture, and that their proliferation and differentiation capacity decrease with time in culture (Wagner *et al*., 2008). Therefore, analysis of the senescence status of MSCs is an important part of the overall assessment of the suitability of the cells for therapeutic purposes. Recently, the mechanisms underlying replicative senescence in stem cells have gained attention. Detailed investigations at the molecular level have revealed two major pathways for the induction of cellular senescence. One is dependent on telomere length, is mediated by the p53 and p21 cell signaling pathway and also encompasses the DNA-damage response mechanism (Shibata *et al.,* 2007). In contrast, a telomere-independent mechanism, typically activated by oxidative stress, is mediated by the Erk-p38$^{\mathrm{MAPK}}$ signaling pathway (Muller, 2009). There is evidence that this process also involves accumulation of cell cycle inhibitor proteins like p16$^{\mathrm{INK4a}}$ and heat shock proteins (Shibata *et al.,* 2007).

Real time PCR helps simultaneous measurement of gene expression in many different samples (Fink *et al.,* 1998). Real time PCR is really useful technique when only small numbers of cells are available (Higuchi *et al.,* 1993). Compared to conventional quantification methods such as northern blot, RT PCR, Real time PCR is highly sensitive, reproducible and has large dynamic range. To carry out real time PCR, the equipment itself is expensive and requires use of expensive reagents. Furthermore, to get conclusive results one should have an in-depth knowledge about the technique. Several groups have published protocols for single-cell gene expression analysis by real time PCR. Some researchers used cells 3-10 times larger than typical mammalian cells such as muscle fiber, neuron etc. (Eberwine *et al.,* 1992; Esumi *et al.,* 2008) and most of these protocols involved mRNA isolation and two step PCR (Esumi *et al.,* 2006; Nolan *et al.,* 2006). RNA isolation from single cell is challenging and it becomes difficult to get accurate gene expression measurement because of variability in yield for different samples (Taniguchi *et al.,* 2009). Recently, an improved one tube real time PCR protocol was proposed for analyzing single-cell gene expression in small mammalian cells (Li *et al.,* 2010). One tube real time PCR reduces the risk of contamination and promotes high reliability. Major error in real time PCR comes from pipetting and PCR processes (Taniguchi *et al.,* 2009).

Flow cytometry is a laser based technology that is applicable for cell counting, cell sorting, and biomarker detection. The cells are rendered differentially fluorescent and incorporated into a small liquid stream illuminated by a laser beam (Brown and Wittwer, 2000). The cells pass sequentially through the beam, and fluorescent light from the cells gives rise to electrical signals which are then analyzed by a computer. The most important feature of flow cytometry is that it analyses cells separately based on measurement of each particle within the suspension while in most scientific procedures measurements are taken as a average value for the whole population (Nunez, 2001). In addition several parameters can be measured on numbers of cells within a few minutes. The properties measured include a particle’s relative size, granularity, and fluorescence intensity. Any suspended particle or cell from 0.2-150 micrometers in size is suitable for analysis (Brown and Wittwer, 2000). Flow cytometry is a very useful tool and is used worldwide. Its importance in scientific research and diagnosis is increasing. It is now used routinely in many labs to detect the presence of specific surface and intracellular markers, measure metabolic activity and DNA content, among many other uses (Nunez, 2001). With cell sorting, applications range from the separation of large numbers of cells for functional studies or chromosomes for preparing gene libraries to the direct cloning of single rare transfected or hybridoma cells into each well of a tissue culture plate (Jayat and Ratinaud, 1993). Flow Cytometry is also used for the analysis of various cellular compounds including nucleic acids, proteins, cell organelles etc. DNA content in cells can be analysed using FACS. DNA specific dyes are used for staining the cells and the emitted fluorescence is proportional to DNA content (Jayat and Ratinaud, 1993). Cell distribution histograms are then used to analyse cells in different phases of cell cycle.

Immunostaining is a valuable biochemical tool for detecting specific antigens from proteins to infectious agent and specific cellular population in cells/tissues (Brandtzaeg, 1998). Because of its sensitivity, specificity and reproducibility, immunostaining has become one of the important technique in molecular biology with broad applications. Before performing immunostaining, fixation of cells or tissues is necessary. Fixation helps to preserve cellular details enabling accurate diagnosis and assessment (Stadler *et al.,* 2013). Ethanol 95% is considered as most efficient fixative. However, most laboratories use methanol for fixation as it is relatively cheaper. Before using antibodies, blocking solution such as normal serum, BSA (0.1% to 5%) is applied over fixed cells or tissues to prevent nonspecific binding of antibodies (Stadler *et al.,* 2013). A secondary antibody is used after the application of primary antibody which aids in the detection of target antigen by directly binding to it. These secondary antibodies are labeled with fluorophores or enzymes that forms chromogenic end product in the presence of specific substrate.

1.3 Objectives

The broad objective of this investigation is to understand the effect of different concentrations of serum as well as glutamine on ageing of bone marrow derived Mesenchymal stem cells.

The specific objectives of this study are:

1. To study signs of ageing on the basis of cell morphology and population doubling time
2. To optimize the RT-PCR reaction parameters and use the optimized parameters for cDNA synthesis using lysates containing RNA prepared from both early and late passage bone marrow derived Meenchymal stem cells grown in different concentrations of serum as well as different concentrations of glutamine
3. To use syntheisised cDNA for quantification of expression level of different genes including p21, ki67, APE and HTR using real time PCR
4. To analyze the presence of ageing specific markers viz. ki67 and p21 in early and late passage cells by immunostaining
5. To use Flow cytometry to determine the amount of reactive oxygen species produced in cells in response to different concentrations of serum as well as glutamine

1.4 Scope of the study

Like somatic cells, Mesenchymal stem cells have a limited life span in vitro. After a limited number of cell divisions, there occur morphological changes in Mesenchymal stem cells which can be characterized by enlarged and irregular cell shape with many actin stress fibers. Ultimately, these cells show reduced differentiation potential and impairment of their functions upon prolonged in vitro culture (Bonab *et al.,* 2006; Noer *et al.,* 2007). Hayflick limit is supposed to reflect the ageing process of whole organism. Stem cells are involved in renewal of tissues of any organism and impairment of their function results in ageing (Ho *et al.,* 2005). In addition, senescence of MSCs might limit their clinical applications. It is therefore necessary to analyse in vitro senescence in MSC to evaluate its potentiality in basic research as well as in cellular therapy. Furthermore, it is necessary to find out their distribution in vivo and study their therapeutic mechanisms as well as optimize their use in regenerative medicine strategy.

One of the important factors of ageing is cellular damage which occurs due to different types of stress. Many studies show that stress which develops due to starvation extends the lifespan of organisms. The genes that regulate resistance to starvation may affect ageing. Hayflick and Moorehead found out that the process of ageing occurs due to improper functioning of the cells (Hayflick and Moorehead, 1980). With ageing, changes in cell organelles, membranes and genetic material could be observed. In an experiment, mice raised in calorically restricted diet showed increased lifespans and a delay in the development of ageing related physiologic and pathologic changes (Lee *et al*. 1999). In addition, it has been found that calorie restriction induces expression of genes that participate in stress resistance including genes that regulate glycolysis, gluconeogenesis and DNA repair. Thus, calorie restriction causes an increase in energy metabolism which is responsible to delay ageing. Furthermore, a class of protein called Sirtuins has been found to counteract ageing in yeast, *Caenorhabditis elegans*, and *Drosophila (*Blander and Guarente, 2004). SIR2 gene encoding sirtuin protein in yeast is believed to suppress toxic rDNA formation as well as manage damage caused by oxidative stress (Sinclair and Guarente 1997). Sirtuins are found to be located within mitochondria and in their intermembrane space (Aguilaniu *et al.,* 2003). Mitochondria supply all the necessary biological energy of the cell so sirtuins are believed to play vital role in life prolongation during nutrient deficiency. The mechanism by which life of cells prolong is unclear. Sirt5, a type of sirtuin protein located in intermembrane space of mitochondria has been found to modify the protein cytochrome C and contribute to malfunctioning of apoptosis but its precise function is still unclear (Aguilaniu *et al.,* 2003). The scope of the present study is to observe how cells respond to higher and lower concentrations of serum and glutamine and relate the response to ageing. Morphological analysis along with analysis of ageing related markers are done by quantification of genes using real time PCR, quantification of protein using immunostaining techniques, analysis of cell cycle and estimation of ROS using FACS.

1.4.1 Quantification of expression level of different genes using real time PCR

Expression levels of different genes are assessed by real time PCR. With ageing there occurs change in morphological characteristics of MSC. Along with this expression of senescence associated genes like P21, APE increases in late passages while expression of proliferation related genes such as ki67 decreases (Morrison *et al.,* 1998). These changes in expression pattern of genes can be assessed by real time PCR. Two quantification types are followed in real time PCR. In relative quantification, reference gene is taken and based on it, relative expression of target gene is quantified. In another type, absolute quantification is done on the basis of an internal or an external calibration curve (Morrison *et al.,* 1998; Pfaffl, 2001). In present study, relative quantification is done. Internal control genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), b-actin, b-2 microglobulin, 18S ribosomal RNA are used to ensure the integrity of every steps of the real time PCR process (Thomas *et al.,* 2000; Marten *et al.,* 1994). These internal controls are housekeeping genes that are essential for cell survival and are believed to show stable expression in various tissues. The amount of template is thus determined by calculating the amount of changes in target sequence as compared to the control.

1.4.2 Qualitative analysis of proteins using immunostaining

In present study, quantification of proliferation marker-Ki67 and senescence marker-p21 is done by immunostaining. The cells fixed in 70% methanol are treated with primary and fluorochrome bound secondary antibody under appropriate condition. The primary antibodies used in immunostaining can be monoclonal or polyclonal. The monoclonal antibodies are developed from hybrids and are relatively more specific as it provides antibodies against a single antigen epitope. The polyclonal group is developed from immunization of animals such as rabbit, goat, mouse, monkey etc. and provides antibodies against number of epitopes.

1.4.3 Estimation of reactive oxygen species using FACS

Cell permeable fluorescent and chemiluminescent probes are used as one of the simplest technique to determine ROS. 2'-7'Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell. It has several advantages over other techniques developed. It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time (Sarkar *et al*., 2005). In this study, on the basis of stained and unstained control, total percentage of stressed cells is determined.

1.5 Research Hypothesis

Null hypothesis: When bone marrow derived Mesenchymal stem cells are cultured in nutrient deprived condition, ageing is delayed.

Alternate hypothesis: When bone marrow derived Mesenchymal stem cells are cultured in nutrient deprived condition, ageing is not delayed.

CHAPTER 2: LITERATURE REVIEW

2.1 Mesenchymal Stem Cells (MSCs)

Caplan first proposed the concept of mesenchymal stem cell (Caplan *et al*., 1994). Friedenstein and his colleagues in 1979 discovered that bone marrow contained mesenchymal stem cell along with hematopoietic stem cells. Friedenstein *et al* described Mesenchymal stem cells (MSCs) as a population of adherent cells showing fibroblast like appearance and exhibiting non phagocytic properties. They found out that these cells can be isolated from whole bone marrow aspirates and can be cultured in plastic dishes as mesenchymal stem cells show plastic adherence property. Later the term mesenchymal stem cell was validated by the contribution of many other laboratories, in reference to work done by Friedenstein and Owen (Gao *et al*., 2001). MSCs are therefore defined as adherent, fibroblast like cells that differentiate to osteoblasts, adipocytes, and chondrocytes in vitro (Dominici *et al.*, 2006).

Dominici *et al* (2006) has proposed minimal criteria to define multipotent mesenchymal stem cell. It states that if the cells are considered to be the population of MSCs, it should be plastic adherence in very specific culture condition and should be expressing specific surface antigen such as CD105, CD73 and CD90 and most importantly, these cells should have multipotent differentiation potential (Dominici *et al*., 2006).

Mesenchymal stem cells were mainly extracted from bone marrow but there have been additional sources for MSCs including: muscle, dermis, trabecular bone, adipose tissue, periosteum, pericyte, blood, synovial membrane and possibly other cell lineage. Basically, mesenchymal stem cells possess two important characteristics i.e. the capability to differentiate into characteristic end stage cell types such as adipocytes, chondrocytes, osteoblasts, tendon, muscle and other connective tissues (Pittenger *et al*.,1998; Mackey *et al*., 1998; Anker *et al*., 2003) and second, is to possess immunoregulatory properties because of which it can be used as regenerative medicine (Thejaswi *et al*., 2012;Uccelli *et al*., 2008).

Mesenchymal stem cells are regarded as an excellent candidate for cell therapy because these cells are easily accessible, isolation techniques are easier and expandable to required quantity in relatively short period of time (Colter *et al*., 2000; Sekiya *et al*., 2002). In addition, Mesenchymal stem cells can be cryopreserved with minimal loss of potency (Lee *et al*., 2005). Besides this, MSCs have been used for regenerative therapies in heart and various other organs where MSCs are believed to secrete beneficial cytokines and growth factors enhancing regeneration of damaged organ (Li and Ikehara, 2013; Patel *et al*., 2013).

2.1.1 Sources of Mesenchymal stem cells

Mesenchymal stem cells have been isolated from different sources including bone marrow (Friedstein *et al*., 1976), adipose tissue (Eirin *et al*., 2012), placenta (Fukuchi *et al*., 2004), dental pulp (Huang *et al*., 2009), synovial membrane (Hermida Gomez *et al.,* 2011), peripheral blood (Tondreau *et al*., 2005), periodontal ligament (Park *et al*., 2011), endometrium (Schwab *et al*., 2008) and umbilical cord (Bakash *et al.*, 2005).

MSCs have been identified in other different sources like fetal blood but its frequency is found to be very low (Campagnoli *et al*., 2001). Some laboratories have been able to grow MSCs from umbilical cord blood also (Erices *et al*., 2000; Sarugaser *et al*., 2005) while other laboratories are working on it. These researches show that different techniques have been developed till date for isolation and expansion of mesenchymal stem cells from different sources.

2.1.2 Functions of mesenchymal stem cell

It has been proposed that mesenchymal stem cells are the in vivo precursors of the hematopoietic microenvironment in bone marrow that regulate hematopoiesis (Friedstein *et al*., 1966). MSCs are isolated from all fetal hematopoietic sites even before these sites are colonized by HSC (Hematopoietic stem cells) (Mendes *et al*., 2005) which provide strong evidence that MSCs have a key role in providing modulatory signals to HSC *ex vivo* (Dexter *et al*., 1977).

One of the most remarkable characteristic of MSCs is their immunomodulatory property. It has been shown that Mesenchymal stem cell suppresses proliferation of lymphocytes by inhibiting activation of T cell, proliferation of B cell and differentiation of dendritic cells (Thejaswi *et al*., 2012; Uccelli *et al*., 2008). Production of immunostimulatory cytokines is also altered by altering the cytolytic potential of natural killer cells (Thejaswi *et al*., 2012; Uccelli *et al*., 2008). The ability of MSCs to secrete huge range of soluble mediators like interleukin 10, prostaglandin e2, nitric oxide and transforming growth factor β (TGFβ) partially explains the immunomodulatory property of Mesenchymal stem cells (Meirelles *et al*., 2009).

MSC are believed to show “trophic activity”as they are capable of producing variety of factors of biological significance in vitro such as neuroregulatory peptides and cytokines (Caplan *et al*., 2007). In addition, it has been found that migration of marrow stromal progenitors into a degenerating muscle, forms a fully differentiated muscle fiber (Ferrari *et al*., 1998) which confirms the migratory ability of MSCs specific sites.

2.1.3 Characterisation of MSCs

Specific markers have not yet been developed for the exclusive identification of Mesenchymal stem cells. Ability of mesenchymal stem cells to adhere to plastic dishes under standard culture condition (Sekiya *et al*., 2002) and a fibroblast like morphology are taken as characteristic markers for MSCs (Prockop, 1997). Proliferative capacity of Mesenchymal stem cells to differentiate into colony forming units and to various other lineages is also taken as an indicator for being mesenchymal stem cells (Pittenger *et al*., 1999).

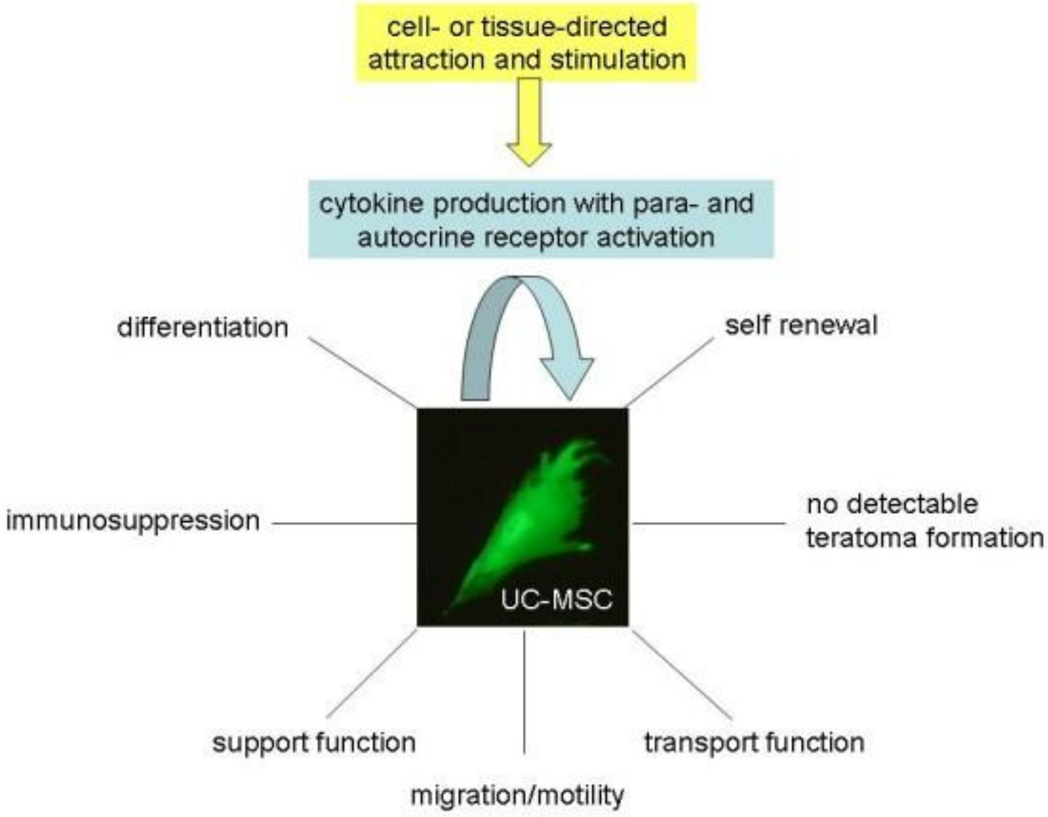


Figure 2.1: Major characteristics of MSC and their functions *in vitro* and *in vivo* (Mendes *et al*., 2005)

Other methods were also developed to identify mesenchymal stem cells. Martinez and his colleagues used magnetic beads to show that human MSCs express the neural ganglioside GD2. They used murine monoclonal antibody against GD2 and an allophycocyanin-conjugated donkey anti-mouse secondary antibody with corresponding magnetic beads. Fluorescent antibody cell sorting with specific antibodies was also done to find out specific MSC markers (Fickert *et al*., 2003). In 1980s, an antibody specific to MSC cell surface antigen was recognized by Simmons *et al* (1991). Furthermore, It was proposed that MSCs should express CD105 (known as endoglin and originally recognized by the MAb SH2), CD73 (SH3 or SH4), CD90 (Thy-1), CD166, CD44, and CD29 (Caplan *et al*., 1994; Pittenger *et al*., 1999) to be mesenchymal stem cells.

It has been shown that human mesenchymal stem cells do not express hematopoietic markers like CD14, CD31, CD34 and CD45 (Dominici *et al*., 2006). Moreover, MSCs express a large number of adhesion molecules like ALCAM (CD166), the hyaluronate receptor (CD44) and ICAM-1 (CD54), growth factor and cytokine receptors, integrins and additional markers like Tetraspan (CD9) or Thy-1 (CD90) (Deans *et al*., 2000).

2.1.4 Multilineage potential of MSCs

Mesenchymal stem cells possess the ability to differentiate in to various lineages. Osteogenic differentiation of MSCs can be promoted under the influence of dexamethasone, β-glycerol phosphate, ascorbate in the presence of 10% v/v FBS. Quantitative assays showed increase in alkaline phosphatase activity, and calcium accumulation was evident which increased over time (Anker *et al*., 2003).

Moreover, chondrogenic differentiation capacity of MSCs has been described by many groups using medium supplemented with transforming growth factor β1, ascorbate-2-phosphate and dexamethasone. The differentiated cells showed secretion of glycosaminoglycan (GAG) by immune-histological staining and also were seen to express typical genes of the chondrogenic lineage (Mackey *et al*., 1998). In addition, to promote adipogenic differentiation, mesenchymal stem cells were treated with 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin. Induction was evident by the accumulation of lipid rich vacuoles within cells and expression of peroxisome proliferation activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL) and the fatty acid-binding protein aP2 (Pittenger *et al*., 1998). Human MSCs can also be differentiated into partially functional hepatocyte-like cells in the presence of liver specific growth factor like hepatocyte growth factor, fibroblast growth factors (Beshad *et al*., 2011).

2.2 Ageing

Sames and Stolzing (2005) defined ageing as ‘‘the sum of major restrictions or limitations in regenerative mechanisms of multicellular organisms”. In other words, it can be defined as the progressive decline in the resistance to stress and other cellular damages, causing a gradual loss of cellular functions.

Hayflick *et al*. (1976) suggested that a biological clock contained within a cell contribute to ageing. He discovered that embryonic tissue derived human cell can divide only for a finite number of times in culture and further suggested that certain mechanisms occurring in cells limit their replicative potential and causes cellular senescence. This limitation was termed as Hayflick limit. Accordingly, different stages of cell culture have been summarized into 3 different phases.

Phase I: This phase represents the primary culture. Cells from the explant multiply to cover the surface of the culture.

Phase II: This phase represents the period when cells divide in culture. Once the culture flask becomes confluent, the cells must be subcultivated to continue cell growth. The culture medium is removed, cells are washed with PBS and harvested using digestive enzyme, trypsin. The cells are resuspended in growth medium and added to two or more culture flask. These cells adhere to the plastic dish and start dividing. These cells become confluent in few days depending upon their growth rate and are again subcultured.

Phase III: This phase represents the period when cells start dividing slowly and eventually stops dividing, though they may or may not die. The occurrence of growth arrest after a period of normal cell proliferation is known as the Hayflick limit (Phase III).

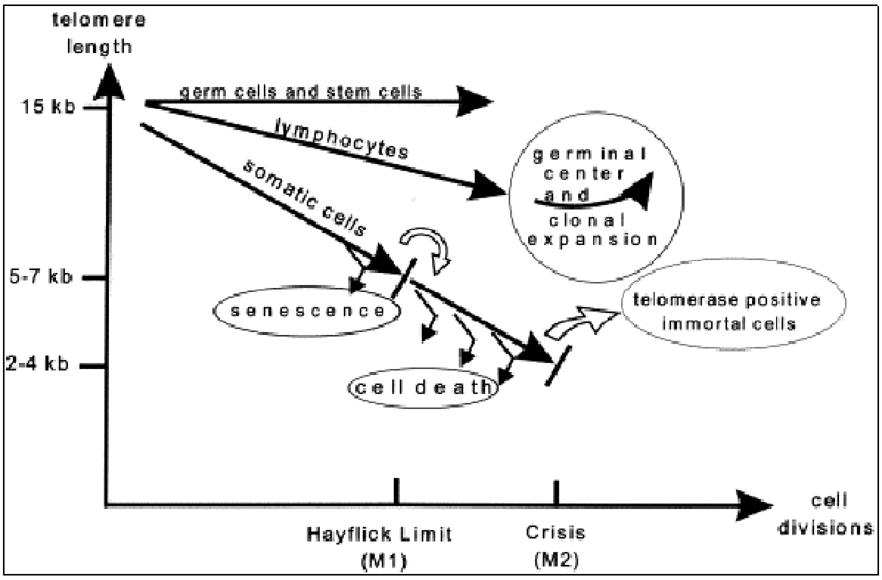


Figure 2.2: Hayflick's limit in cellular proliferation and senescence (Hayflick, 1979)

Hayflick (1979) put forward a hypothesis in order to find out the possible reasons of the transition to phase III. He hypothesized that ''the finite lifetime of diploid cell strains in vitro may be an expression of ageing or senescence at the cellular level.'' There occurs shortening of telomeres with each division of a cell which ultimately causes cells to reach their "Hayflick limit". Senescence is often equated with replicative senescence (Campisi *et al.*, 2000). Replicative senescence is defined as an essentially irreversible arrest of cell division that limits the proliferation of cells (Hayflick and Moorhead, 1961). Besides replicative senescence, stress induced premature senescence also occurs in cells in response to many stress, including abnormal activation of oncogenes, damage to chromatins and DNA, oxidative stress and inadequate culture conditions and this type of stress is totally independent of the telomere length and number of cell divisions (Merz et al, 1969). However, both replicative and stress induced senescent cells demonstrate similar changes morphologically and similar pattern of regulation of cell cycle (Thomas *et al.*, 1997;Hayflick *et al.*, 1979).

2.2.1 Replicative senescence

Replicative senescence is determined by the number of cumulative population doubling that a cell makes before entering in to a senescent phase (Hayflick *et al.*,1976; Goldstein and Singal,1974).Shortening of telomeres causes cells to reach their "Hayflick limit". Hayflick limit is a period of apparently normal cell proliferation observed until cell division stops (Hayflick *et al*., 1979). This limitation of cell division that causes cells to become senescent is called replicative senescence. Replicative senescence can be characterized by altered morphology, decreased growth rate and impaired functional characteristics (Thomas et al., 1997). It has been proposed that replicative senescence occurs as a result of moderate stress on cell population while a larger amount of stress causes apoptosis and again more stress causes necrosis (Soti *et al*., 2003).

2.2.2 Premature cellular senescence

Premature senescence occurs as a result of variety of conditions other than shortening of telomere. Generally, cells are isolated from an organism and are allowed to grow in an artificial environment which causes a total shift in concentrations of nutrients and growth factors, presence of ambient oxygen levels and other various factors. These factors cause a type of culture shock and hence induce senescence. This type of senescence is called stress induced senescence. Mutant HRAS (HRAS v12) is an oncogene which when introduced into mammalian cell lines causes immortalization but when introduced alone in primary cells leads to arrest in the cell cycle and shows phenotypic resemblance to cells suffering from replicative senescence (Serrano et al., 1997). This type of senescence is defined as oncogene induced senescence in vitro. In addition, there is another type of senescence called tumor suppressor loss induced senescence in vitro which occurs as a result of loss of a tumor suppressor gene like phosphate and tensin homolog (PTEN) and NF1. It was proposed that the depletion of PTEN and NF1 causes senescence in mice and human cell (Sabin and Anderson, 2011).

2.3 Ageing of Mesenchymal Stem Cells

During ageing, there occur various changes in physiological, functional and molecular parameters. There occurs mitochondrial dysfunction, shortening of telomeres, accumulation of DNA damage. Along with this, impairments of stem cell function are also major causes of ageing (Linton and Dorshkind, 2004). Stem cells maintain tissue homeostasis and impairment of their functions leads to organ failure.

It has been reported that in humans and rodents, when hematopoietic stem cell becomes aged, they lose their differentiation potential and start differentiating into myeloid cells (Morrison *et al*., 1996; Linton and Dorshkind, 2004; Pang *et al*., 2011). Muscle stem cell in mice and human has been found to show impaired functions and decreased proliferation rate (Kadi *et al*., 2004; Collins *et al*., 2007; Bortoli *et al*., 2003; Conboy *et al*., 2005). In hematopoietic stem cells, mesenchymal progenitors also serve as one of the niche cells (Omatsu *et al*., 2010). This is why the depletion of mesenchymal progenitors in both bone marrow and skeletal muscle leads to loss of hematopoiesis and loss of skeletal muscle mass in the uninjured condition (Roberts *et al*., 2013). Mesenchymal stem cells possess immunomodulatory properties which can be explained by their ability to secrete huge range of soluble mediators (Meirelles *et al*., 2009). These mediators are also involved in maintaining tissue homeostasis keeping intact the function and number of stem cells (Caplan and Dennis, 2006; Le Blanc and Ringden, 2007). Thus, it can be concluded that any alteration in MSCs properties can direcly contribute to the development of ageing.

Normal osteoblast demonstrates similar process of ageing in culture. During 30-35 population doublings, these cells showed decreased cell division and growth rate, remarkable changes in morphology and reduced synthesis of DNA, RNA and proteins (Kassem *et al.*, 1997). It has been suggested that MSCs begin to lose their stem cell properties from the moment they are cultured *in vitro* and are applicable for cell and gene therapy at early stages of culture (Bonab *et al.*, 2006).

Like several other cell types, human mesenchymal stem cells also demonstrates characteristically distinguishable irreversible growth arrest, higher level of sensitivity to cellular injury and altered gene expression with increased expression of proteins including p21, p53, p16 and p19 (Zhu *et al.*, 2005).

2.3.1 Observable markers of ageing in MSCs

2.3.1.1 Changes in morphology

It has been reported that aged mesenchymal stem cell do not exhibit spindle shaped morphology as in very early passages (Dexter *et al.*, 1977). Cells in early stages of culture form big colonies composed of spindle shaped cells while that of late stages form small colonies with broad, flattened cells (Liu *et al.*, 2004). These cells are bigger in size, spread further and also contain more actin stress fibers (Dexter *et al.*, 1977; Stenderup *et al.*, 2003; Mauney *et al.*, 2004). Cells in early culture when becomes confluent contain higher cellular density than in late stage cultures despite the fact that senescent cells are more sensitive to cell-cell contact inhibition (Bayreuther *et al.*, 1988). Besides these facts, presence of debris in the medium and granularity of cytoplasm is also considered as one of the significant marker for ageing *in vitro* (Smith and Lincoln, 1984).

2.3.1.2 Alteration in proliferation and differentiation potential

Ageing of cells can be characterized by diminishing division capacity. Total CFU numbers in aged MSC is found to decrease resulting in reduction of average colony size (Chen, 2002; Globerson, 1997; Prinz *et al.*, 1999). For mesenchymal stem cells *in vitro*, maximal population doubling is found to be between 30 and 40 (Banfi *et al.*, 2000). Dexter and his group showed significant decline in the growth rate of MSC from aged donors as well (Dexter *et al.*, 1977). It has been discussed that MSCs possess the capacity to differentiate into various types of lineages but it seems to change with age. Meunier and his group (1971) reported that aged MSC lost their osteogenic potential and gained adipogenic potential which was later termed as ‘adipogenic switch’ (Ross *et al.*, 2000).

2.3.2 Diagnostic markers of ageing in MSCs

2.3.2.1 Ki67

The pki67 protein is a nucleolar protein associated with cell proliferation (Gerdes *et al*., 1983, 1984). It is present in cells at active stages of cell cycle showing the interdependency of cell proliferation and pKi67 expression in cell growth and division. This correlation of cell proliferation and pki67 expression makes this protein an excellent marker for the determination of growth fraction of given population and to diagnose tumour development. pKi67 was originally identified by Gerdes *et al* (1983) using a monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line.

During interphase of cell cycle, the ki67 protein is confined in the nuclear cortex and in the dense fibrilar components of the nucleolus whereas during mitosis these proteins become localized along the periphery of the condensed chromosomes (Isola *et al*., 1990; Verheijen *et al*., 1989). It has been reported that in proliferating cells, a fraction of pKi67 is found at sites linked to rRNA transcription which shows the role of pKi67 in early steps of rRNA synthesis (Bullwinkel *et al.*, 2006). The functions of these proteins during interphase are not clear but it has been suggested that during mitosis these proteins could be involved in the condensation and decondensation of chromosomes or in the protection and stabilization of chromosomes. Also the localization of these proteins along the periphery of chromosomes could be a mechanism to carry out symmetrical distribution of nucleolar proteins between daughter cells (Starborg *et al*., 1996)**.** The human ki67 mRNA sequence encoding the ki67 antigen was recently isolated and characterized, and as the result of an alternative splicing process it was shown to encode two different proteins with calculated molecular masses of 359 kDa and 320 kDa (Gerdes *et al*., 1991; Schluter *et al*., 1993).

Ki67 is established as an appropriate marker for detection of ageing in cultured cells. It has been shown that 56% ki-67 positive human dermal fibroblast were found at fourth passage, compared to only 30% at passage 38 with decrease in this percentage for further passages (kill *et al*., 1996; Bridger *et al*., 2000). Furthermore, cell cycle analysis was carried out which showed the presence of ki67 in all active phases of cell cycle G1, S, G2 and mitotic phase but was found to be absent in quiescent or resting cells in G0 phase (Gerdes *et al*., 1984). Comparative analysis of ki67 antigen staining in different phases of cell cycle showed that the staining begins to increase during S phase, further increases in G2 phase and becomes highest in metaphage (du Manoir, 1991; Bruno and Darzynkiewicz, 1992; Braun *et al*., 1988). Moreover, it was reported that during anaphase and telophase the ki67 staining starts to decrease (Braun *et al*., 1988; Starborg *et al*., 1996).

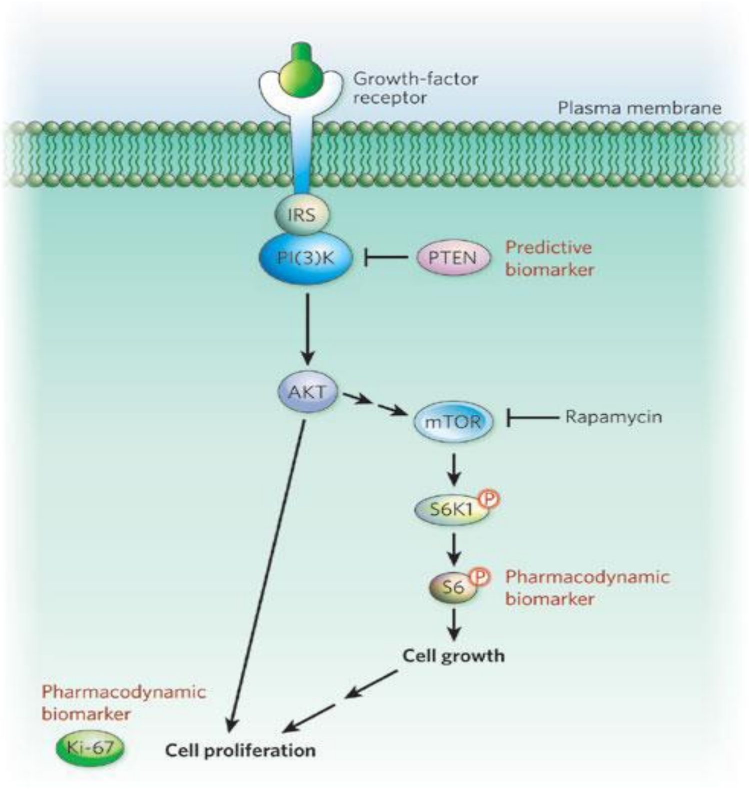


Figure 2.3: Ki67 activation pathway (Sawyers *et al*., 2008)

2.3.2.2 P21

Progression of the cell through different phases of the cell cycle requires sequential activation of cyclin/cdk complexes (Sherr *et al*., 1993; Ekholm *et al*., 2000). Activated cyclin/cdk complexes inactivate retinoblastoma protein (Rb) family through phosphorylation that is a negative regulator of cell cycle progression from G1 to S phase. This leads to induction of E2F regulated gene expression and cell proliferation. The Cyclin dependent kinase inhibitor p21 binds and inhibit the activity of cyclin/cdk complexes and hence negatively regulates cell cycle progression (sherr *et al*., 1995, 2000). The protein p21 inhibits cell cycle progression primarily through the inhibition of CDK2 activity which plays important role not only in the phosphorylation of Rb and activation of E2F dependent gene expression but also for release of replication origins and activation of proteins involved in DNA synthesis (Zhu *et al*., 2005).

The tumor suppressor protein p53 is found to regulate the expression of the p21 gene (El-Deiry *et al*., 1993, Xiong *et al*., 1993). Therefore p21 is considered as the key effector of cell cycle arrest which is induced by the activation of p53 pathway. A study was made in tumor cells under treatment and it was reported that p21-negative cell lines induces fewer senescence-associated genes compared with p21-positive cell lines (Chang *et al*., 2002). Simultaneously, another group found that an induction of p21 in fibrosarcoma cells not only led to growth arrest but was also involved in the induction of genes with paracrine activities (Chang *et al*., 2000).

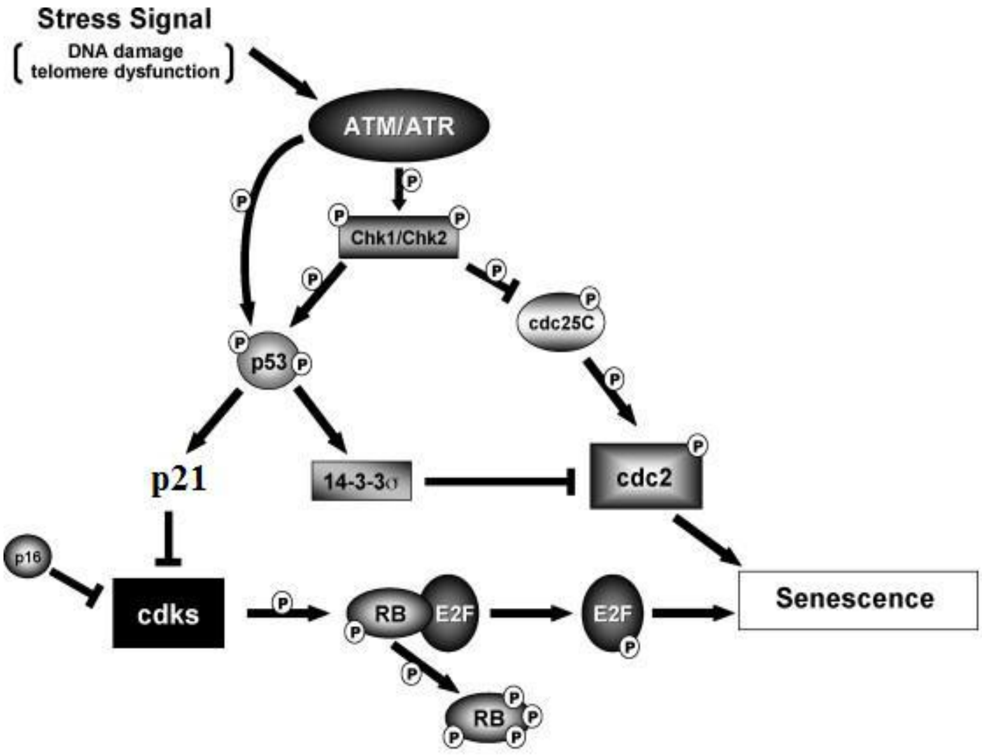


Figure 2.4: p21 activation pathway (Morgan et al, 1995)

2.3.2.3 Reactive Oxygen Species (ROS)

Reactive oxygen species consists of superoxide anions, hydrogen peroxide and hydroxyl radicals. ROS are generated in the cells by the mitochondrial respiratory chain as byproducts of aerobic respiration and various other catabolic and anabolic processes (Poyton *et al*., 2009). Other than mitochondrial respiration, a number of cytosolic enzymes are able to generate ROS (Lambeth *et al*., 2004). For example, plasma membrane associated enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces superoxide from oxygen using electron from NADPH (Quinn *et al*., 2006). ROS are also produced in response to different environmental stimuli such as growth factors, inflammatory cytokines, ionizing radiation, UV, chemical oxidants, chemotherapeutics, hyperoxia, toxins, and transition metals (Liu *et al*., 2009; Norbury *et al*., 2001).

Harman *et al.* (1956) proposed that accumulation of deleterious effects to DNA, proteins and other molecules caused by free radicals is one of the main reason of ageing, and the ability of an organism to cope with cellular damage induced by ROS determines organism's lifespan. ROS are considered as one of the causative factors of ageing as they react with DNA, protein and lipid molecules generating oxidative damage (Harman *et al*., 1956). Oxidative stress occurs because of an imbalance between production of free radicals/ROS and antioxidants (Reuter *et al*., 2010). Mammalian cells have developed complicated defense mechanisms to counteract cellular damage caused by ROS. For example, DNA lesions produced by ROS are corrected by DNA repair pathways such as base excision repair, nucleotide excision repair, mismatch repair and double strand break repair (Wilson *et al*., 2003; Maynard *et al*., 2009). Hematopoietic stem cells (HSCs) in aged mice become deficient in DNA repair pathways and shows increased sensitivity to ROS with diminished self renewal and functional capacities (Rossi *et al*., 2007). Antioxidant defense mechanisms such as antioxidative enzymes, tocopherol, tocotrienols, ascorbic acid, carotenoids, polyphenols, lipoic acids etc. are also present in cells to balance reactive oxygen species (Papas *et al*., 1999).

Mitochondrial genome regulates synthesis of ATP and RNAs required for protein translation as well as it encodes proteins for oxidative phosphorylation (Falkenberg *et al*., 2007). Mitochondrial DNA (mtDNA) consists of only one noncoding region that is called D-loop or the displacement loop which plays important role in replication and transcription of mitochondrial DNA (Kasamatsu *et al*., 1971). It has been reported that mitochondrial DNA of aged human and animal tissue shows point mutation in D-loop and additionally deletion of mtDNA has also been detected so far (Piko *et al*., 1988; Calloway *et al*., 2000). Deletion of mtDNA is thought to be generated during repair of damaged mtDNA (Krishan *et al*., 2008). In order to correct mtDNA damage a double strand break is made (Krishen *et al*., 2008) which causes annealing of free single strand regions with homologous sequences on other single stranded mtDNA or within noncoding regions (Harber, 2000). Further processing is done by ligation and degradation of the remaining exposed single strands which results in the formation of an intact mitochondrial genome having a deletion (Krishan *et al*., 2008). Furthermore, it has been reported that the most common deletion is a 4977-bp deletion occurring commonly in muscle and brain (Linnane *et al*., 1992; Hayakawa *et al*., 1992) showing even higher frequency of deletion in brain, heart and skeletal muscle with age (Meissner *et al*., 2008). The abundance of mtDNA also declines with age in various tissues of human and rodent (Welle *et al*., 2003).

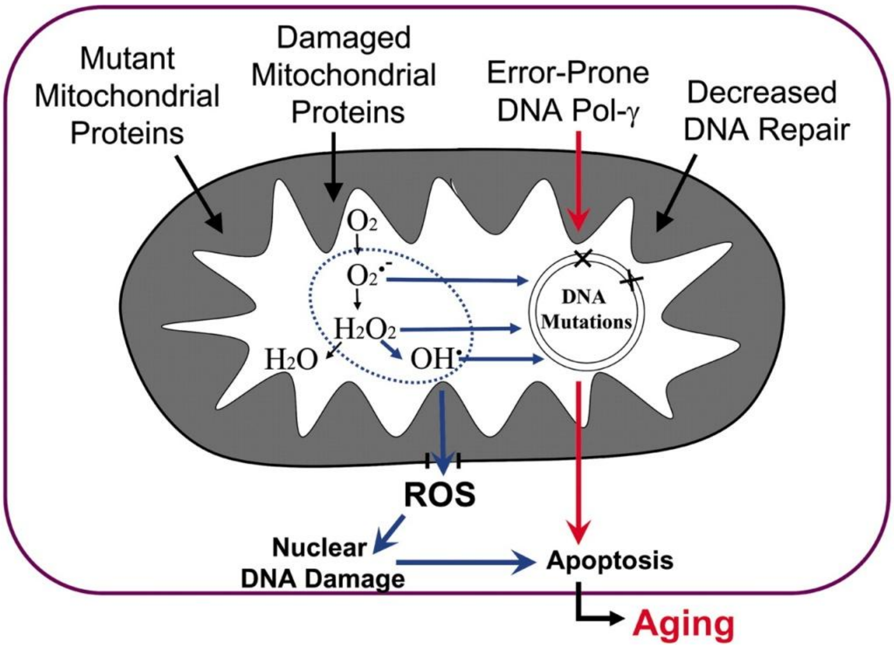


Figure 2.5: Mitochondrial DNA damage and ageing (Source: Loeb *et al*., 2005).

It is found that oxidative damage in mitochondrial DNA (mtDNA) is more pronounced than in nuclear DNA. This fact shows that mitochondria is more susceptible to oxidative damage and are thought to play important role in ageing both as major producer and primary target of ROS (Harman *et al*., 1972; Richter *et al*., 1992; Miquel *et al*., 1980). Spleen, liver and brain of aged mice showed higher number of the permeability transition pores in inner mitochondrial membrane. These structures are associated with cell necrosis and apoptosis (Mather and Rottenberg, 2000; Rottenberg and Wu, 1997). Mice with mitochondrial DNA polymerase lacking Proof reading activity showed early ageing phenotypes, reduced lifespan and mtDNA mutation (Trifunovic *et al*., 2004). These evidences suggest that mitochondria and ageing are closely linked to each other.

2.3.2.4 Senescence associated Beta-galactosidase (SA-β-GAL)

Senescence associated Beta-galactosidase (SA-β-GAL) is a most commonly used senescence biomarker because of the simplicity of the assay and specificity of the reaction (Dimri *et al*., 1995; Debacq-Chainiaux *et al*., 2009). Beta-D-galactosidase is an enzyme localized in the lysosome that cleaves a number of naturally occurring substrates such as gangliosides, glycoproteins and glycosaminoglycans (Suzuki *et al*., 1995). Beta-D-galactosidase localized in lysosomes of mammalian cell is regarded as specific subtrate for detecting senescence. Lysosome is an acidic organelle so it has been shown that beta galactosidase activity is higher at pH between 4.0 and 4.5 while lower at pH 6.0 (Zhang *et al*., 1994). For the detection of senescence associated beta galactosidase the cells should be frozen instantly to preserve enzymatic activity (Debacq-Chainiaux *et al*. 2009).

It has been shown that organs of aged human and other animals show enhanced SA-β-GAL activity (Dimri *et al*., 1995; Mishima *et al*., 1999; Pendergrass *et al*., 1999; Sigal *et al*., 1999; Melk *et al*., 2003). Furthermore it has been found that beta galactosidase activity is detectable at pH 6 in cultured cells undergoing senescence but it is not detectable in proliferating cells (Dimri *et al*., 1995). These findings suggest that SA-β-GAL enzyme is a good marker for detecting cells undergoing senescence. Furthermore, it has been reported that MSCs in late passages show increase in beta gal activity while no significant difference has been observed in MSCs from young and aged donors (Stenderup *et al*., 2003).

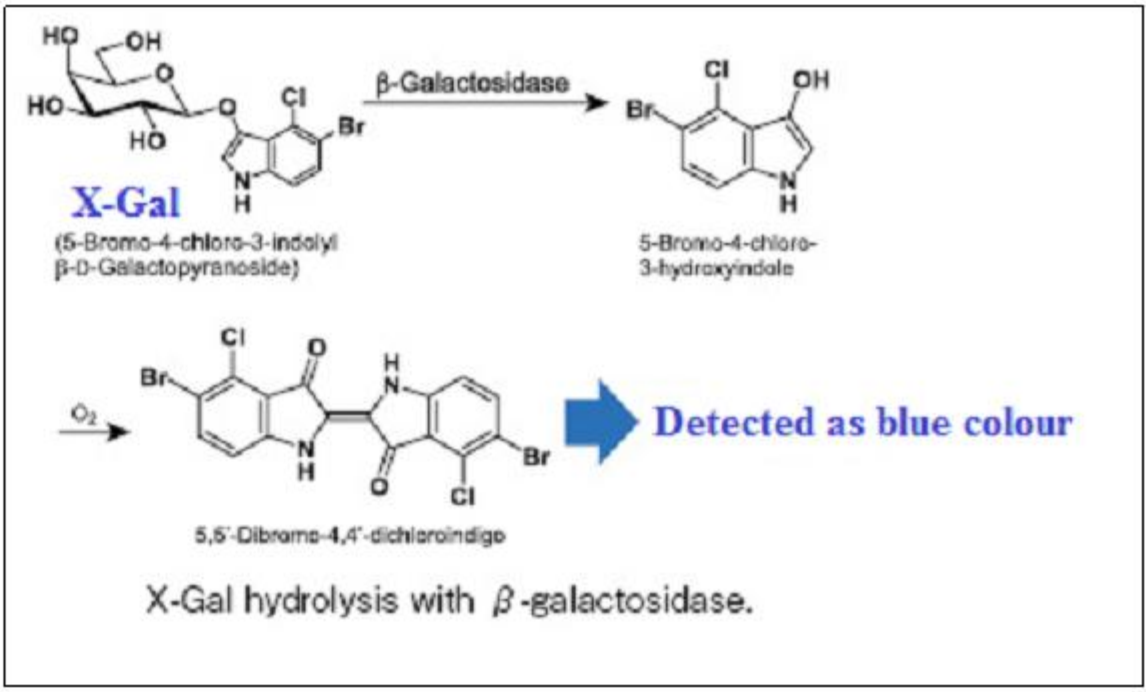


Figure 2.6: X-gal hydrolysis by beta galactosidase (Debacq-Chainiaux *et al*., 2009)

2.4 Media and supplements for MSCs in culture

Although mesenchymal stem cells can be found in many tissues in the body, such as bone marrow, fat, and muscle, they are mainly dormant and present in small quantities. For clinical use, such as tissue engineering or cell-based therapies, most strategies require that adult MSCs are isolated and expanded to (tens of) million cells (Schop *et al.*, 2009). To expand stem cells in a controlled, reproducible, and cost-effective way, extensive research is being performed on the development of bioreactors. Essential parameters for the optimization of an expansion process in a bioreactor are growth, metabolism, and growth inhibitors (Sotiropoulou *et al.,* 2006). To the best of our knowledge, the metabolism of human MSCs and the growth inhibition of human MSCs by metabolites have not been described in detail before. For the expansion of MSCs in culture several different media compositions are generated as per the need of the researcher. Some of them are Mesenchymal Stem Cell Basal Medium (MSCBM;

Cambrex) which is a widespread and suitable medium used in MSC cultivation, but it is extremely difficult to use generally for clinical treatment because of its unclear traceability and cost. Dulbecco's Modified Eagle's Medium (D-MEM) is also used for the expansion of MSCs. Apart from these, another media called Iscove's Modified Dulbecco's Media (IMDM) which is a highly enriched synthetic media well suited for rapidly proliferating, high-density cell cultures is used by supplying the appropriate levels of serum.

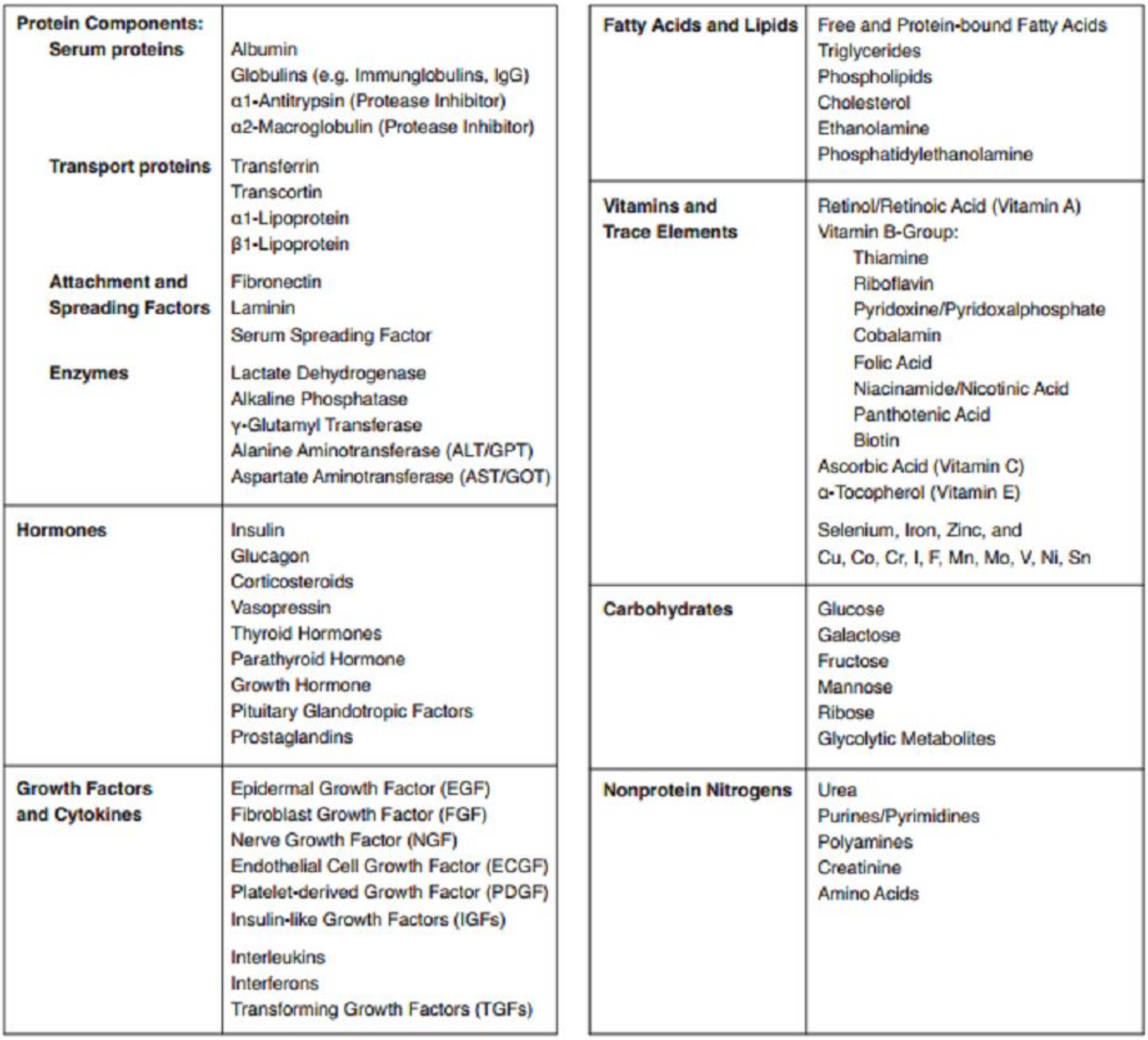
2.4.1 Role of FCS in *in-vitro* MSC culture

Animal serum is an extremely complex mixture of a large number of constituents, low and high molecular weight biomolecules, with different, physiologically balanced growth promoting and growth-inhibiting activities (Jung *et al.*, 2012). Serum is blood without any cells, platelets or clotting factors. Fetal bovine serum (FBS) is a cocktail of most of the factors required for cell attachment, growth and proliferation and is thus used as an almost universal growth supplement effective for most types of human and animal cells (Schop *et al.*, 2009). In MSC culture FCS is used as a serum-supplement, it is considered to be a rich source of nutrients. It has low levels of antibodies and more number of growth factors. The globular protein called bovine serum albumin forms the major component in FCS.

Serum functions as a buffering agent (Cifrian *et al.*, 1996) in addition it also offers protection against certain “cytotoxic” agents by unknown mechanisms. BSA present in serum acts as a carrier for lipids provides bulk of the protein for culture and also acts as a sink for proteases, like trypsin. The in vitro life span of cultured human fibroblasts has been found to vary significantly with differing lots of serum (Schneider *et al.,* 1978).

The following summarizes the immense role of serum in cell culture as serum provides:

- growth factors and hormones
- binding and transport proteins
- attachment and spreading factors
- additional amino acids, vitamins and trace elements
- fatty acids and lipids
- protease-inhibitors
- “detoxification” (due to binding and inactivation)
- (colloid) osmotic pressure
- reduction of shear stress



Protein Components: **Serum proteins**	Albumin Globulins (e.g. Immunglobulins, IgG) α1-Antitrypsin (Protease Inhibitor) α2-Macroglobulin (Protease Inhibitor)
Transport proteins	Transferrin Transcortin α1-Lipoprotein β1-Lipoprotein
Attachment and Spreading Factors	Fibronectin Laminin Serum Spreading Factor
Enzymes	Lactate Dehydrogenase Alkaline Phosphatase γ-Glutamyl Transferase Alanine Aminotransferase (ALT/GPT) Aspartate Aminotransferase (AST/GOT)
Hormones	Insulin Glucagon Corticosteroids Vasopressin Thyroid Hormones Parathyroid Hormone Growth Hormone Pituitary Glandotropic Factors Prostaglandins
Growth Factors and Cytokines	Epidermal Growth Factor (EGF) Fibroblast Growth Factor (FGF) Nerve Growth Factor (NGF) Endothelial Cell Growth Factor (ECGF) Platelet-derived Growth Factor (PDGF) Insulin-like Growth Factors (IGFs) Interleukins Interferons Transforming Growth Factors (TGFs)

Fatty Acids and Lipids	Free and Protein-bound Fatty Acids Triglycerides Phospholipids Cholesterol Ethanolamine Phosphatidylethanolamine
Vitamins and Trace Elements	Retinol/Retinoic Acid (Vitamin A) Vitamin B-Group: Thiamine Riboflavin Pyridoxine/Pyridoxalphosphate Cobalamin Folic Acid Niacinamide/Nicotinic Acid Panthotenic Acid Biotin Ascorbic Acid (Vitamin C) α-Tocopherol (Vitamin E) Selenium, Iron, Zinc, and Cu, Co, Cr, I, F, Mn, Mo, V, Ni, Sn
Carbohydrates	Glucose Galactose Fructose Mannose Ribose Glycolytic Metabolites
Nonprotein Nitrogens	Urea Purines/Pyrimidines Polyamines Creatinine Amino Acids

Figure 2.7: Components of FBS (Sotiropoulou *et al.,* 2006)

2.4.2 Role of glutamine in *in-vitro* MSC culture

For general cell culture, glucose and glutamine are the two most important nutrients for the generation of cellular energy (adenosine -triphosphate, ATP), which is necessary for cell growth and maintenance (Schop *et al.,* 2009). Glucose, the primary source of ATP for mammalian cells, is utilized either by oxidative phosphorylation (yielding about 30–38 mol of ATP per mole glucose) or by anaerobic glycolysis (yielding 2 mol of ATP and 2 mol of lactate per mole glucose). Both the glucose consumption and the lactate production indicate which metabolic route the cells use to produce energy from glucose (Glacken, 1988). Apart from a constituent of proteins (via protein biosynthesis), glutamine is also an important energy source for mammalian cell culture. Glutamine is metabolically deaminated to glutamate (Newsholme and Newsholme, 1989). The glutamate is then converted to α-ketoglutarate by either the transamination pathway yielding alanine or the deamination pathway using glutamate dehydrogenase (GDH) yielding an extra ammonia (Ljuggren and Haggstrom, 1994). α -Ketoglutarate is then converted to pyruvate. The conversion of glutamine to pyruvate is called the glutaminolysis. Pyruvate can next either be converted to lactate or alanine or be completely broken down in the citric cycle.

CHAPTER 3: MATERIALS AND METHODS

3.1 Preparation of antibiotics and chemicals for culture of MSCs

3.1.1 Pen Strep (0.1%)

0.6 grams of Penicillin G (Sigma) was weighed along with 0.5 grams of Streptomycin sulfate (Gibco). The mixture was dissolved in 100mL of PBS followed by filtration. It was stored in 4^{o}C for further use.

3.1.2 Hydrocortisome

0.72 grams of hydrocortisome (Gibco) was weighed. It was dissolved in about 5mL of DMSO (DMSO was added slowly to dissolve the powder). After it was dissolved completely, the final volume was adjusted to 20mL by ethanol.

3.1.3 Trypsin EDTA (0.1%)

100 mg of EDTA and 110mg of Trypsin (Sigma) was weighed and dissolved in 100 mL of PBS. The solution was filtered and stored in 4^{o}C for further use. It was pre warmed before use.

3.1.4 Composition of RNaST Lysis Buffer

RNase Inhibitor	2µg/µL
NaCl	0.135 M
Tris-HCL	9 mM (pH 8.0)
Dithiothreiotol	4.5 mM

3.2 Preparation of culture media

3.2.1 Iscove's Modified Dulbecco's Media (IMDM) with different serum concentration

3.2.1.1 Preparation of IMDM stock solution

17.67 grams of IMDM was weighed along with 3.024 grams of Sodium bicarbonate and dissolved in 1 litre of milliq. The mixture was then filtered and stored in 4^{o}C for further use.

3.2.1.2 Preparation of IMDM working solution

To prepare 100 mL of working solution, 100µL of Beta mercaptoethanol, 100 µL of Hydrocortisome and 1mL of Penstrep was taken. The final volume was adjusted to 100mL by IMDM stock solution and was filtered and stored in 4^{o}C.

3.2.1.3 Preparation of IMDM with different serum concentration

To prepare IMDM working solution with different concentrations of serum, IMDM working solution was taken and to it different volume of serum i.e. FBS (Fetal Bovine Serum) and MSC-FBS in the ratio 1:1 was mixed as shown below:

Table 3.1: Preparation of 50mL of IMDM solution with different concentration of serum

Percentage of serum	**Volume of IMDM working solution (mL)**	**Volume of serum (mL)**
20%	40	10
15%	43.5	7.5
10%	45	5
5%	47.5	2.5
2.5%	48.75	1.25
0.5%	49.75	0.25

3.2.2 IMDM with different glutamine concentration

3.2.2.1 Preparation of IMDM stock solution without glutamine

17.67 grams of glutamine (Gibco) free IMDM was weighed and to it 3.024 grams of sodium bicarbonate was added. The mixture was dissolved in milliq and the final volume was adjusted to 1 litre. The solution was filtered and was stored in 4^{o}C for further use.

3.2.2.2 Preparation of IMDM working solution without glutamine

To prepare 100 mL of working solution, 15mL of FBS was taken and to it 100µL of Beta mercaptoethanol, 100 µL of Hydrocortisome and 1mL of Pen strep was added. The final volume was adjusted to 100mL by stock solution.The solution was filtered and stored in 4^{o}C for further use. The media was pre-warmed before use.

3.2.2.3 Preparation of IMDM with different concentration of glutamine

From 200mM glutamine solution (100X), 4mM glutamine containing medium was prepared. Other dilutions were made using previously prepared glutamine free media and 4mM glutamine containing media as shown below:

Table 3.2: Preparation of 50mL of IMDM solution with different concentration of serum

Concentration of Glutamine (mM)	Volume of glutamine free media (mL)	Volume of 4mM glutamine containing media(mL)
4	---	50
2	25	25
1	37.5	12.5
0.5	43.25	6.25

0.25	46.875	3.125
0	50	---

3.3 Processing Bone Marrow cells

A human fetus was obtained from hospital after informed consent. Sample processing was done as soon as possible. The bone on shoulder joint and hip joint was cut. The skin and muscle was cleared off very carefully. Both the bones were cut with scissors on elbow joint and knee joint to make the bones open from both ends. Phosphate buffer saline and pen strep (1%) was taken in a 10 mL syringe and entire 10 mL was flushed through the cleared bone into a petridish. Again another petridish was taken and the same process was repeated till entire bone turned white and all the content was flushed out. The flushed out cells were plated in six-well plate containing IMDM with six different concentrations of serum as well as with six different concentrations of glutamine. These plates were incubated at 37°C with 5% CO_2 and the media was changed on every alternate day.

3.4 Cell culture and estimation of population doubling

Bone marrow cells were cultured in six different concentrations of serum as well as glutamine containing IMDM media with 1% Penicillin Streptomycin, 0.1% Hydrocortisome and 0.1% Beta-mercaptoethanol. The plates were incubated at 37^{o}C with 5% CO2. Once culture reached confluence, the cells were trypsinized (0.1% trypsin) and counted using a haemocytometer. 50,000 cells were were plated accordingly in each different concentration of serum as well as glutamine for next passage while the remaining cells were used for immunostaining and preparation of lysate. Cells were viewed in Nikon Eclipse TS100 microscope. The total number of cells seeded and harvested was noted down and population doubling was calculated.

$$\text{Population doubling (P.D)} = \frac{\log N - \log n}{\log 2}$$

where, N= No of cells harvested

n= No of cells seeded

$$\text{Population doubling time} = \frac{\text{P.D}}{\text{Number of days}}$$

3.5 Preparation of Lysate

When the adherent cells in culture dish became 80% confluent, the cells were trypsinised (0.1% trypsin) for 5-7 minutes (until the cells were detached from the culture plate) and resuspended in respective concentrations of serum or glutamine containing medium. Total number of cells were counted using haemocytometer and 50,000 cells per plate was plated for next passage. Remaining cells were cultured in a separate plate and after 6 hours the media was aspirated and the plate was washed with PBS. 200 µL of chilled 1X PBS/90mm dish was added and the cells were scrapped out using cell scrapper (BD Falcon). It was then centrifuged (Eppendorf centrifuge) at 2000g for 10-15 mins. Supernatant was gently discarded and to the pellet 10 µL of RNaST buffer (Applied Bioscience) was added. The pellet was dissolved in the buffer by gently vortexing the content. It was then immediately transferred to dry ice for immediate freezing. Eppendorf tubes containing lysate was then stored at -80°C for further use.

3.6 Real Time PCR

3.6.1 cDNA synthesis

One cell PCR method was used in this study. The lysate prepared above (containing RNA) was directly used for cDNA synthesis of Ki67, P21 and APE. The real time PCR reaction conditions were optimized by varying many parameters such as oligo dT concentration, hexamer concentration, dNTP concentration (Invitrogen), dTT concentration. Finally, the concentrations of all these parameters were optimized.

Table 3.3: Reaction parameters for cDNA synthesis of respective genes

S.N.	Components	Concentration	Volume/single reaction
1.	Lysate	----	2 µL
2.	OligodT	0.5 µg/µL	1 µL
3.	Hexamer	0.5 µg/µL	1 µL
4.	dNTPs	10mM	1 µL
5.	MilliQ		8 µL
		Total volume	13 µL

The tubes were incubated at 65°C for 5 mins and were put back in ice. Rests of the components were then added to the mixture and the final volume was made to 20µL.

Table 3.4: Final mixture for cDNA synthesis

S.N.	Components	Concentration	Volume/single reaction
1.	First strand buffer	5X	4 µL
2.	dTT	0.1mM	1 µL
3.	RNase H	5000U/mL	1 µL
4.	Reverse transcriptase	1000U/mL	1 µL

3.6.2 PCR cycling condition for cDNA synthesis

Short spinning was done to mix the components properly and following PCR cycling condition was set up.

Table 3.5: PCR cycling condition for cDNA synthesis (CCMB optimized protocol)

Steps	**Process**	**Temperature**	**Time**
1.	Priming	50°C	50 minutes
2.	Extension	75°C	15 minutes
3.	Hold	22°C	5 minutes

3.6.3 Real time PCR

3.6.3.1 Optimization of primers for real time PCR

The no template control (NTC) matrix was used for the optimization of primers for test genes (P21, Ki67 and APE) along with endogenous reference genes (TBP and B2M). For each optimizations, forward primer (3 µL), reverse primer (3 µL), MilliQ (9 µL) and SYBR green Master Mix (15 µL) were added and reaction was done under ABI Real time PCR instrument in triplicates.

Table 3.6: Primer optimization using NTC matrix

Forward/ Reverse	**2.5pM**	**5Pm**	**7.5pM**	**10pM**	**12.5pM**	**15pM**
2.5pM	2.5F 2.5R	5F 2.5R	7.5F 2.5R	10F 2.5R	12.5F 2.5R	15F 2.5R
5pM	2.5F 5R	5F 5R	7.5F 5R	10F 5R	12.5F 5R	15F 5R
7.5pM	2.5F 7.5R	5F 7.5R	7.5F 7.5R	10F 7.5R	12.5F 7.5R	15F 7.5R
10pM	2.5F 10R	5F 10R	7.5F 10R	10F 10R	12.5F 10R	15F 10R
12.5pM	2.5F 12.5R	5F 12.5R	7.5F 12.5R	10F 12.5R	12.5F 12.5R	15F 12.5R
15pM	2.5F 15R	5F 15R	7.5F 15R	10F 15R	12.5F 15R	15F 15R

On the basis of NTC matrix, different parameters like background noise/contamination, primer dimer formation etc were checked using ABI Real time PCR instrument and the primer concentrations which showed undetermined values were selected as optimized primer concentrations. The optimize sets for forward and reverse primers for each gene are given below:

Table 3.7: Optimized primer concentrations for test and reference genes

S.N	Test and Reference genes	Forward primer (pM)	Reverse primer (pM)
1	P21	2.5	5
2	Ki67	2.5	2.5
3	APE	2.5	7.5
4	TBP	5	7.5
5	B2M	2.5	2.5

Primers (Invitrogen) used in Real Time PCR and their sequences are listed below:

Table 3.8: List of primers used in Real Time PCR

S.N	Primer name	Primer Sequence (5'-3')
1.	Ki67-Forward	TGACCCTGATGAGAAAGATCAA (22 bp)
	Ki67-Reverse	ACGTCCAGCATGTTCTGAGG (20 bp)
2.	P21-Forward	TTAGCAGCGGAACAAGGAGT (20 bp)
	P21-Reverse	AGCCGAGAGAAAACAGTCCA (20 bp)
3.	APE-Forward	CGGAATTCCATGCCGAAGCGTGGGAAAA (28 bp)
	APE-Reverse	CGCAAGCTTTCACAGTGCTAGGTATAGGG (29 bp)
4.	TBP-Forward	TGCACAGGAGCCAAGAGTGAA
	TBP-Reverse	CACATCACAGCTCCCCACCA (20 bp)
5.	B2M-Forward	GGCTATCCAGCGTACTCCAA (20 bp)
	B2M-Reverse	GATGAAACCCAGACACATAGCA (22 bp)

3.6.3.2 Procedure

Optimized primer concentrations for each gene (P21, Ki67 and APE) were obtained from the above NTC reaction and master mix was prepared for quantitative analysis of respective genes using Real time PCR along with endogenous reference genes TBP (TATA binding protein) and B2M (Beta-2-microglobulin). The reaction mix for test genes, reference genes and control was prepared for triplicate reaction as follows:

Table 3.9: Reaction parameters for Real Time PCR

Components	Volume for Test/Reference	Volume for Control (NTC)
cDNA Template	3µL	---

Forward primer	3 µL	3 µL
Reverse primer	3 µL	3 µL
SYBR green Master Mix	15 µL	15 µL
MilliQ	6 µL	9 µL
Total	30 µL	30 µL

The SYBR green Mater Mix (Takara) consists of SYBR green I dye, Ampli Taq Gold DNA polymerase, dNTPs, passive reference dye, optimized buffer components. Excitation maxima and emission maxima for SYBR green are 494nm and 521nm respectively.

30 µL of Real Time PCR reaction mix prepared above was pipetted into three wells each containing 10 µL in a 384 well plate. For each gene, reaction was done in triplicates to avoid pipetting errors. No template control for each gene was also prepared for triplicate reaction. A clear thin optical film was used to cover the contents of the 384 well plate to prevent evaporation and cross contamination by mixing of the components to other wells. The plate was short spinned at 500 rpm for 30-40 seconds prior to loading the plate to ABI Real time PCR instrument. Real time PCR cycling parameters was set using SDS 2.3 version real time PCR program and detector for sample identification was also set.

3.6.3.3 Real time PCR cycling condition

The following PCR conditions were set in the SDS 2.3 version real time PCR program.

Table 3.10: Cycling conditions for Real-Time PCR

Stage 1	Stage 2	Stage 3	Stage 4
50°C 02:00	95°C 10:00	95°C 00:15 60°C 00:30 72°C 00:30	95°C 10:00 60°C 00:30 95°C 10:00
		Repeat 40 times	Dissociation stage

When the PCR run was complete, the data obtained was saved in a folder and the 384 well plate was removed from the instrument. Further calculations were done manually using the following formula.

ΔCt=Ct Test sample-Ct Reference

$\Delta\Delta$Ct=Ct Test sample-Ct Control

3.7 Amido Black staining

3.7.1 Preparation of Amido black stain

To prepare 100 mL of amido black solution, 0.1 gm of amido black was weighed and to it 40mL of methanol and 10mL of acetic acid was added and the volume was adjusted to 100 mL with MilliQ.

3.7.2 Preparation of Destaining solution

To prepare 100mL of destaining solution, 90mL methanol was mixed with 2mL of acetic acid and the volume was made to 100mL by adding 8mL MilliQ.

3.7.3 Preparation of Elution buffer

To prepare 50mL of elution buffer, 25mL of 50% ethanol was mixed with 50mM NaOH and 4µL of 0.1mM EDTA adjusting the final volume to 50 mL with MilliQ.

3.7.4 Procedure

The protein samples (lysate) were spotted on nitrocellulose membrane using slot blot apparatus and were dried at room temperature for 5 minutes. The membrane was made wet with double distilled water and Amido black stain was added. It was then incubated for 10-30 mins. The membrane was washed 3 times with double distilled water, each time for 5 mins. The membrane was then kept in destaining solution for 10 minutes to remove unbound stain. The membrane was rinsed with double distilled water and air dried. The membrane was cut precisely and kept in elution buffer (900µL) and was kept for vortexing 1600-1700 rpm for 30 minutes. Absorbance of the samples at 630nm was taken using spectrophotometer and standard graph was plotted. From this graph the concentration of protein was determined.

3.8 Immunostaining

3.8.1 Fixation

The cells were grown in coverslips in respective percentage of serum as well as glutamine containing media for 6-7 hours at 37^{o}C, 5% CO2. The media was aspirated and the cells in cover slip were covered with chilled 70% methanol in order to fix the cells for 10 minutes at room temperature. Fixative was aspirated and the cells were covered with PBS and stored at 4^{o}C.

3.8.2 Staining

To the cell sample in cover slip, blocking solution (3% BSA) was applied and was incubated for 20 minutes. Meanwhile primary antibody from Abcam (mouse raised anti-p21 and goat raised anti-Ki67) was prepared by diluting it in 1% BSA in the ratio of 1:200. The blocking solution was aspirated and diluted primary antibody was applied. It was incubated in room temperature for 2-3 hours. It was rinsed with 1X PBS, keeping for 5 minutes in each wash. Fluorochrome conjugated secondary antibody from Abcam (donkey raised anti-mouse anti-p21 and donkey raised anti-goat anti-Ki67) diluted in the ratio of 1:500 in 1% BSA was applied and was incubated for an hour at room temperature in dark .It was rinsed with 1X PBS, keeping for 5 minutes each time. The cover slips were then transferred to slide (upper surface facing downward) containing glycerol along with 1X DAPI. The mountant was allowed to fix for some time and the slide was observed under fluorescent microscope (Zeiss Axioplan 2).

3.9 X-Gal Staining for Beta-Galactosidase

3.9.1 Preparation of X-gal Dilution buffer

160mg Crystalline Potassium Ferricyanide (Sigma #P-8131, FW 329.2) of 5mM concentration,20mg magnesium chloride of 2mM concentration(sigma #M-8266,FW 95.21) was weighed and dissolved in 100 mL PBS. It was mixed well and stored at 4^{o}C, protected from light. The solution was warmed at 37^{o}C prior to use.

3.9.2 Preparation of X-gal stock solution (4% in DMF)

20mg X-gal (Boehringer Mannheim #745-740) was weighed and dissolved in 0.5mL DMF (N, N Dimethylformamide). It was stored at -20 C,protected from light.

3.9.3 X-gal Working solution

X-gal dilution buffer was pre warmed to prevent precipitation of X-gal. Then X-gal stock solution was diluted in the ratio of 1:40 in pre warmed X-gal dilution buffer.

3.9.4 X-Gal Staining

The cells were grown in coverslips in respective percentage of serum as well as glutamine containing media for 6-7 hours at 37^{o}C with 5% CO2.The media was aspirated and the cells in cover slip was covered with cold formalin(2% formaldehyde in PBS) in order to fix the cells, for 10 minutes at room temperature. Fixative was aspirated and the cells were covered with PBS.

The cover slip was washed with 3 changes in PBS for five minutes each and then rinsed in distilled water. The cover slips were then incubated in X-gal working solution at 37^{o}C for 12-16 hours. Incubation was done in humidified chamber to prevent cover slips from drying. The cover slips were then rinsed with PBS with two changes in PBS. It was then rinsed with distilled water briefly. The cover slip was counter stained with nuclear fast red for 3-5 minutes and rinsed with distilled water. The cover slip was directly mounted with aqueous mounting solution.

3.10 Estimation of Reactive oxygen species (ROS) by flow cytometry

BM zero passage cells were cultured in 6 different concentrations of serum. After the cells was 80% confluent, the media was removed from the plate. The cells were washed with PBS to remove media and cells were harvested using trypsin EDTA. These cells were suspended in serum containing media and centrifuged at 1600 rpm for 4minutes. 1mL of media was added to each tube and the cells were counted using hemocytometer.50,000 cells were plated in six-well plate for next passage and incubated at 37^{o}C with 5% CO2. Rest of the cells were cultured in the same plate and incubated at 37^{o}C with 5% CO2 for 24hours.

After 24hours, media was aspirated from each plate and PBS wash was given. BS with DCFDA (50mM) was then added to the cells. To 5mL of PBS, 2µL of DCFDA (50mM) was added. The plates were then incubated for 10 minutes at 37^{o}C. All this was done in dim light. The plates were then washed with PBS. The cells were harvested using Trypsin-EDTA and was suspended in PBS after blocking the process with serum containing medium.PBS suspended cells were then centrifuged at 1600 rpm for 4 minutes. The pellet was resuspended in 500 µL of PBS. The cells were then acquired and analyzed on BD FACS Array bioanalyzer. Excitation maxima and emission maxima for DCFDA are 495nm and 529nm respectively.

BM zero passage cells were also cultured in 6 different concentration of glutamine viz. 4mM, 2mM, 1mM, 0.5mM, 0.25mM glutamine containing media and one in glutamine free medium. Control was also kept for all these concentrations. Same procedure described above was applied for estimation of reactive oxygen species.

CHAPTER 4: RESULTS

4.1 Population Doubling (PD) and Growth curve

The bone marrow derived Mesenchymal cells were grown in IMDM media with different concentrations of serum (0.5 to 20%) as well as glutamine (0 to 4mM). The total numbers of cells were counted on haemocytometer for each passage and the number of days taken by the culture to become confluent was also noted. Population doubling (PD) and population doubling time was calculated by using the formula mentioned in section 3.5 of materials and methodology.

Table 4.1: Cell count and Population doubling estimation with different serum concentrations

% of serum	No. of passage	No. of cells plated(n)	No. of cells harvested(N)	Time in days	Population Doubling	Population Doubling Time
20%	1p	30000	825000	6	4.781	0.796
	2p	50000	810000	6	4.018	0.669
	3p	50000	730000	7	3.868	0.552
	4p	50000	375000	6	2.907	0.484
15%	1p	30000	510000	6	4.087	0.681
	2p	50000	750000	6	3.907	0.651
	3p	50000	500000	7	3.322	0.474
	4p	50000	250000	7	2.322	0.331
10%	1p	30000	366666	7	3.611	0.515
	2p	50000	187500	12	1.907	0.158
5%	1p	30000	112500	7	1.907	0.272
	2p	50000	97500	15	0.963	0.064
2.50%	1p	30000	225000	13	2.907	0.223
	2p	50000	67500	13	0.433	0.033
0.50%	1p	30000	50000	6	0.737	0.122

Bone marrow derived Mesenchymal stem cells grew faster in IMDM containing higher concentrations of serum (20% and 15%) compared to cells grown in IMDM with lower concentrations of serum (10%, 5%, 2.5% and 0.5%) as shown by total cell count before & after each passage and number of days to become confluent (Table 4.1). This was also supported by the population doubling time and growth curve (Table 4.1 and figure 4.1). The population doubling time of the cells grown in higher concentration of serum is also higher than the population doubling time of the cells grown in lower concentrations of serum. Cells cultured in IMDM with 20% and 15% grew upto fourth passage, after which these cells became senescent while cells cultured in IMDM with 10%, 5%, 2.5% and 0.5% grew only upto second passage except for 0.5% which grew for only one passage and became senescent and began to die.

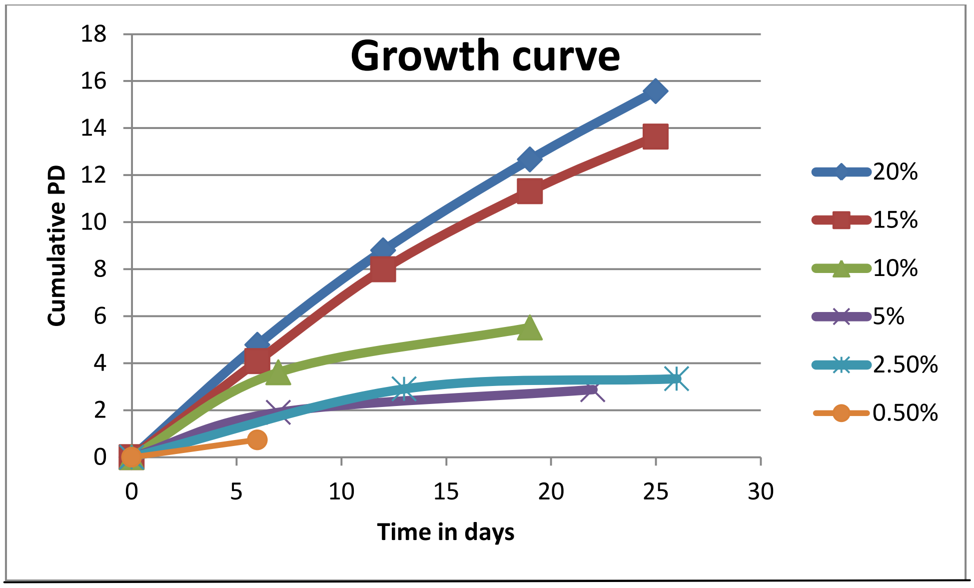


Fig 4.1: Growth curve of bone marrow derived Mesenchymal stem cells grown in different concentration of serum

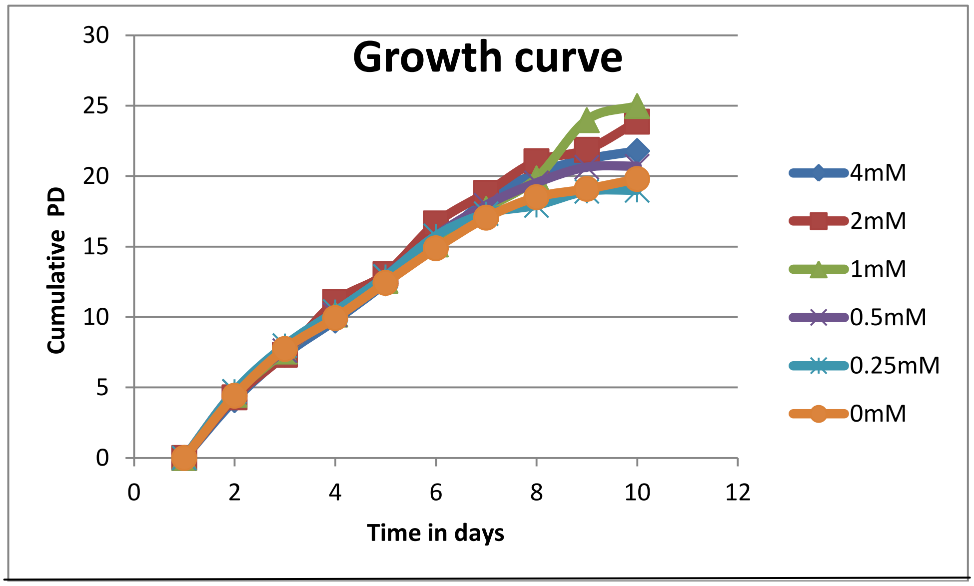


Fig 4.2: Growth curve of bone marrow derived MSCs grown in different concentration of glutamine

Bone marrow derived Mesenchymal stem cells grew almost similarly in IMDM containing glutamine irrespective of the concentrations of glutamine used (0mM to 4mM) as shown by total cell count at the beginning and end of each passage and number of days to become confluent (Table 4.2). This was also supported by the population doubling time and growth curve (Table 4.2 and figure 4.2). The population doubling times were also comparable for cells at same passage number for each glutamine concentrations which meant that the cells took similar number of days to become confluent. The cells grew upto ninth passage irrespective of glutamine concentration in the media and became senescent and began to die.

Table 4.2: Cell count and Population doubling estimation with different glutamine concentrations

Conc of glutamine	No of passage	No of cells plated(n)	No of cells harvested(N)	Time in days	Population Doubling	Population Doubling Time
4mM	1p	35000	600000	6	4.099	0.683
	2p	50000	463888	4	3.213	0.803
	3p	50000	270833	7	2.437	0.348
	4p	50000	311666	7	2.640	0.377
	5p	50000	375000	6	2.906	0.484
	6p	50000	400000	7	2.999	0.428
	7p	50000	187500	8	1.906	0.238
	8p	50000	100000	7	0.999	0.142
	9p	50000	75000	5	0.584	0.116
2mM	1p	35000	691666	6	4.304	0.717
	2p	50000	405555	4	3.019	0.754
	3p	50000	675000	7	3.754	0.536
	4p	50000	197500	7	1.981	0.283
	5p	50000	600000	6	3.584	0.597
	6p	50000	225000	7	2.169	0.309
	7p	50000	237500	8	2.247	0.280
	8p	50000	87500	7	0.807	0.115
	9p	50000	200000	5	1.999	0.400
1mM	1p	35000	783333	6	4.484	0.747
	2p	50000	419166	4	3.067	0.766
	3p	50000	308333	7	2.624	0.374
	4p	50000	262500	7	2.392	0.341
	5p	50000	300000	6	2.584	0.430
	6p	50000	287500	7	2.523	0.360
	7p	50000	225000	8	2.169	0.271
	8p	50000	875000	7	4.129	0.589
	9p	50000	100000	5	0.999	0.200
0.5mM	1p	35000	916666	6	4.710	0.785
	2p	50000	375277	4	2.907	0.726
	3p	50000	291666	7	2.544	0.363
	4p	50000	337500	7	2.754	0.393
	5p	50000	362500	6	2.857	0.476
	6p	50000	225000	7	2.167	0.309
	7p	50000	150000	8	1.584	0.198
	8p	50000	107500	7	1.104	0.157
	9p	50000	52500	5	0.071	0.014
0.25mM	1p	35000	930000	6	4.731	0.788
	2p	50000	474200	4	3.245	0.811
	3p	50000	266666	7	2.415	0.345
	4p	50000	280000	7	2.485	0.355
	5p	50000	366666	6	2.874	0.479
	6p	50000	150000	7	1.584	0.226
	7p	50000	75000	8	0.584	0.073
	8p	50000	100000	7	0.999	0.142
	9p	50000	52500	5	0.071	0.014

4.2 Morphology of Bone Marrow derived MSCs

Change in cell morphology is regarded as one of the important sign of ageing. Mesenchymal stem cells in-vitro show varied morphology on long term culture. In this study cells were grown in different concentrations of serum (0.5 to 20%) as well as glutamine (0 to 4mM). For all treatments, the cells in early passages showed thin spindle shaped morphology with no damage in cell structure (figure 4.3 and figure 4.4). As these cells entered into senescence, the cells for all concentrations of serum and glutamine looked bigger in size, spread further (became longer) and contained more podia and actin stressed fibers. In addition, the culture dishes of late passage cells also contained cell debris floating in the culture medium.

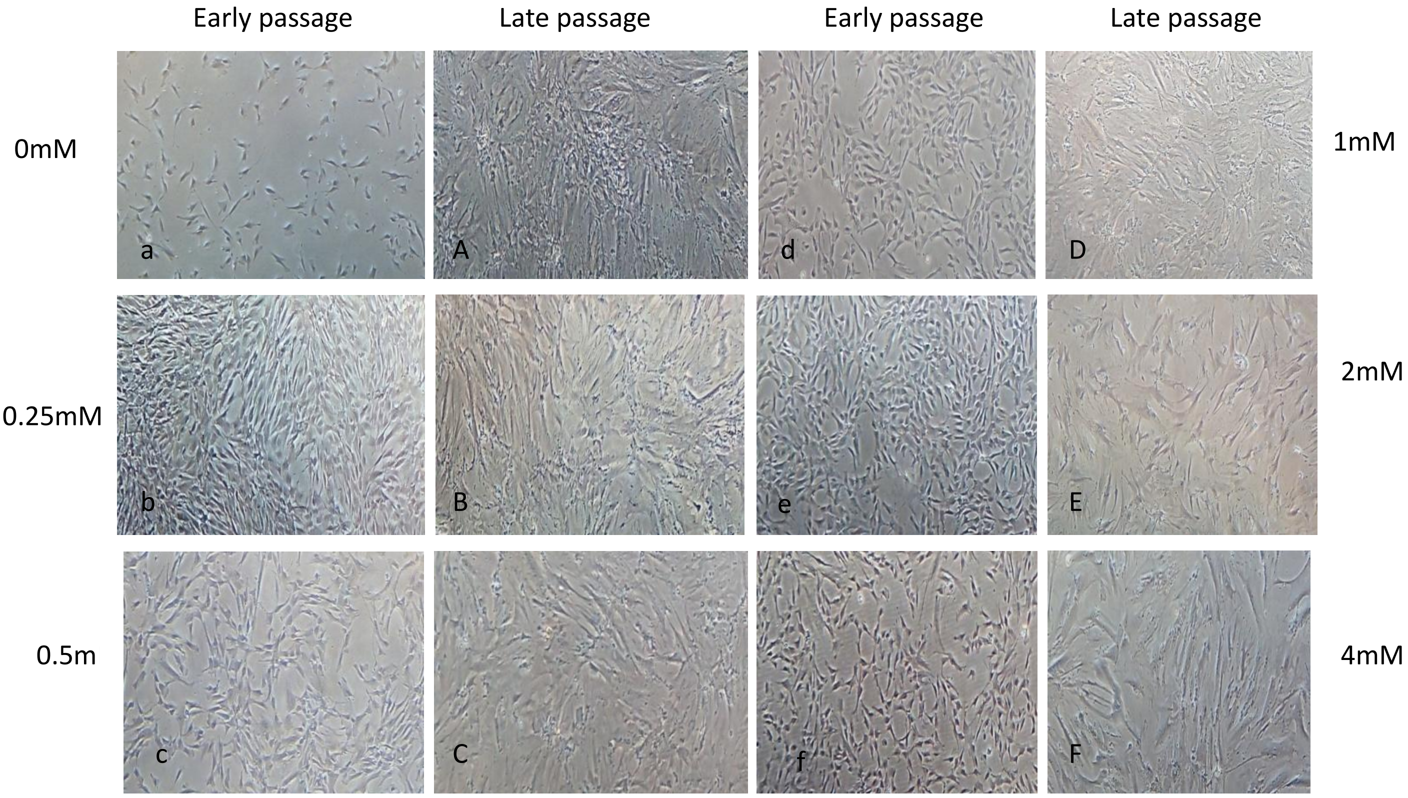


Figure 4.3: Morphological changes in bone marrow cells cultured in different concentrations of glutamine in early and late passage. Early and late passages are represented by small letters and capital letters respectively.

The cell density for cells grown in different concentrations of glutamine was almost similar for early and late passages of respective concentrations (figure 4.3). For all concentrations of glutamine (0-4mM), the cells in late passage (only after sixth passage) showed damaged morphology as compared to early passage.

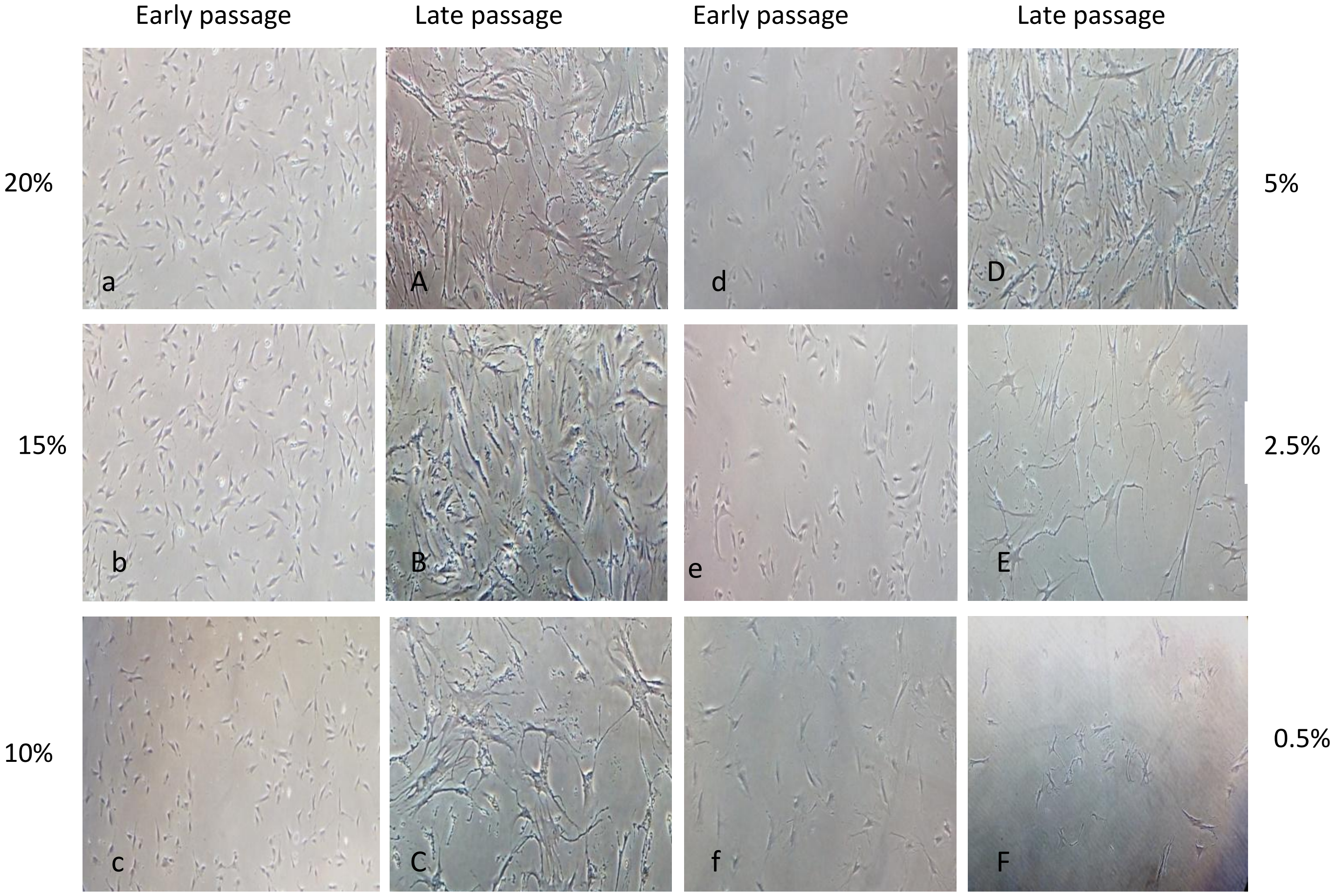


Figure 4.4: Morphological changes in bone marrow cells cultured in different concentrations of serum in early and late passage. Early and late passages are represented by small letters and capital letters respectively.

The cell density was decreased significantly from cultures grown in higher concentration of serum viz. for 20% and 15% compared to lower concentrations viz. 10%, 5%, 2.5% and 0.5% as observed in figure 4.4. Cells grown in 2.5% and 0.5% of serum showed flattened morphology from second passage and cell number decreased rapidly. Cells grown in 5% and 10% of serum showed flattened morphology after second passage and cell number also decreased significantly. Cells grown in 15% and 20% of serum showed signs of aging from fourth passage onwards.

4.3 Immunostaining for Ki67 and p21 proteins

The expressions of proliferation marker Ki67 and senescence marker p21 were tested using immunostaining technique in Axioimager 2 fluorescent microscope. The filters were chosen according to the flourochrome conjugated secondary antibody used in the study. FITC filter was used to detect Ki67 and red filter was used for p21. DAPI was used to stain nucleus so that the cells could be focused under fluorescent microscope and then the filters were changed to observe the expression of p21 and Ki67 in different passages of bone marrow derived Mesenchymal stem cells grown in IMDM with varying concentrations of glutamine and serum.

The expression of Ki67 was observed as green fluorescent in cells at both early and late passages of Mesenchymal stem cells irrespective of the glutamine concentration used whereas the expression of p21 (red fluorescent in cells) was not observed in any cells in either early or late passage. Merged image of all stains also supported this observation (figure 4.5 to 4.8).

The expression of Ki67 was also observed cells at both early and late passage of all serum varying IMDM media as green fluorescent in cells (figure 4.9 to 4.12) whereas the expression of p21 was observed as red fluorescent even in early passages of lower serum containing media (photoplates 7 and 11 of figure 4.10). The senescence associated protein p21 kept on expressing in late passage cells grown at lower serum containing media (figure 4.11 and 4.12)

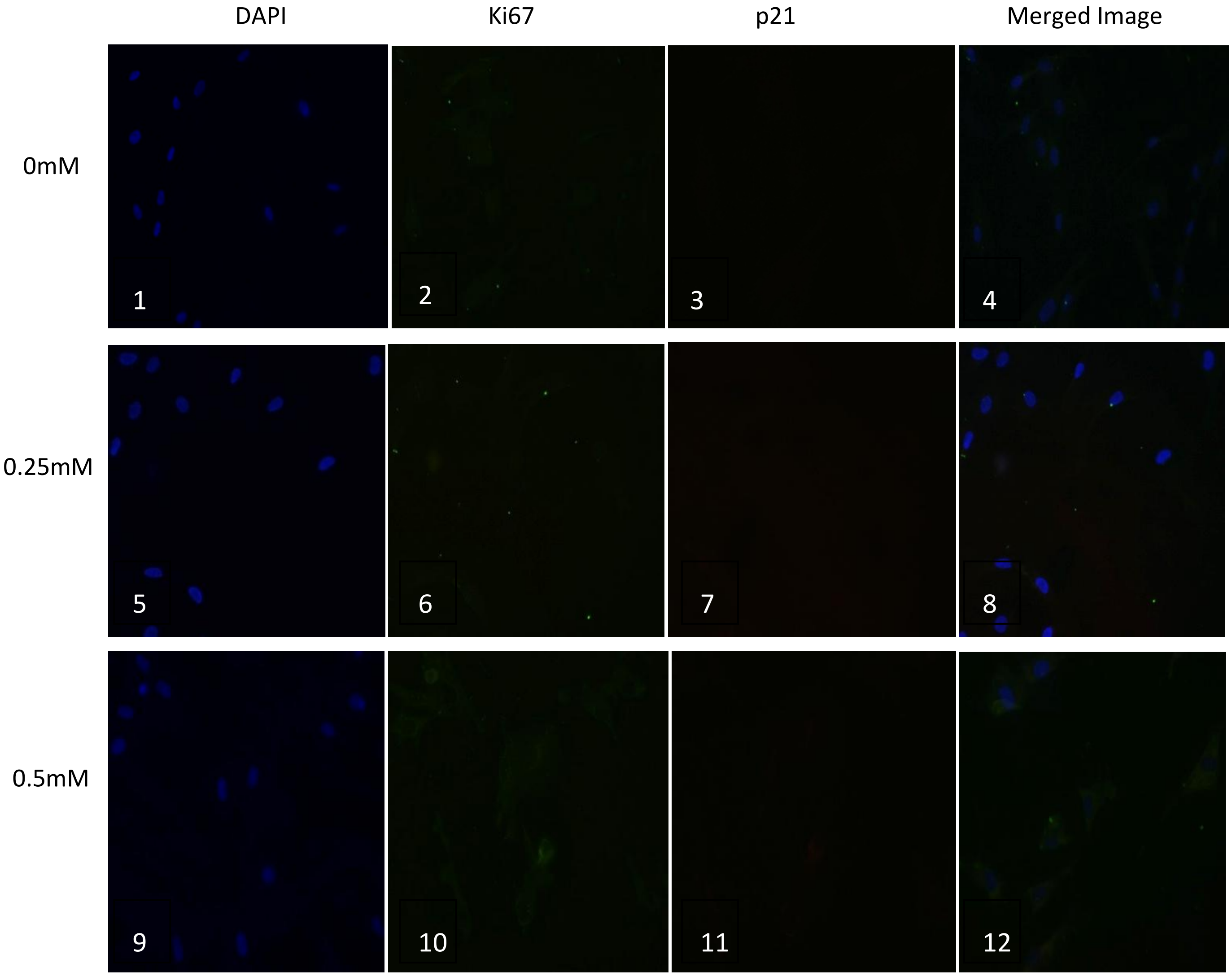


Figure 4.5: Immunostaining of bone marrow cells grown in different concentrations of **glutamine** during their **early passage.** Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0mM** glutamine concentration respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0.25mM** glutamine concentration respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0.5mM** glutamine concentration respectively.

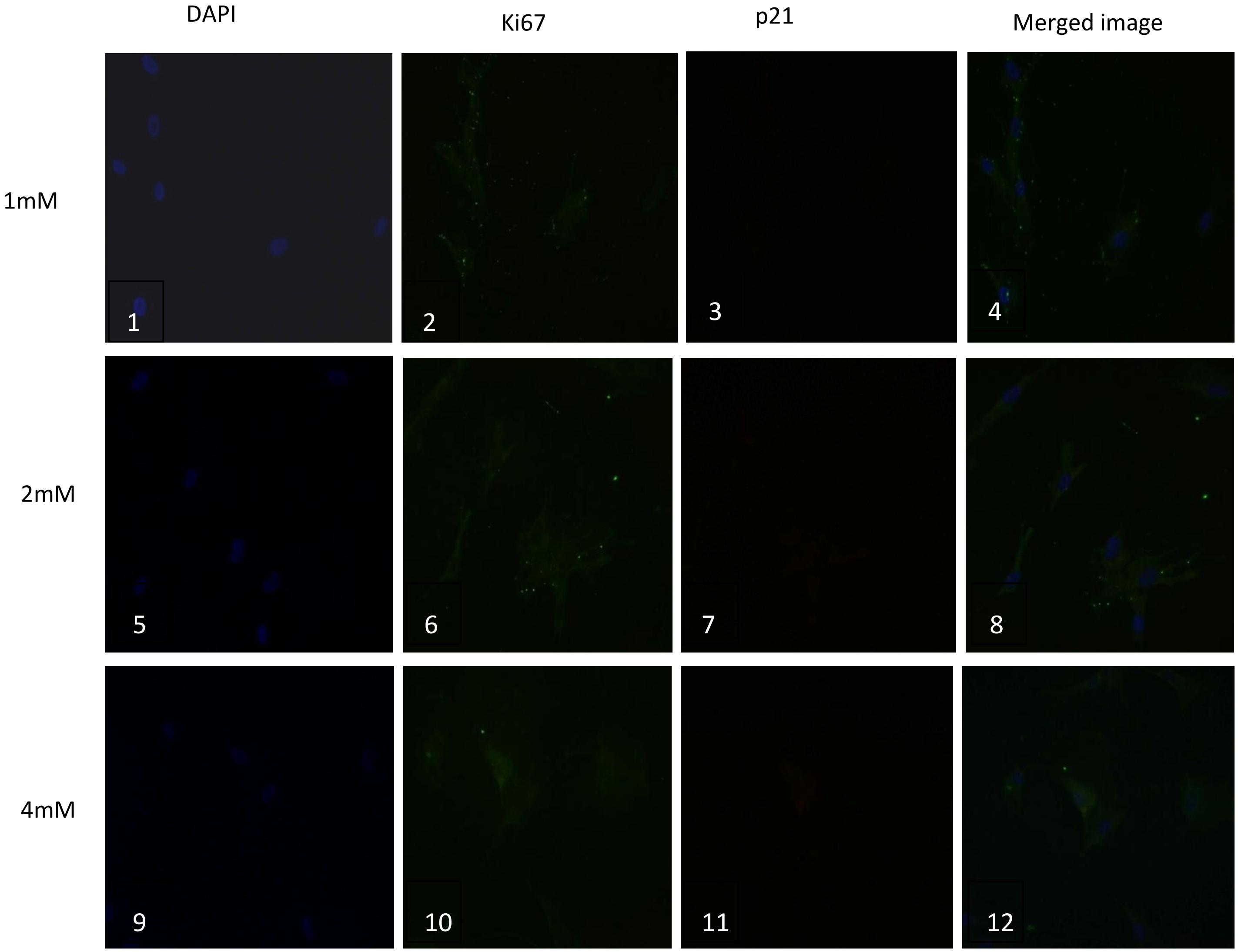


Figure 4.6: Immunostaining of bone marrow cells grown in different concentrations of **glutamine** during their **early passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **1mM** glutamine concentration respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **2mM** glutamine concentration respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **4mM** glutamine concentration respectively.

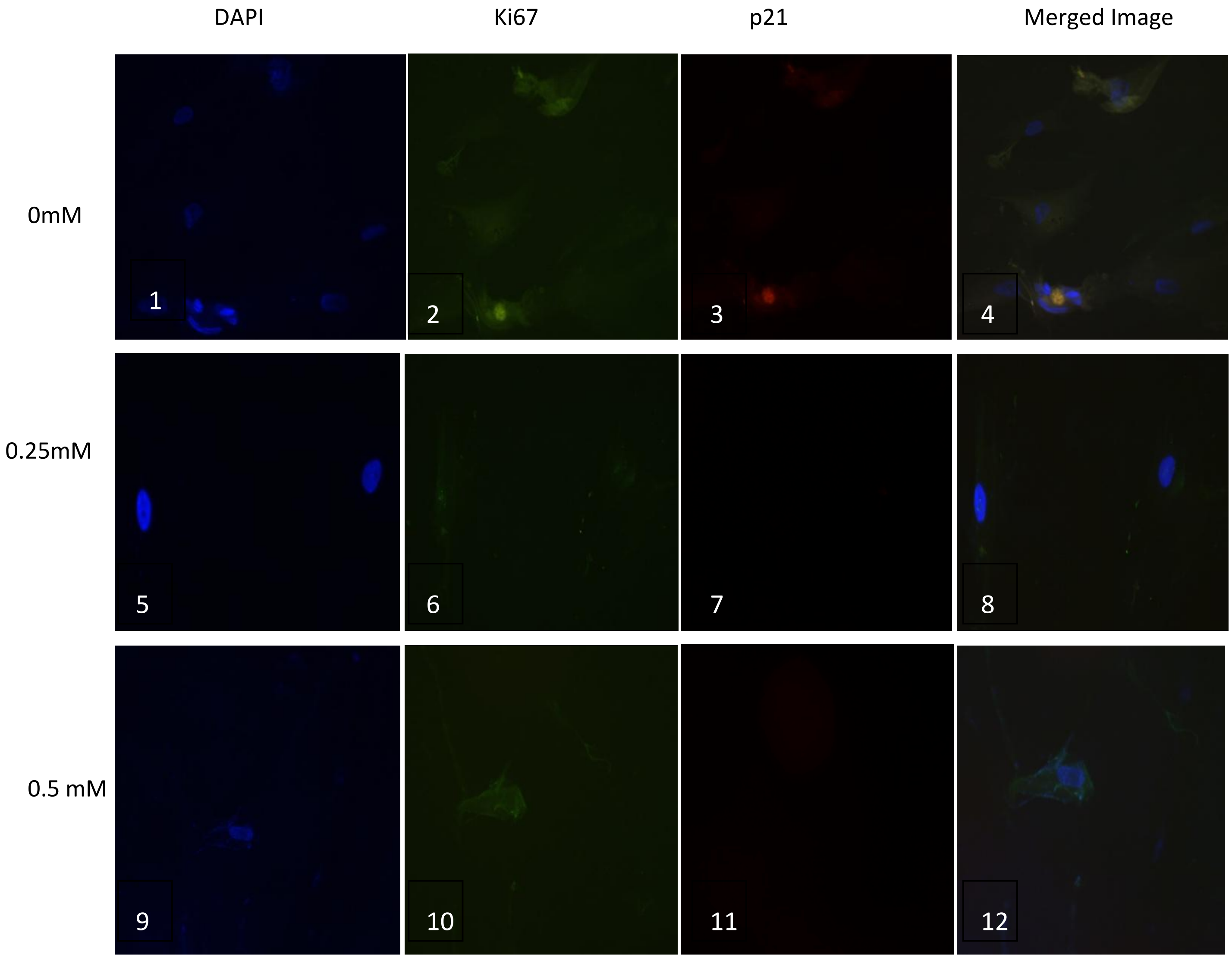


Figure 4.7: Immunostaining of bone marrow cells grown in different concentrations of **glutamine** during their **late passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0mM** glutamine concentration respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0.25mM** glutamine concentration respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **0.5mM** glutamine concentration respectively.

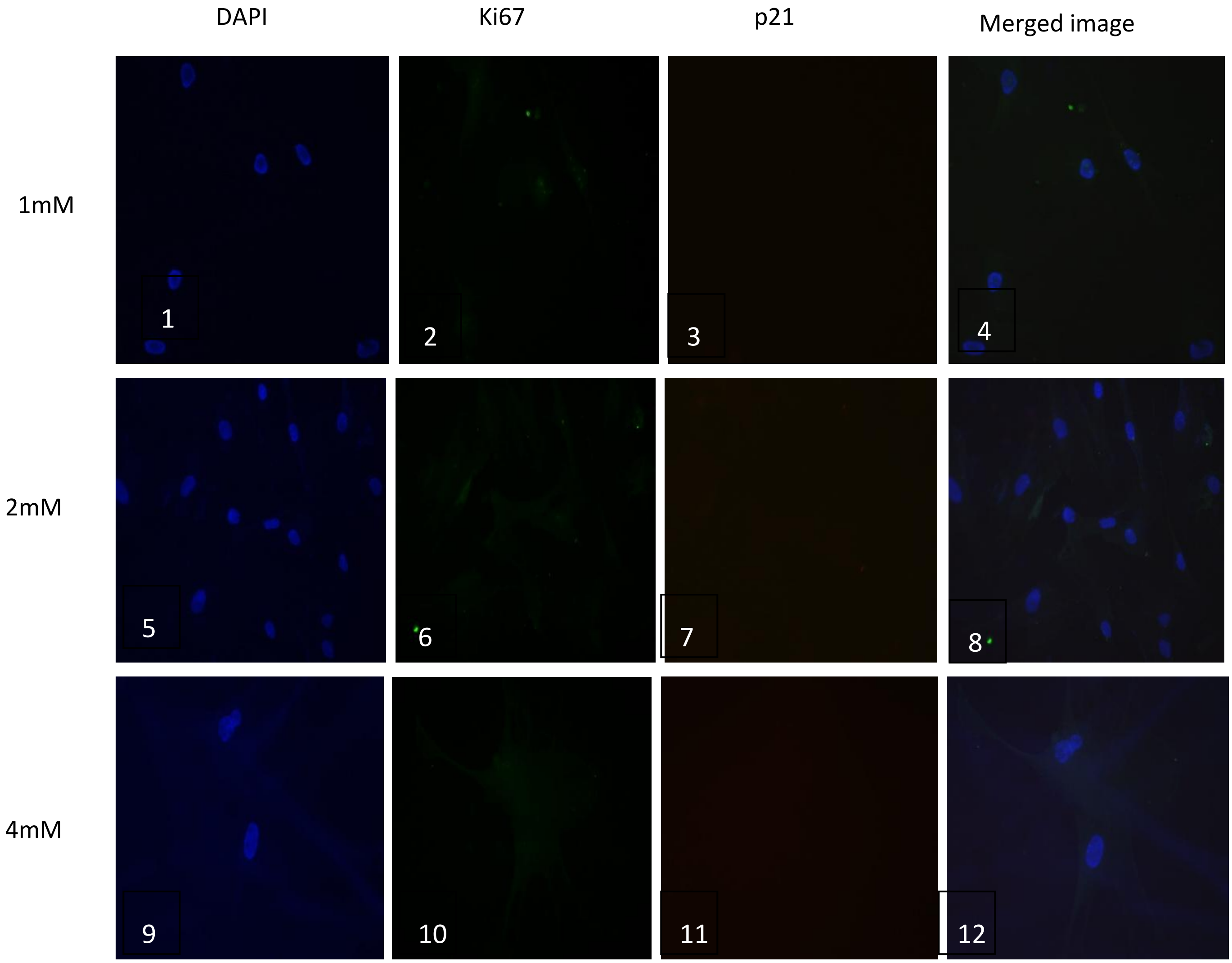


Figure 4.8: Immunostaining of bone marrow cells grown in different concentrations of **glutamine** during their **late passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **1mM** glutamine concentration respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **2mM** glutamine concentration respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **4mM** glutamine concentration respectively.

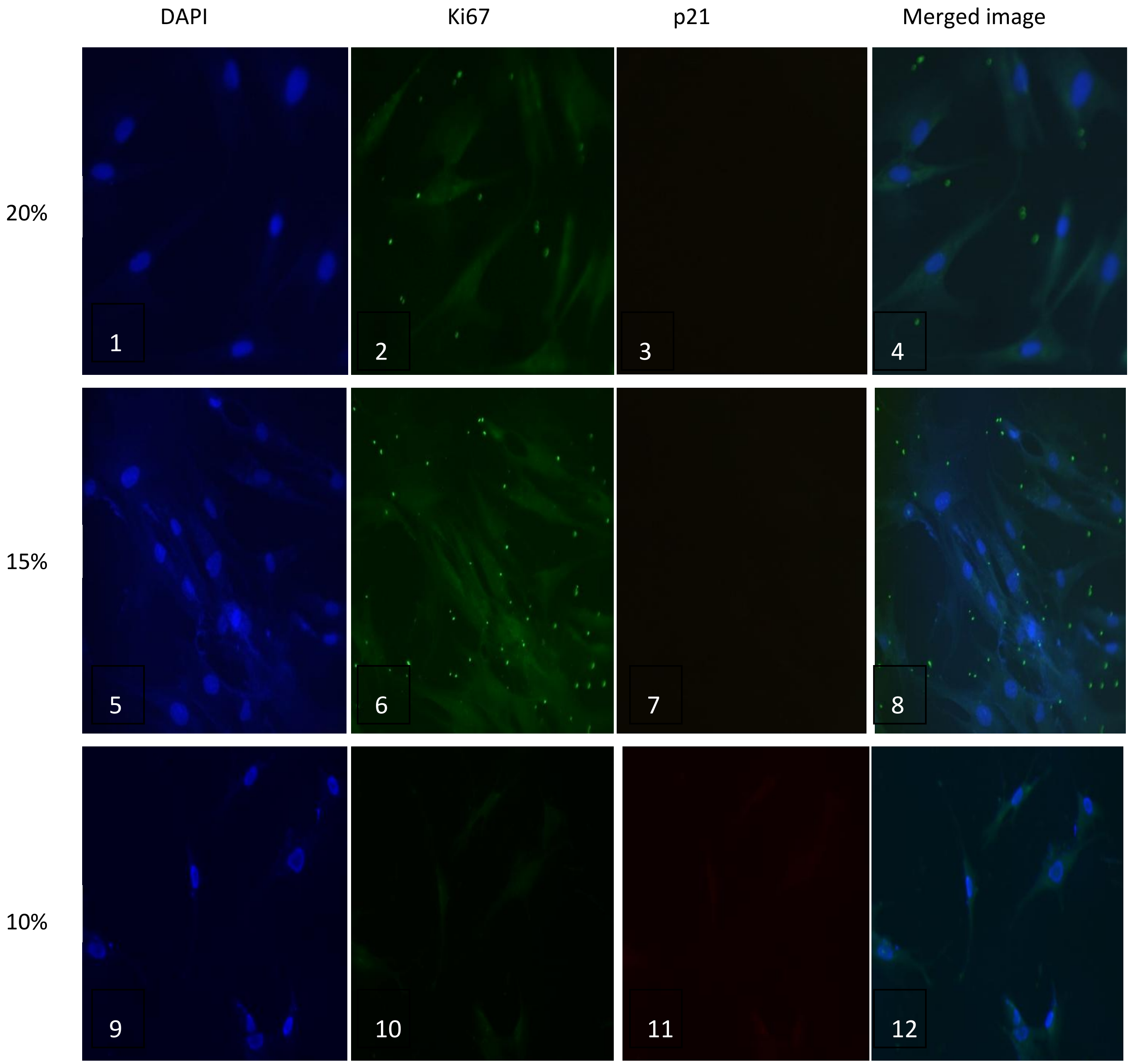


Figure 4.9: Immunostaining of bone marrow cells grown in different concentrations of **serum** during their **early passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **20%** serum respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **15%** serum respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **10%** serum respectively.

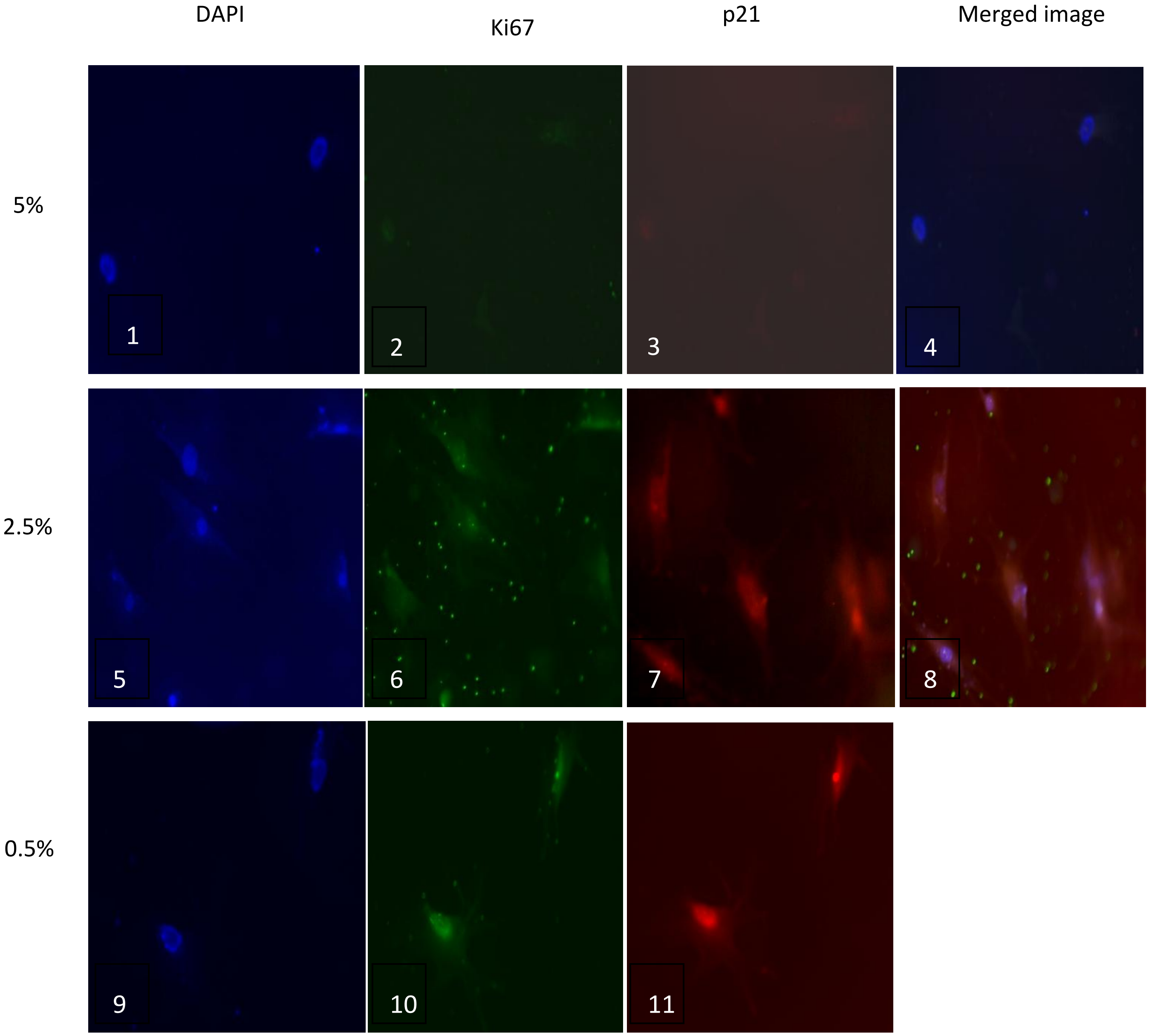


Figure 4.10: Immunostaining of bone marrow cells grown in different concentrations of **serum** during their **early passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **5%** serum respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **2.5%** serum respectively. Figure no 9 to 11 represents for DAPI stained, Ki67 stained and p21 stained image for bone marrow derived Mesenchymal stem cells grown in **0.5%** serum respectively.

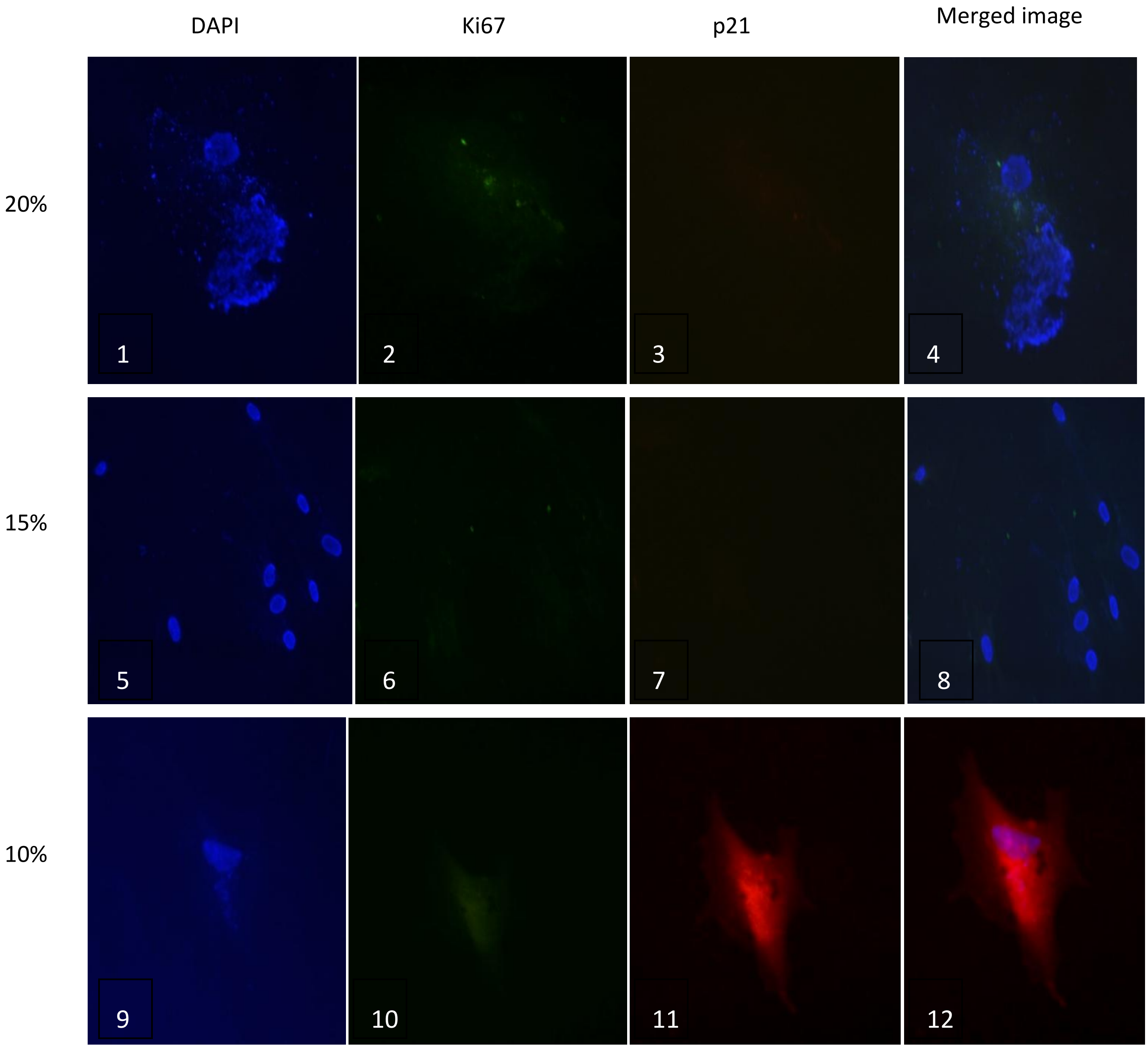


Figure 4.11: Immunostaining of bone marrow cells grown in different concentrations of **serum** during their **late passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **20%** serum respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **15%** serum respectively. Figure no 9 to 12 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **10%** serum respectively.

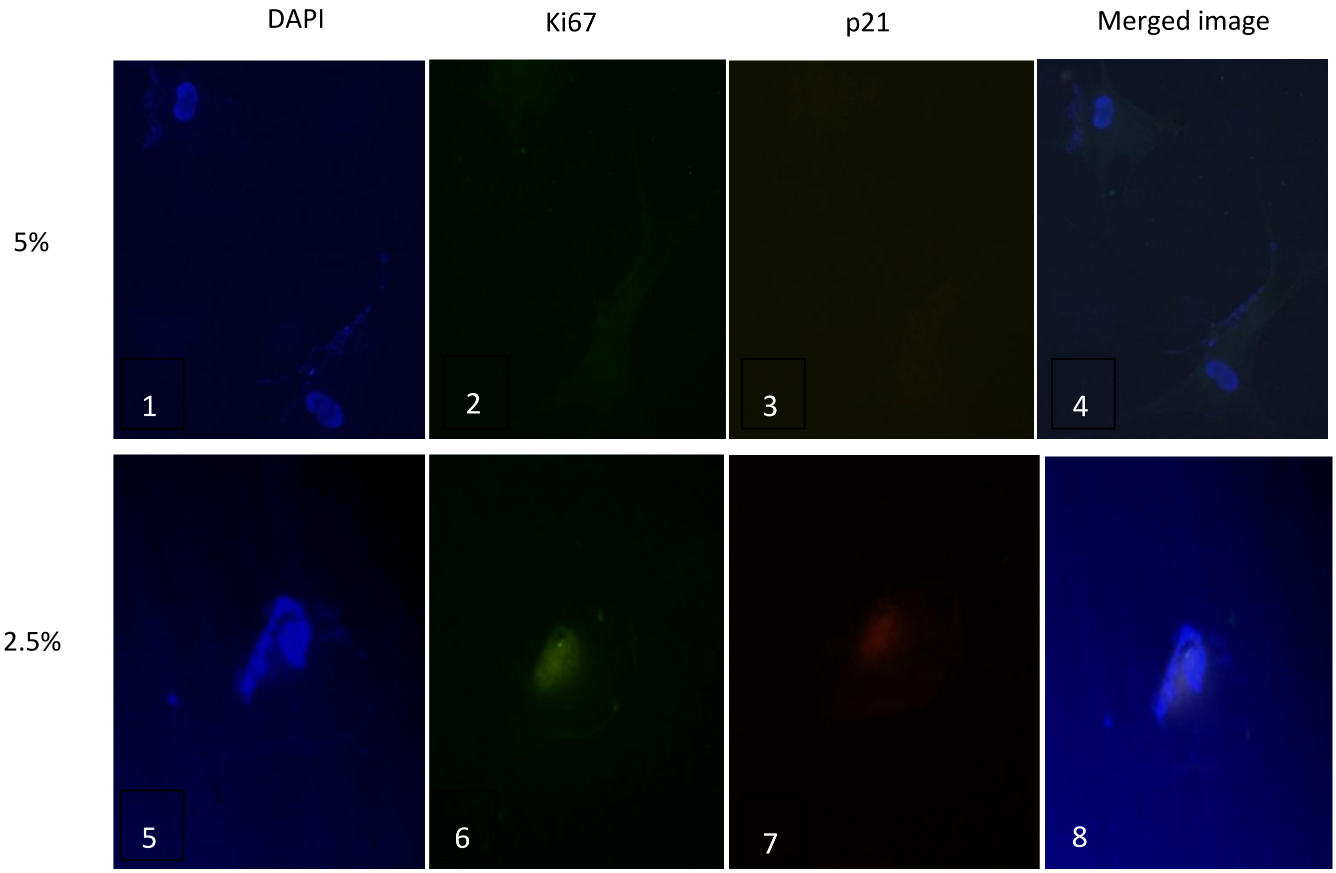


Figure 4.12: Immunostaining of bone marrow cells grown in different concentrations of **serum** during their **late passage**. Figure no 1 to 4 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **5%** serum respectively. Figure no 5 to 8 represents for DAPI stained, Ki67 stained, p21 stained and merged image for bone marrow derived Mesenchymal stem cells grown in **2.5%** serum respectively.

4.4 Senescence associated beta-gal staining

Senescence associated beta gal is a most commonly used biomarker of aging. Beta galactosidase is produced in aged cells. X-gal along with potassium ferrocyanide, potassium fericyanide and magnesium chloride is used for beta gal staining. Beta galactosidase produced in aged cells cleave X-gal present in the stain and gives blue colour. In this study, cells of early and late passage are stained and are related to aging. The cells in early passage do not show any stain while that of late passage shows blue stain of beta galactosidae in their cytoplasm. Figures 4.13 and 4.14 represent beta gal staining for serum (15%) and glutamine (2mM) in their early and late passages. No change in beta gal staining and hence no difference in blue color was observed in the cytoplasm of MSCs in all concentrations of serum and glutamine. Hence, any comparative analysis of ageing in response to serum and glutamine starvation could not be done.

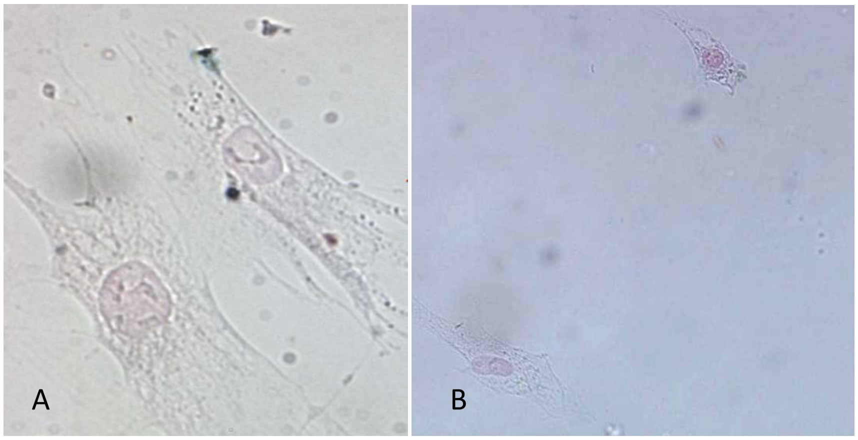


Figure 4.13: Beta-gal staining of cells cultured serum and glutamine in their early passage. A symbolizes 15% serum and B symbolizes 2mM glutamine

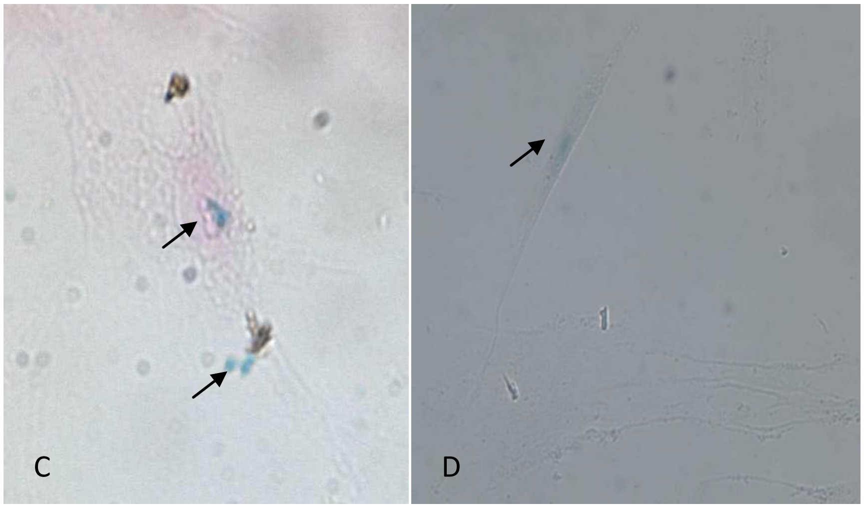
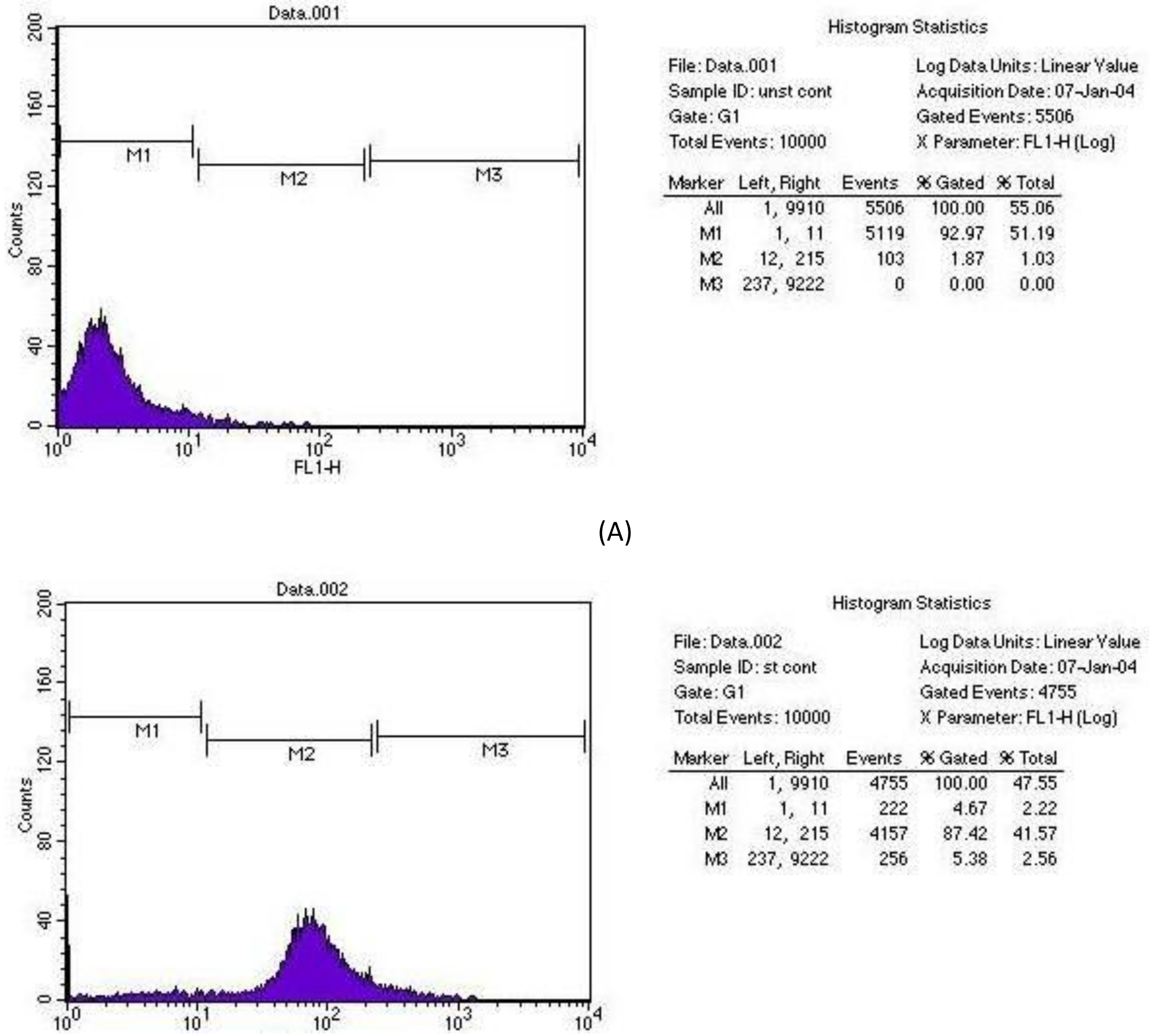


Figure 4.14: Beta-gal staining of cells cultured glutamine in their late passage. Black arrow denotes the region of stained beta galactosidase in aged cells. C symbolizes 15% serum and D symbolizes 2mM glutamine

4.5 FACS for estimation of ROS

DCFDA (2’7’- dichlorofluorescein diacetate) measures the reactive oxygen species produced in cell. DCFDA diffuses into cell where it is deacetylated by the cellular esterase to a non-fluorescent compound. ROS present in the cells oxidizes the non fluorescent compound to 2’,7’-dichloroflurescein (DCF) giving fluorescence. The amount of fluorescence produced is detected by the FL1 detector.



Histogram Statistics

File: Data.001
Sample ID: unst cont
Gate: G1
Total Events: 10000
Log Data Units: Linear Value
Acquisition Date: 07-Jan-04
Gated Events: 5506
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	5506	100.00	55.06
M1	1, 11	5119	92.97	51.19
M2	12, 215	103	1.87	1.03
M3	237, 9222	0	0.00	0.00

(A)

Histogram Statistics

File: Data.002
Sample ID: st cont
Gate: G1
Total Events: 10000
Log Data Units: Linear Value
Acquisition Date: 07-Jan-04
Gated Events: 4755
X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	4755	100.00	47.55
M1	1, 11	222	4.67	2.22
M2	12, 215	4157	87.42	41.57
M3	237, 9222	256	5.38	2.56

(B)

Figure 4.15: Histogram showing control unstained (A) and control stained (B) population of cells

The above histogram shows the total percentage of stressed cells. M1 represents unstained population and M2 represents stained population. M3 represents the total percentage of stressed population. On the basis of population marked in stained control cells, the cells falling outside the gate are counted as high ROS producing stressed cells and total percentage of stressed population is determined.

Table 4.3 (A): Percentage of unstained cells, stained cells and cells with an increased production of ROS grown in different concentrations of serum

No of passage	Percentage of cells								
	20% serum			15% serum			10% serum		
	M1	M2	M3	M1	M2	M3	M1	M2	M3
1p	9.61	88.00	0.05	4.63	91.05	0.07	10.95	83.71	3.91
3p	15.64	81.38	2.34	15.86	79.60	4.18	17.35	66.03	15.53
5p	14.58	72.63	10.88	23.51	59.36	14.65	------	------	------

Table 4.3 (B): Percentage of unstained cells, stained cells and cells with an increased production of ROS grown in different concentrations of serum

No of passage	Percentage of cells					
	5% serum			2.5% serum		
	M1	M2	M3	M1	M2	M3
1p	16.20	80.84	0.87	17.72	79.72	0.02
2p	21.84	63.12	11.92	24.76	60.41	13.61

In this study, estimation of reactive oxygen species was done for each passage of bone marrow derived Mesenchymal stem cells grown in IMDM with different serum concentrations (Table 4.3 A & B). The percentage of stressed cells i.e cells producing enchanced level of reactive oxygen species increased with increase in passage number for each serum concentrations. The cells grown in IMDM containing lower concentration of serum (2.5%, 5% and 10%), the ROS accumulated in the cells even after first passage. Thus, the serum starved condition could not help the cells to prolong ageing.

4.6 Amido black staining

Amido black staining was done to determine the concentration of protein in cell lysate which was used to determine the value of relative gene expression in real time PCR. Amido black is a very sensitive technique as it can be used to determine even small concentrations of protein in sample.

Table 4.4: Concentration of protein in cells cultured in different concentrations of serum

S.N.	Passage	Concentration of serum(%)	Protein concentration (µg/µl)
1.	Early passage	20	1.478261
		15	2.51087
		10	0.047826
		5	1.26087
2.	Late passage	20	0.619565
		15	0.51087
		10	0.51087
		5	0.326087

The protein concentration of cells cultured in IMDM containing different concentrations of serum is found to be greater in early passage as compared to cells in late passage (Table 4.8). This variation is obtained because the density of cells in early passage is higher than that of late passage.

Table 4.5: Concentration of protein in cells cultured in different concentrations of glutamine

S.N.	Passage	Concentration of glutamine(mM)	Protein concentration (µg/µl)
1.	Early passage	4	0.934783
		2	0.902174
		1	0.934783
		0.5	0.934783
		0.25	0.619565
		0	0.678351
2.	Late passage	4	0.826087
		2	1.054348
		1	0.630435
		0.5	0.76087
		0.25	0.826087
		0	0.76087

The protein concentration of cells cultured in IMDM containing different concentrations of glumanine is found to be almost comparable between cells in early passage and late passage (Table 4.9). This result was also supported by cell count. The density of cells cultured in different concentrations of glutamine was also found to be almost similar in each passage for respective concentrations of glutamine.

4.7 Real time quantification

Real time PCR was done to quantify the relative expression of ageing related genes viz. Ki67 (proliferative marker), p21 (senescent marker) and APE (apurinic/ apyrimidinic endonuclease). In this study direct cell to PCR method was used. From the cells cultured in IMDM containing different concentrations of serum and glutamine, cell lysate containing RNA was prepared using RNaST buffer. Protein concentration was determined in the lysate by amido black mehod (table 4.8 and 4.9). cDNA synthesis was then carried out directly from cell lysate containing RNA before doing real time PCR. Expression levels of ki67, p21 and APE were analyzed taking same amount of template for both serum and glutamine.

4.7.1 Relative expression of ageing related markers in varying concentrations of serum containing media

Cells were grown in IMDM containing different concentrations of serum. Relative gene expression of ki67,p21 and APE was then calculated comparing expression of aging related genes viz. ki67, p21 and APE in early and late passage. For real time PCR, for each concentrations of serum (20%, 15%, 10%, 5%, 2.5% and 0.5%) two culture dishes were taken, one containing cells in early passage (First or second passage) and other containing cells in late passage (third passage and so on) for cells grown in IMDM with different concentrations of serum). Following real time PCR for cells cultured in varying concentration of serum containing media, the relative gene expression of respective genes were calculated using the formula mentioned in section 3.6.3.3 of materials and methodology.

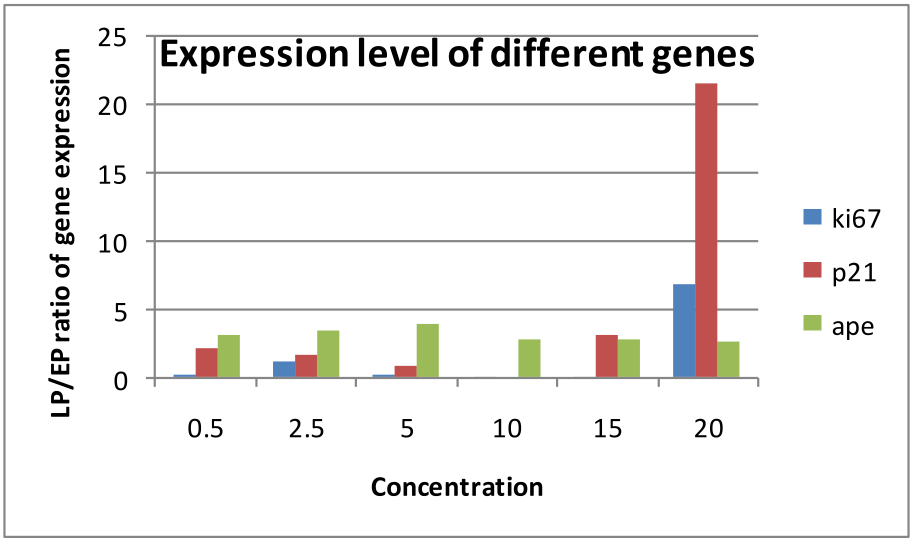


Figure 4.16: Ratio of gene expression level in late and early passage of cells cultured in different concentrations of serum versus concentration of serum. EP represents lower passage (1st & 2nd passage for serum and 1st to 3rd passage for glutamine) and LP represents late passage (3rd & 4th passage for serum and 6th to 9th passage for glutamine).

Ki67 was found to be expressed highly in late passage cells grown in higher concentrations (20% and 15%) of serum than late passage cells grown at lower concentrations (0.5%, 2.5% and 5%) of serum which suggested that the cells at late passage of higher serum containing media are actively proliferating whereas the cells at late passage for lower serum concentration i.e. serum starved condition has reduced proliferating activity suggesting those cells were entering senescent. The expression of base excision repair gene APE (apurinic/ apyrimidinic endonuclease) seems to be increasing in cells from higher concentration (20% serum containing media) to cells in lower concentration (0.5% serum containing media) (figure 4.16). This shows that the rate of DNA damage in cells cultured in IMDM containing lower concentrations of serum(10%, 5%, 2.5% and 0.5%) is higher as compared to high concentrations (20% and 15%). DNA damage causes expression of APE to enhance the repair of damage. Thus, it can be said that the cells cultured in lower concentrations of serum aged faster which do not support the hypothesis of this study i.e. serum starvation is not helping to delay the aging process.

4.7.2 Relative expression of ageing related markers in varying concentrations of glutamine containing media

Cells were grown in IMDM containing different concentrations of glutamine. Relative gene expression of ki67,p21 and APE was then calculated comparing expression of aging related genes viz. ki67, p21 and APE in early and late passage. For real time PCR, for each concentrations of glutamine (4mM,2mM,1mM, 0.5mM, 0.25mM and 0) two culture dishes were taken, one containing cells in early passage (First or second passage) and other containing cells in late passage (third passage and so on) for cells grown in IMDM with different concentrations of serum). Following real time PCR for cells cultured in varying concentration of glutamine containing media, the relative gene expression of respective genes were calculated using the formula mentioned in section 3.6.3.3 of materials and methodology.

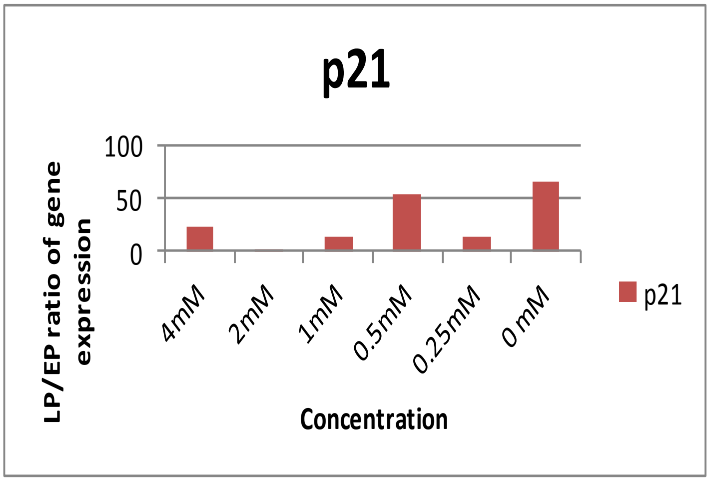


Figure 4.17: Ratio of expression of p21 in early and late passage of cells cultured in different concentrations of glutamine versus concentration of glutamine. EP represents lower passage (1st & 2nd passage for serum and 1st to 3rd passage for glutamine) and LP represents late passage (3rd & 4th passage for serum and 6th to 9th passage for glutamine).

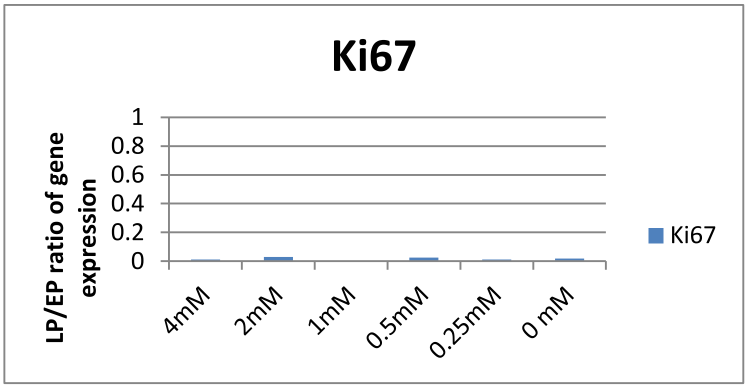


Figure 4.18: Ratio of expression of ki67 in early and late passage of cells cultured in different concentrations of glutamine versus concentration of glutamine. EP represents lower passage (1st & 2nd passage for serum and 1st to 3rd passage for glutamine) and LP represents late passage (3rd & 4th passage for serum and 6th to 9th passage for glutamine).

The cells cultured in lower concentrations of glutamine (0 to 0.5mM) seems to express more senescent marker p21 in their late passage as compared to late passage cells in higher concentrations of glutamine (figure 4.17). The expression level of proliferation marker Ki67 was found to be higher in early passage than that of cells in late passage irrespective of glutamine concentration as LP/EP ratio of Ki67 was near to zero in all concentrations. However the ratio of expression of Ki67 in late passage to early passage was almost equal in all concentrations of glutamine (figure 4.18). The findings implied that the cells were actively proliferating even after activation of p21 in late passage cells grown in lower glutamine concentrations which suggested that the cells in late passage cultured in glutamine starved condition (0-0.5 mM) showed delay in aging like cells cultured in higher glutamine concentrations.

CHAPTER 5: DISCUSSIONS

5. Discussion

Mesenchymal stem cells are regarded as an excellent candidate for cell therapy because these cells are easily accessible, isolation techniques are easier and expandable to required quantity in relatively short period of time. Because of these properties, Mesenchymal stem cells (MSCs) are rapidly emerging as a clinically-viable cell therapy so it is necessary to analyze *in vitro* senescence in MSCs. It has been reported that in humans and rodents, when hematopoietic stem cell becomes aged, they lose their differentiation potential (Morrison *et al*., 1996). A number of starvation studies are done in animal models and it has been found to be effective in prolongation of life. For example: mice raised in calorically restricted diet showed increased lifespan and a delay in the development of ageing related physiological and pathological changes (Lee *et al*. 1999). McCay *et al* first presented that calorie restriction retards ageing and extends life span (McCay *et al*., 1935). In another study, normal rodent cell cultured in little or no serum medium showed unlimited life span (Lee *et al*., 1999). In the present study, bone marrow derived Mesenchymal stem cells were grown in conditions of restricted nutrient and growth factors by varying the concentrations of serum and glutamine in the medium. The main idea of the study was to compare markers of ageing such as changes in morphology of cells, population doubling/growth curve, reactive oxygen species production, protein and gene expression with respect to different concentrations of serum and glutamine.

5.1 Growth curve and morphology analysis

The morphology of bone marrow derived MSCs were studied throughout the culture along with population doubling and growth curve of the MSCs grown in different concentrations of serum and glutamine. The cell count showed that the MSCs grew faster in IMDM containing higher concentrations of serum as compared to lower concentrations whereas the MSCs grew almost similarly irrespective of glutamine concentration. MSCs grew only upto fourth passage even in higher concentrations of serum (15% and 20%) while all MSCs cultured in different concentrations of glutamine (0 to 4 mM) reached upto ninth passage showing prolongation of ageing even in glutamine restricted conditions. The signs of ageing of MSC can be studied on the basis of morphology as well. It has been shown that long term culture of fibroblast cells showed granular cytoplasm along with debris in the medium (Smith and Lincoln, 1984). MSCs grown for long term also shows ageing signs like these fibroblast cells. In this study, for cells grown in lower concentrations of serum such morphological abnormalities was observed from second passage while for cells grown in different concentrations of glutamine showed such abnormalities only in late passage i.e. >6th passage. The cell density of MSCs (in figure 4.3 and 4.4) were also reduced significantly in serum restricted IMDM (0.5%, 2.5%, 5% and 10%) compared to those of higher serum concentrations (15% and 20%) whereas the cell density of MSCs grown in glutamine restricted IMDM (0, 0.25mM, 0.5mM and 1 mM) was comparable to those of IMDM containing higher glutamine concentrations (2mM and 4mM). These observations show that glutamine starvation is causing a delay in onset of ageing.

5.2 Immunostaining of ageing related markers

Further analysis of ageing of Mesenchymal stem cells under different concentrations of serum and glutamine was done by immunostaining and senescence associated beta gal staining. Immunostaining was performed to find out the expression of ageing markers namely, ki67 and p21. The expression of p21 was observed in cells even in early passage for lower concentrations of serum containing IMDM which continued to express in late passage as well while serum enriched cells did not show any expression even in late passage. For all glutamine concentrations, the cells did not show expression of p21, both in early and late passage. The Ki67 was observed to be expressed in both early and late passages of all concentrations of serum as well as glutamine. Ki67 is regarded as an excellent marker for detection of proliferation (Gerdes *et al*., 1983, 1984) and the cyclin dependent kinase inhibitor p21 is found to inhibit the activity of cyclin/cdk complexes, negatively regulating cell cycle progression (Sherr *et al*., 1995, 2000). Up-regulation of p21 is observed in aged MSCs which indicates the onset of pro-apoptotic activities (Campisi, 2005). Thus, ki67 acts as proliferation marker and p21 acts as a potent marker for detecting senescence. The findings of this study suggested that serum starved cells became senescent earlier than glutamine starved cells which means that there was delay in ageing in glutamine starved cells. Senescent associated beta galactosidase is also regarded as one of the most commonly used technique to detect senescence (Dimri *et al*., 1995). In this study, cells in late passage showed presence of blue stain in their cytoplasm which indicates the presence of beta gal activity while cells in early passage were found to be negative.

5.3 Estimation of reactive oxygen species by Flow cytometry

Reactive oxygen species (ROS) play a critical and fundamental role in ageing (Harman, 1956). ROS is found to be increasing in aged cells so it is regarded as one of the important factor of ageing (Harman, 1956). In this study, ROS was determined for each passage of cells cultured in different concentrations of serum. The percentage of stressed cells was found to be increasing in late passages compared to early passages cells irrespective of the concentration of serum used in the medium. ROS was accumulated earlier from second passages for lower concentrations of serum while increased ROS was noted only at late passages for cells grown in higher concentrations of serum. As in previous findings, serum starved cells fail to extend ageing and stopped dividing from second passage as compared to higher serum concentrations. The 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe used in this study is commonly employed dye to detect ROS accumulated in cells and may react with several ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite. The cell-permeant DCFDA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterases. Upon oxidation by ROS, the nonfluorescent DCFDA is converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF) (Hassani and Dupuy, 2013).

5.4 Real Time PCR analysis

Real time PCR was also employed for the determination of relative gene expression of ageing specific markers like p21, Ki67 and APE (apurinic/apyrimidinic endonuclease). Real time PCR helps simultaneous measurement of gene expression in many different samples (Fink *et al*., 1998). Direct Cell to PCR is really useful technique when only small numbers of cells are available (Higuchi *et al*., 1993). Compared to conventional quantification methods such as northern blot and RT PCR, Real time PCR is highly sensitive, reproducible and has large dynamic range. Quantitative real time PCR was done to compare expression of ageing related genes such as ki67, p21 and APE in early and late passage cells grown in IMDM with different serum and glutamine concentrations. The present study showed decline of Ki67 expression and a significant increase in APE expression in late passage when moving from higher serum concentrations to lower serum concentrations suggesting reduced proliferation as represented by lower Ki67 activity and increased DNA repair mechanisms as Apurinic/apyrimidinic endonuclease activity was enhanced. Both of these are signs of ageing. The expression of p21 was found to be high in late passage cells grown at lower concentrations of glutamine than higher concentrations of glutamine but the ratio of expression of Ki67 in late passage and early passage remains unchanged in all glutamine concentrations. This finding showed that late passage MSCs in glutamine starved condition are still proliferating like early passage MSCs even when senescent associated marker p21 has enhanced expression. This may be due to some unknown mechanism by which the effect of p21 activation has been balanced when there is no or very less glutamine in the medium.

The overall findings of present study confirm the role of glutamine starvation in delay of ageing in bone marrow derived Mesenchymal stem cells where as serum deprivation seems to enhance the ageing process. The role of glutamine starvation in delaying ageing has not been done separately in past and the present study is one of the preliminary studies and hence the underlying mechanism remains to be explored. In case of serum starvation, it has been found that serum starvation causes death of human MSCs *in vitro* even after 24 hours. Although the exact mechanisms underlying hMSC death due to serum deprivation still remain to be elucidated but serum deprivation was found to induce caspase dependent apoptosis like (nuclear shrinkage, chromatin condensation, decrease in cell size, and loss of membrane integrity) (Zhu *et al*., 2006; Potier *et al.,* 2006) which supports present finding. To gain a deep insight of the effect of serum and glutamine starvation on ageing, gene knockout experiments have to be performed and in vivo studies should also be done using mice models involving other ageing related markers as well.

CHAPTER 6: CONCLUSIONS

6. Conclusions

Mesenchymal stem cells (MSCs) are self-renewing, clonal precursors of nonhematopoietic tissues, which can be cultured in vitro while retaining the potential to give rise to chondrogenic, osteogenic, adipogenic, neural and cardiomyocyte phenotypes (Zhu et al., 2006). The possibility of isolating and expanding autologous mesenchymal stem cells (MSCs) and driving them toward numerous phenotypes, has opened new avenues for repairing lost or damaged tissues. The possible clinical applications include MSCs either in the form of cell suspensions to restore cardiac or cerebral function after tissue ischemia, or in association with a biocompatible scaffold for repairing cartilage, bone and adipose tissues (Potier et al., 2006). Senescence limits the proliferation of normal human Mesenchymal stem cells in culture. This fact limits the application of mesenchymal stem cell in cellular therapy and regenerative medicine. Starvation studies in animal model have been found to be effective in prolonging life. In present study, bone marrow derived Mesenchymal stem cell cultured in glutamine starved condition gives more promising result than serum starved condition. Population doubling calculated for lower concentrations of glutamine showed small variation from that of higher concentrations. The morphological abnormalities could be observed only in late passage for all different concentrations but proliferation capacity of glutamine starved cells was higher as these cells grew upto ninth passage comparing to serum starved condition which grew only upto second passage. In addition, immunostaining results for MSCs cultured in different concentrations of glutamine showed presence of proliferation marker ki67 both in early and late passage but senescent marker p21 was almost undetectable. MSCs grown at lower concentration of serum showed p21 expression in early passages which meant that the cells were entering senescence in early passage but this was not seen in case for MSCs grown at lower glutamine concentrations. The cells in late passage for all concentrations of serum and glutamine showed positive result for beta gal staining. Reactive oxygen species was found to be accumulated in late passage as compared to early passage in all serum concentrations. However, cells in lower concentrations of serum accumulated ROS earlier (second passage) than cells grown in higher serum concentrations (fifth passage). Though, the level of p21 expression in cells cultured at different concentrations of glutamine was found to be increased in late passage as shown by real time PCR, the ratio of expression of ki67 in late passage to early passage was found to be almost equal irrespective of the concentrations of glutamine used. This suggested that glutamine starved cells continued proliferating even after p21 was activated.

Thus, cells cultured in different concentrations of serum did not show any delay of senescence. In fact, cells cultured in lower concentrations of serum showed major signs of ageing like morphological abnormalities, decreased proliferation, increased expression of senescent associated marker p21, accumulation of reactive oxygen species as compared to cells grown in higher concentrations of serum. Glutamine limitation was found to be effective in delaying ageing of cells as compared to serum starved condition. Further studies need to be carried out to establish role of glutamine in delaying senescence and to gain a clear insight of mechanism of ageing in response to nutrient starvation.

Recommendations

The present investigation is a preliminary work in the field of molecular and cellular study in the scenario of serum and glutamine starvation, Hyderabad and hence represents a baseline work for further research. Based on the results of this investigation, following recommendations are suggested:

1) This study is restricted to only two ageing related markers namely p21 and Ki67. Detailed study of serum and glutamine starvation should also be done using other ageing related markers like TERT, hTR and length of telomeres etc.
2) Our study showed that Ki67 level was unchanged whereas p21 activity was suppressed in glutamine starvation of MSCS. Hence, the interaction of Ki67 and p21 should also be studied separately.
3) The present study was performed *in vitro* only. *In vivo* studies should also be done to understand a real scenario of serum and glutamine starvation.
4) Involvement of p21, Ki67 and other various markers in ageing with respect to nutrient starvation can be explored more after conducting experiments that depicts functionality aspects of such markers in knockout mice.
5) The present study is based on molecular and cellular analysis, metabolomics can also be performed to gain a deep understanding of the underlying mechanism of ageing in nutrient starvation.

References

Alberio R, Campbell KH and Johnson AD (2006) Reprogramming somatic cells into stem cells. *Reproduction*, **132**: 709-720

Ancelin K, Brunori M, Bauwens S, Koering CE, Brun C, Ricoul M, Pommier JP, Sabatier L, Gilson E (2002) Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol. Cell. Biol.* **22**: 3474-3487

Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C,Kruisselbrink AB, van Bezooijen RL (2003) Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica*, **88**: 845-852

Avilion AA, Piatyszek MA, Gupta J (1996) Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Research*, **56**: 645-650

Baksh D, Yao R, Tuan RS (2007) Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells,* **25**: 1384-1392.

Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R (2000) Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. *Experimental Hematology*, **28**: 707-715

Bestilny LJ, Brown CB, Miura Y (1996) Selective inhibition of telomerase activity during terminal differentiation of immortal cell lines. *Cancer Research,* **56**: 3796-3802

Blackburn H (1991) Structure and function of telomeres. *Nature,* **350**: 569-572

Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B (2006) Aging of mesenchymal stem cell in vitro. *BMC Cell Biology*, **7** (14): 21-27

Brandtzaeg P (1998) The increasing power of immunohistochemistry and immunocytochemistry. *Journal of Immunological Methods*, **216**: 49-67.

Braun N, Papadopoulos T, Muller-Hermelink HK (1988) Cell cycle dependent distribution of the proliferation-associated Ki-67 antigen in human embryonic lung cells. *Virchows Arch B Cell Pathol Incl Mol Pathol*, **56**: 25-33.

Bridger JM, Boyle S, Kill IR, Bickmore WA (2000) Re-modelling of nuclear architecture in quiescent and senescent human fibroblasts. *Current Biology*, **10** (3): 149-152

Broccoli D, Smogorzewska A, Chong L, de Lange T (1997) Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nature Genetics*, **17**: 231-235

Brown M and Wittwer C (2000) Flow Cytometry: Principles and Clinical Applications in Hematology. *Clinical Chemistry,* **46** (8): 1221-1229

Bruno RD and Smith GH (2010) Functional Characterization of Stem Cell Activity in the Mouse Mammary Gland. *Stem Cell Rev and Rep*, **122**: 1-10

Bruno S and Darzynkiewicz Z (1992) Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Proliferation*, **25**: 31-40.

Bullwinkel J, Baron-Lohr B, Gerdes J (2006) Ki67 Protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. J. cell physiol, **206**: 624-635

Calloway CD, Reynolds RL, Herrin GL, Anderson WW (2000) The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. *American Journal of Human Genetics*, **66** (4): 1384-1397

Campagnoli C, Roberts IA, Kumar S (2001) Identification of mesenchymal stem/progenitor cells in human first trimester fetal blood, liver and bone marrow. *Blood*, **98**: 2396-2402

Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell*, **120**: 513-522.

Caplan AI (1994) The mesengenic process. *Clinical Plastic Surgery,* 21: 429-435

Caplan AI (2007) Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell Physiol*., 213 (2): 341-347

Chang BD, Swift ME, Shen M, Fang J, Broude EV, Roninson IB (2002) Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. *Proceedings of the National Academy of Sciences of the United States of America*, **99**: 389-394

Chang BD, Watanabe K, Broude EV (2000) Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proceedings of the National Academy of Sciences of the United States of America*, **97**: 4291-4296

Cifrian E, Guidry AJ, Marquardt WW (1996) Role of milk fractions, serum, and divalent cations in protection of mammary epithelial cells of cows against damage by Staphylococcus aureus toxins. *American Journal of Veterinary Research,* **57** (3): 308-312

Colter DC, Class R, DiGirolamo CM, Prockop DJ (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA,* **97**: 3213-3218

de Lange T, Shiue L, Myers RM (1990) Structure and variability of human chromosome ends. *Mol Cell Biol*, **10**: 518-527

Deans RJ and Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. *Experimental Hematology*, **28** (8): 875-84.

Del Bo R, Bordoni A, Boneschi FM (2002) Evidence and age-related distribution of mtDNA D-loop point mutations in skeletal muscle from healthy subjects and mitochondrial patients. *Journal of the Neurological Sciences*, **202** (1-2): 85-91

Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling proliferation of hematopoietic stem cells in vitro. *J. Cell Physiol.,* 91 (4): 335-344

Dominici M, Hofmann TJ, Horwitz ME (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. *Cytotherapy,* **8**: 315-317

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. *Cytotherapy*, **8** (4), 315-317

du Manoir S, Guillaud P, Camus E, Seigneurin D, Brugal G (1991) Ki-67 labeling in postmitotic cells defines different Ki-67 pathways within the 2c compartment. *Cytometry*, **12**: 455-463

Eirin A, Zhu XY, Krier JD (2012) Adipose tissue-derived mesenchymal stem cells improve revascularization outcomes to restore renal function in swine atherosclerotic renal artery stenosis. *Stem Cells,* 30: 1030-1041

Ekholm SV, and Reed SI (2000) Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. *Current Opinion in Cell Biology*, **12***:* 676-684

Erices A, Congett P, Minguell JJ (2000) Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematology,* **109**: 235-242

Fairall L, Chapman L, Moss H, de Lange T, Rhodes D (2001) Structure of the TRFH dimerization domain of the human telomeric proteins TRF1 and TRF2. Mol Cell, **8**: 351-361

Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. *Annual Review of Biochemistry*, **76**: 679-699

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J (1995) The RNA component of human telomerase. *Science*, **269**: 1236-1241.

Ferrari G, de Angelis CG, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998) Muscle regeneration by bone marrow derived myogenic progenitors. *Science,* 279: 1528-1530

Fickert S, Fiedler J, Brenner RE (2003) Identification, quantification and isolation of mesenchymal progenitor cells from osteoarthritic synovium by fluorescence automated cell sorting. *Osteoarthr. Cartilage*, **11**: 790-800.

Fink L, Seeger W, Ermert L, Hanze J, Stahl U, Grimminger F, Kummer W, Bohle RM (1998) Real-time quantitative RT-PCR after laser-assisted cell picking. *Nature Methodology,* **4**: 1329-1333

Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV (1966) Osteogenesis in bone marrow cells. *J. Embryol. Exp. Morphol,* 16: 381-390

Fukuchi Y, Nakajima H, Sugiyama D (2004) Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells,* 22: 649-658

Glacken M W (1988) Catabolic control of mammalian cell culture. *Biotechnology,* **6**: 1041-1053

Goldstein S and Singal DP (1974) Senescence of cultured human fibroblasts: mitotic versus metabolic time. *Experimental Cell Research*, **88** (2), 359-364

Greider CW (1996) Telomere length regulation. *Annu Rev Biochem*, **65**: 337-365.

Haber JE (2000) Partners and pathways-repairing a double-strand break. *Trends in Genetics*, **16** (6): 259-264

Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during aging of human fibroblasts. *Nature*, **345**: 458-460

Harley CB, Vaziri, H, Counter C, Allsopp RC (1992) The telomere hypothesis of cellular aging. *Exp. Gerontol.* **27**: 375-382

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, **11** (3): 298-300

Harman D (1972) The biologic clock: the mitochondria? *Journal of the American Geriatrics Society*, **20** (4): 145-147

Harrington L, Zhou W, McPhail T, Oulton R, Yeung DS, Mar V, Bass MB, Robinson MO (1997) Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.,* **11**: 3109-3115

Hassani RAE and Dupuy C (2013) Detection of intracellular reactive oxygen species. *Bioprotocol*, **3** (1): 1-6

Hastie ND, Dempster M, Dunlop MG (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature*, **346**: 866-868

Hayakawa M, Hattori K, Sugiyama S, Ozawa T (1992) Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. *Biochemical and Biophysical Research Communications*, **189** (2): 979-985

Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research*, **37**: 614-636

Hayflick L (1976) The cell biology of human aging. *The New England Journal of Medicine*, **295** (23), 1302-1308

Hayflick L (1979) The cell biology of aging. *J Invest Dermatol,* **73** (1): 8-14

Henderson ER, Blackburn EH (1989) An overhanging 39 terminus is a conserved feature of telomeres. *Mol Cell Biol*, **9**: 345-348

Hermida-Gomez T, Fuentes-Boquete I, Gimeno-Longas MJ (2011) Quantification of cells expressing mesenchymal stem cell markers in healthy and osteoarthritic synovial membranes. *J Rheumatol,* **38**: 339-349

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysisreal-time monitoring of DNA amplification reactions. *Biotechnology*, **11**: 1026-1030

Ikebe C and Suzuki K (2014) Mesenchymal Stem Cells for Regenerative Therapy: Optimization of Cell Preparation Protocols. *BioMed Research International,* **10**: 20-31

Jayat C and Ratinaud MH (1993) Cell cycle analysis by flow cytometry: Principles and applications. *Biol Cell,* **78**: 15-25

Jung S, Panchalingam KM, Rosenberg L, Behie LA (2012) Ex Vivo Expansion of Human Mesenchymal Stem Cells in Defined Serum-Free Media. *Stem Cells International,* **23**: 41-62

Kang MK, Swee J, Kim RH, Baluda MA, Park NH (2002) The telomeric length and heterogeneity decrease with age in normal humanoral keratinocytes. *Mech Ageing Dev*., **123**: 585-592

Karlseder J, Smogorzewska A, de Lange T (2002) Senescence induced by altered telomere state, not telomere loss. *Science,* **295**: 2446-2449

Kasamatsu H, Robberson DL, Vinograd J (1971) A novel closed-circular mitochondrial DNA with properties of a replicating intermediate. *Proceedings of the National Academy of Sciences of the United States of America*, **68** (9): 2252-2257

Kassem M, Ankersen L, Eriksen EF, Clark BFC, Rattan SIS (1997) Demonstration of cellular aging and senescence in serially passaged long term cultures of Human Trabecular Osteoblast. *Osteoporosis International*, **7**: 514-524

Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR, Jefferson RA (1997) Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol. Genet.*, **6**: 2011-2019

Kill IR (1996) Localisation of the Ki-67 antigen within the nucleolus: Evidence for a fibrillarin-deficient region of the dense fibrillar component. *J Cell Sci*, **109** (6): 1253-1263

Krishnan KJ, Reeve AK, Samuels DC (2008) What causes mitochondrial DNA deletions in human cells? *Nature Genetics*, **40** (3): 275-279

Kristnamurthy J (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature*, **443**: 443-457

Lambeth JD (2004) NOX enzymes and the biology of reactive oxygen. *Nature Reviews Immunology*, 4 (3): 181-189

Lee MW, Yang MS, Park JS, Kim HC, Kim YJ, Choi J (2005) Isolation of mesenchymal stem cells from cryopreserved human umbilical cord blood. *International Journal of Hematology,* **81**: 126-130

Li M and Ikehara S (2013) Bone-marrow-derived mesenchymal stem cells for organ repair. *Stem Cells International*, **13**: 1-8

Li Y, Thompson H, Hemphill C, Hong F, Forrester J, Johnson RH, Zhang W, Meldrum DR (2010) An improved one-tube RT-PCR protocol for analyzing single-cell gene expression in individual mammalian cells. *Anal Bioanal Chem,* **397**: 1853-1859

Linnane AW, Zhang C, Baumer A, Nagley P (1992) Mitochondrial DNA mutation and the ageing process: bioenergy and pharmacological intervention. *Mutation Research*, **275** (3-6): 195-208

Liu J, Qu W, Kadiiska MB (2004) Role of oxidative stress in cadmium toxicity and carcinogenesis. *Toxicology and Applied Pharmacology*, **238** (3): 209-214

Ljuggren J and Haggstrom L (1994) Catabolic control of hybridoma cells by glucose and glutamine limited fed batch cultures. *Biotechnology Bioengineering*, **44**: 808-819

Loeb LA, Wallace DC, Martin GM (2005) The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. *Proceedings of the National Academy of Sciences of the United States of America*, **102** (52): 18769-18770.

Madrigal M, Rao KS and Riordan NH (2014) A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *Journal of Translational Medicine*, **12**: 260-267

Mather M and Rottenberg H (2000) Aging enhances the activation of the permeability transition pore in mitochondria. *Biochemical and Biophysical Research Communications*, **273** (2): 603-608

McCay CM, Crowel MF, Maynard LA (1935) The effect of retarded growth upon the length of the life span and upon the ultimate body size. *Journal of Nutrition*, **10**: 63-79

McElligott R, Wellinger RJ (1997) The terminal DNA structure of mammalian chromosomes. *EMBO Journal*, **16**: 3705-3714

Meirelles LS, Fontes AM, Covas DT, Caplan AI (2009) Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Review.* **20**: 419-427

Meissner C, Bruse P, Mohamed SA (2008) The 4977 bp deletion of mitochondrial DNA in human skeletal muscle, heart and different areas of the brain: a useful biomarker or more? *Experimental Gerontology*, **43** (7): 645-652

Mendes SC, Robin C, Dzierzak E (2005) Mesenchymal progenitor cells localize within hematopoietic sites through ontogeny. *Development*, 132: 1127-1136

Merz GS, Ross JD (1969) Viability of human diploid cells as a function of in vitro age. *J Cell Physiol*, **74** (3): 219-222.

Miquel J, Economos AC, Fleming J, Johnson Jr JE (1980) Mitochondrial role in cell aging. *Experimental Gerontology*, **15** (6): 575-591

Morgan DO (1995) Principles of CDK regulation. *Nature (Lond.),* **374**: 131-134

Morrison T, Weis JJ, Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques,* **24**: 954-962.

Moyzis RK, Buckingham JM, Cram LS (1988) A highly conserved repetitive DNA sequence (TTAGGG)n, present at the telomeres of human chromosomes. *Proceedings of the National Academy of Sciences of the United States of America*, **85**: 6622-6626

Muller M (2009) Cellular senescence: molecular mechanisms, in vivo significance, and redox considerations. *Antioxidant Redox Signaling,* **11**: 59-98

Nakamura TM, Morin GB, Chapman KB (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science*, **277**: 955-959

Newsholme P and Newsholme EA (1989) Rates of utilization of glucose, glutamine and oleate and formation of end-products by mouse peritoneal macrophages in culture. *Biochemisty,* **211**: 227-234

Norbury CJ and Hickson ID (2001) Cellular responses to DNA damage. *Annual Review of Pharmacology and Toxicology*, **41**: 367-401

Nunez R (2001) DNA measurement and cell cycle analysis by flow cytometry. *Current Issues on Molecular Biology*, **3** (3): 67-70

Olovnikov AM (1973) A theory of marginotomy: The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *Journal of Theoretical Biology*, **41**: 181-190

Papas AM (1999) Diet and antioxidant status. In: Papas AM, editor. Antioxidant status, diet, nutrition, and health. Boca Raton, Fla: CRC Press. Page 89-105

Park JC, Kim JM, Jung IH (2011) Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: In vitro and in vivo evaluations. *J Clin Periodontol,* **38**: 721-731

Patel DM, Shah J, Srivastava AS (2013) Therapeutic potential of mesenchymal stem cells in regenerative medicine. *StemCells International*, **13**: 25-40

Pfaffl MW (2001) Development and validation of an externally standardised quantitative insulin like growth factor-1 (IGF-1) RT–PCR using LightCycler SYBR Green I technology. In Meuer S, Wittwer C and Nakagawara K (eds), *Rapid Cycle Real-time PCR, Methods and Applications.* Springer Press, Heidelberg, Germany, Page 281–291

Piko L, Hougham AJ, Bulpitt KJ (1988) Studies of sequence heterogeneity of mitochondrial DNA from rat and mouse tissues: evidence for an increased frequency of deletions/additions with aging. *Mechanisms of Ageing and Development*, **43** (3): 279-293

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science*, **284**: 143-147

Potier E, Ferreira E, Meunier A, Sedel L, Logeart D, Petite H (2007) Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue engineering*, **13** (6): 1325-1331

Pournasr B, Mohamadnejad M, Bagher M, Aghdami N, Shahsavani M, Malekzadeh R, Baharvand H (2011) In Vitro Differentiation of Human Bone Marrow Mesenchymal

Stem Cells into Hepatocyte-like Cells. *Archives of Iranian Medicine,* Volume **14** (4): 244-253

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*, **276**: 71-74

Quinn MT, Ammons MCB, DeLeo FR (2006) The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. *Clinical Science*, 111 (1): 1-20

Ralston A and Rossant J (2010) The genetics of induced pluripotency. *Reproduction* **139:** 35-44

Richter C (1992) Reactive oxygen and DNA damage in mitochondria. *Mutation Research*, **275** (3-6): 249-255

Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL (2007) Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*, **447** (7145): 725-729

Rottenberg H and Wu S (1997) Mitochondrial dysfunction in lymphocytes from old mice: enhanced activation of the permeability transition. *Biochemical and Biophysical Research Communications*, **240** (1): 68-74

Sabin RJ and Anderson RM (2011) Cellular Senescence - its role in cancer and the response to ionizing radiation. *Genome Integrity*, **2**: 7-15

Sacchetti B, Funari A, Michienzi S, Di CS, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell,* **131**: 324-336.

Sarkar M, Varshney R, Chopra M and Dwarakanath BS (2005) Flow-Cytometric analysis of reactive oxygen species in peripheral blood mononuclear cells of patients with thyroid dysfunction. *Cytometry Part B (Clinical Cytometry)*, **70B**: 20-23

Sart S, Schneider YJ, Agathos SN (2009) Ear mesenchymal stem cells: an efficient adult multipotent cell population fit for rapid and scalable expansion. *Journal of Biotechnology*, **139** (4): 291-299

Sarugaser R, Lickorish D, Baksh D (2005) Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors. *Stem Cells*, **23**: 220-229

Schneider EL, Braunschweiger K, Mitsui Y (1998) The effects of serum batch on the in vitro life spans of cell cultures derived from old and young human donors. *Experimental Cell Research*, **115**: 47-52

Schop D, Janssen FW, Rijn LDS, Fernandes H, Bloem RM, Bruijn JD, van Dijkhuizen-Radersma R (2009), Growth, Metabolism, and Growth Inhibitors of Mesenchymal Stem Cells. *TISSUE ENGINEERING (Part A),* **15** (8): 1877-188

Schwab KE, Hutchinson P, Gargett CE (2008) Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. *Human Reproduction*, **23**: 934-943.

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells*, **20**: 530-541

Shelton DN, Chang E, Whittier PS, Choi D, Funk WD (1999) Microarray analysis of replicative senescence. *Current Biology*, **9** (17): 939-945.

Sherr CJ (1993) Mammalian G1 cyclins. *Cell*, **73***:* 1059-1065

Sherr CJ (2000) The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research,* **60**: 3689-3695

Sherr CJ and Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, **13**: 1501–1512

Shibata KR, Aoyama T, Shima Y, Fukiage K, Otsuka S, Furu M, Kohno Y, Ito K, Fujibayashi S, Neo M, Nakayama T, Nakamura T, Toguchida J (2007) Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. *Stem Cells*, **25**: 2371-2382

Simmons PJ, TorokStorb B (1991) Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO1. *Blood*, 78 (1): 55-62

Smith JR and Lincoln DW (1984) Aging of cells in culture. *Int Rev Cytol,* **89**: 151-77

Smith JR and Lincoln DW (1984) Aging of cells in culture. *International Review on Cytology*, **89:** 151-77

Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T (2000) Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol,* **20**: 1659-1668

Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.* **20**: 1659-1668.

Sotiropoulou P, Perez SA, Salagianni M, Baxevanis C, Papamichail M (2006) Characterization of the Optimal Culture Conditions for Clinical Scale Production of Human Mesenchymal Stem Cells. *Stem Cells,* **24**: 462-471

Stadler C, Rexhepaj E, Singan VR, Murphy RF, Emma Lundberg (2013) Immunoflurescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. *Nature methods*, **10**: 315-323

Starborg M, Gell K, Brundell E, Hoog C (1996) The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. *J Cell Sci*, **109**: 143-153.

Starborg M, Gell K, Brundell E, Höög C (1996) The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. *Journal of Cell Science*, **109**: 143-153

Thejaswi K, Amarnath M, Srinivas G, Jerald MK, Avinash RT, Singh S (2012) Immune Modulatory Responses of Mesenchymal Stem Cells from Different Sources in Cultures and In Vivo. *Cell & Tissue Transplantation & Therapy,* **4**: 1-13

Thomas D. Schmittgen, Brian A. Zakrajsek “Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR’ J. Biochem. Biophys. Methods 46 (2000) 69-81

Thomas E, Al-Baker E, Dropcova S, Denyer S, Ostad N, Lloyd A, Kill IR, Faragher RGA (1997) Different Kinetics of Senescence in Human Fibroblasts and Peritoneal Mesothelial Cells. *Exp Cell Res,* **236** (1): 355-358

Tondreau T, Meuleman N, Delforge A (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: Proliferation, Oct4 expression, and plasticity. *Stem Cells,* **23**: 1105-1112.

Trifunovic A, Wredenberg A, Falkenberg M (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature*, 429 (6990): 417-423

Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. *Nature Review Immunology,* **8**: 726-736

Vaziri H and Benchimol S (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Current Biology*, **8** (5): 279-282

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008) Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS.One, 52: 23-35

Wang E (1995) Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Research*, **55** (11): 2284-2292

Weiner A (1988) Eukaryotic nuclear telomeres: molecular fossils of the RNP world? *Cell,* **52**: 155-157

Welle S, Bhatt K, Shah B, Needler N, Delehanty JM, Thornton CA (2003) Reduced amount of mitochondrial DNA in aged human muscle. *Journal of Applied Physiology*, 94 (4): 1479-1484

Wilson DM, Sofinowski TM, McNeill DR (2003) Repair mechanisms for oxidative DNA damage. *Frontiers in Bioscience*, **8**: 963-981

Wright WE, Tesmer VM, Huffman KE (1997) Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes Dev,* **11**: 2801-2809

Zakian A (1989) Structure and function of telomeres. *Annual Review of Genetics*, **23**: 579-604.

Zhu W, Abbas T, Dutta A (2005) DNA replication and genomic instability. *Adv. Exp. Med. Biol.* **570**: 249-279

Zhu W, Chen J, Cong X, Hu S, Chen X (2006) Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Stem cells*, **24**: 416-425

Appendix 1: Components of FBS (source: Price and Gregory, 1982)

Components	Concentration	Range
Total protein	3.8 g/100 ml	3.2-7.0
Albumin	2.3 g/100 ml	2.0 - 3.6
Endotoxin	0.36 ng/ml	0.01 -10.0
Haemoglobin	11.3 mg/100 ml	2.4 - 18.1
Cholesterol	31 mg/100 ml	12- 63
Fattyacids	……..	……..
Phospholipids	……..	……..
Tri glycerides	……..	……..
Glucose	125 mg/100 ml	85 - 247
Insulin	10 ~U/ml	6-14
Cortisone	0.5 ~g/1 00 ml	<0.1 - 2.3
Trijodo-thyronine	119 ng/100 ml	56 - 223
Thyroxine	12.1 ng/100 ml	7.8-15.6
PTH	1718 pg/ml	85 - 6180
PGE	5.91 nglml	0.5 - 30.5
PGF	12.33 ng/ml	3.8 - 42.0