"BONE MARROW DERIVED MESENCHYMAL STEM CELLS AS A MODEL FOR *IN VITRO* TOXICOLOGICAL EVALUATION"

A THESIS SUBMITTED
BY

RESHMITHA.T.R

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

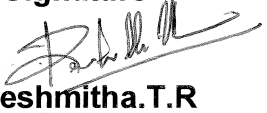
MASTER OF PHILOSOPHY

**SREE CHITRA TIRUNAL INSTITUTE FORMEDICAL SCIENCES AND
THIRUVANANTHAPURAM– 695 012**

DECLARATION

I, **Reshmitha.T.R** hereby declare that I had personally carried out the work depicted in the thesis entitled "**Bone marrow derived mesenchymal stem cells as a model for *in vitro* toxicological evaluation**" under the direct supervision of Dr.P.V.Mohanan, **Toxicology Division,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

Signature



Reshmitha.T.R

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY

THIRUVANANTHAPURAM – 695011, INDIA

(An Institute of National Importance under Govt.of India)

CERTIFICATE

This is to certify that the dissertation entitled **"Bone marrow derived mesenchymal stem cells as a model for *in vitro* toxicological evaluation"** submitted by **Reshmitha.T.R** in partial fulfillment for the degree of Master of Philosophy Technology in Biomedical Research to be awarded by this Institute. The entire work was done by **her** under my supervision and guidance at **Toxicology Division**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram.695012.

Thiruvananthapuram

Date 31/7/2013

Dr.P.V.Mohanan

Signature

The Thesis

Entitled

"Bone marrow derived mesenchymal stem cells as a model for *in vitro* toxicological evaluation"

Submitted

By

RESHMITHA.T.R

For

Master of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FORMEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM

Evaluated and approved

by

Signature

Name of Supervisor

Dr. P.V. Mohanan
ist In Charge, Toxicology Division
iomedical Technology Wing
hitra Tirunal Institute for Medical-
es and Technology (Govt. of India)
ojappura, Trivandrum 695 012
Kerala, India

Signature

Examiner's name and Designation

Dr.ANIL KUMAR. P.R.
Scientist
Division of Implant Biology
Biomedical Technology Wing
Sree Chitra Tirunal Institute for -
Medical Sciences and Technology
Poojappura ,Thiruvananthapuram - 12

ACKNOWLEDGMENT

First of all I thank God, almighty for being with me all throughout and blessing me with good physical and mental health for the fulfillment of this work.

I express my deep sense of gratitude and respect for my guide Dr.P.V.Mohanan, Scientist in charge, Toxicology division, SCTIMST, for his valuable guidance, advice and encouragement from start till completion of this work.

I would like to express my sincere thankfulness to Dr.K.Radhakrishnan, the director SCTIMST, Dr.G.S.Balakrishnan, the head BMT WING and Dr. Sundar jayasingh the Deputy registrar, SCTIMST, Dr.Lissy.k.krishnan, MPhil coordinator for providing me with the necessary facilities from their esteemed institute.

I am very much grateful to Mrs.Geetha.C.S, Remya Bijoy and Dr.Gayathri for their kind advice and valuable suggestions in my work.

I owe my sincere thanks to my friends Reshma.S.Cherian, Leji Babu, Sruthi.S, Syama.S, Anju Mohan, Sheeja Liza Easo who helped me in developing this work as a memorable and enjoyable team effort. I would like to acknowledge my gratitude to Mr.Shaji and Mr.Hari for their support

I would like to express my sincere thanks to all my MPhil friends for their support and encouragement throughout my work.

My sincere gratitude to my parents for their sustained prayers, constant support and encouragement throughout my work.

My heartful thanks to all who have helped me in completing the project by providing valuable information and the support.

Reshmitha.T.R

ABBREVIATION

20-HETE - 20-Hydroxy-5, 8, 11, 14-eicosatetraenoic acid

AHA- alkaline-halo assay

BM- Bone marrow

BMSC-Bone marrow mesenchymal stem cells

BSA -Bovine serum albumin

CA- Chromosomal aberration.

CAT – Catalase.

CFU-F- Colony forming unit –fibroblast

CPDs -cyclobutane pyrimidine dimmers

DAPI -4',6-diamidino-2-phenylindole

DCF- Dichlorofluorescein.

DCFHDA-Dichloro-dihydro-fluorescein diacetate.

DMEM- Dulbecco's modified eagles' medium.

DMSO- Dimethyl sulfoxide.

DNA- Deoxyribonucleic acid.

DSB -double strand break

EDTA- Ethylenediamine-tetraacetic acid.

ESCs- Embryonic stem cells

EtBr- Ethidium bromide.

FBS- Foetal bovine serum.

FHA- Fast halo assay.

GPx -glutathione peroxidase.

GSH –glutathione.

GVHD- Graft versus host disease.

H_2O_2- Hydrogen peroxide.

HSC- Hematopoeitic stem cells

iPS- induced pluripotent stem cells

KCl- potassium chloride.

LM –Low melting.

M: A- Methanol: acetic acid.

MTS -(3-(4,5-dimethylthiazol-2-yl)-5-(3-carbo xymethoxyphenyl)-2-(4-sulfophenyl)-2H-te trazolium).

MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

NaOH- Sodium hydroxide.

NC- Negative control.

PBS- Phosphate Buffer Saline.

PC- positive control.

ROS- Reactive oxygen species.

RT- Room Temperature.

SOD-superoxide dismutase.

SSB- single strand break

Table of contents

Section 1	Title/subtitle	Page no
	SYNOPSIS	1
1	INTRODUCTION	3
1.1	BACKGROUND	3
1.2	REVIEW OF LITERATURE	6
1.3	HYPOTHESIS	18
1.4	OBJECTIVES	19
2	MATERIALS AND METHODS	20
2.1	MATERIALS	20
2.2	EQUIPMENTS	21
2.3	METHODS	23
2.3.1	ISOLATION AND CULTURE OF BMSCs	23
2.3.2	CHARACTERIZATION OF BMSCs	24
2.3.3.	MTT ASSAY	26
2.3.4	ROS DETECTION	27
2.3.5	CHROMOSOMAL ABERRATION	28
2.3.6	DNA LADDERING ASSAY	29
2.3.7	HALO ASSAY	31
3	RESULTS & DISCUSSION	32
3.1	RESULTS	32
3.2	DISCUSSION	49
	SUMMARY & CONCLUSION	51
	BIBILOGRAPHY	53

List of Figures

Fig no	Caption	Page no
Fig:1	Hematopoietic and stromal stem cell differentiation	11
Fig: 2	MSCs and HSCs in bone marrow	11
Fig: 3	Chemical structure of imidazole, a simple aromatic ring	14
Fig: 4	Ball-and-stick model of the imidazole molecule, a nitrogen heterocycle and a simple aromatic ring	15
Fig:5	Schematic representation of MTT reaction	28
Fig: 6	Formation of fluorescent Compound DCF by ROS.	30
Fig: 7	Isolated cells with both HSCs and MSCs (P0 Passage)	37
Fig:8	BMSCs (P3 passage) established in culture with fibroblast shaped morphology	38
Fig: 9	Spindle shaped morphology of BMSCs (passage 3)	39
Fig:10	Expression of CD90 surface marker in BMSCs	40
Fig: 11	Morphological changes induced by different concentration of pure imidazole	41
Fig:12	Dose response of cell viability was detected by MTT assay	42
Fig: 13	Dose response of ROS production by DCFDA	43
Fig:14	No aberration in untreated cell metaphase.	45
Fig:15	chromosomal aberration in 1mg/ml imidazole treated cell metaphase	46
Fig:16	Agarose gel electrophoresis of DNA extracted from BMSCs	48

Fig:17	Halo assay data in Negative control media used	49
Fig:18	Presence of Halo in 1mg/ml imidazole treated cell	50
Fig:19	presence of Halo in 5mg/ml imidazole treated cell	47

List of tables

Table no	caption	Page no
Table :1	chromosomal aberrations in samples	41
Table:2	Concentration of DNA in each sample was estimated using biophotometer	44

SYNOPSIS

Toxicology testing is mainly used to determine the degree of toxicity of a compound can cause damage to a living system. The intention of *in vitro* toxicity testing is to evaluate the toxic effect of chemical on cellular level and thereby which is able to profile a compound action in a cell system

Stem cells are multipotent cells that can divide and differentiate into diverse specialized cell types and it can self-renew to produce more stem cells. Mesenchymal stem cells have the ability to expand many folds in culture while retaining their growth and multilineage potential. The toxicity testing in BMSCs was done with pure imidazole, an organic compound. Imidazole ring exhibit different types of pharmacological and biological activities.

These studies hypothesize that the bone marrow derived mesenchymal stem cells can be used in toxicological testing and thereby replace an organism to certain extent. These cells have a potential application in the field of toxicology. The main objective of this study is that the bone marrow derived mesenchymal stem cells as an alternate model for in vitro toxicological evaluation.

Chapter I of this dissertation include an introduction to the research problem and recent review of literature which include basics of stem cells and mesenchymal stem cells, recent studies in MSCs, basic information about pure imidazole and their recent studies. Some in vitro assays such as trypan blue exclusion assay, MTT assay, oxidative stress detection methods-ROS production and some genotoxic detection methods like chromosomal aberration, DNA ladder assay and halo assay are reviewed with appropriate citation. Finally this chapter describes hypothesis and specific objective of the study.

Materials and methodology adopted for carrying out the studies, which includes materials and equipments used for this work. Method for isolation and culturing of mesenchymal stem cells from murine bone marrow. Characterizations of Bone marrow derived mesenchymal stem cells were done by using their morphology, adherent property and immunophenotyping. Details of cytotoxic and genotoxic studies such as trypan blue assay, MTT assay, ROS production, chromosomal aberration, DNA ladder assay and halo assay are mentioned. Details of the cell culture system, media preparation, cell morphology are elaborated.

The data obtained from different assay done by using BMSCs with pure imidazole resulted that they have both cytotoxic and genotoxic effects. These are analyzed by using MTT assay for cytotoxicity. In oxidative stress, the data obtained was low ROS production high dosage. May be because of antioxidant property and genotoxic effects were confirmed by chromosomal aberration, DNA fragmentation and halo formation around cell nucleus.

Chapter IV summarized the study and conclusions were drawn. The chemical treated BMSCs data shows the toxic level of pure imidazole in cell system. Data revealed that the toxic effects of chemical is similar to *in vivo* system reports. It has to be concluded that BMSCs can be used as an alternate model for in vitro toxicological evaluation.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Toxicology employs multiple scientific disciplines to identify the basic mechanisms of chemical, physical and biological agents that could induce any harmful effect on a target organism. Chemical toxicity has become the subject of concern because of raising apprehension of toxic effects of different chemicals on living organisms. The response of different cells to a variety of chemicals might vary from one another. Highly regulated and complex processes like cell division, cell differentiation, cell migration, DNA replication, proteins distribution and so on, follow a highly orchestrated scheme in a developing organism. All processes are extremely prone to the minute changes, occurring in the cell's environment or inside it. The action of even low quantities of toxic substances inside cells might lead to alteration of processes related to the development of the organism. The various chemical reactivity results in increased production of reactive oxygen species (ROS), including free radicals. ROS and free radical production is one of the primary mechanisms of toxicity; it may result in oxidative stress and consequent damage to proteins, membranes, and DNA. Other factors influenced by toxicity include cellular growth, cell membrane permeability, and mitochondrial membrane potential, morphological changes like cytoskeleton breakdown and cell adhesion properties.

Imidazole is an organic compound with the formula $C_3H_4N_2$. It is a colorless solid that dissolves in water to give mildly basic solution. It is an aromatic heterocycle, classified as a diazole and as an alkaloid. Imidazole is a

five membered, heterocyclic ring, which contains two nitrogen atoms and two double bonds. There are so many compounds which contain Imidazole ring and exhibit different types of pharmacological and biological activities .The imidazole ring is a constituent of several important natural products which including nucleic acid, histidine, histamine, and purine. Imidazole derivatives have occupied a unique place in the field of medicinal chemistry. The inclusion of the imidazole nucleus is an important synthetic strategy in drug discovery. The high therapeutic properties of the imidazole related drugs have encouraged the medicinal chemists to synthesize a large number of novel chemotherapeutic agents

These drugs have broadened scope in remedying various diseases in clinical medicines. Different methods for the synthesis of imidazole and also their various structural reactions offer enormous scope in the field of pharmacology. Imidazoles are a class of antifungal azole derivatives and have a broad spectrum of activities both *in vitro* and *in vivo*. Many histaminergic ligands for histamine H1, H2, and H3 receptors are present in imidazole moiety. These are currently used as tools in pharmacological studies. Imidazole is amphoteric, *i.e.* it can function as both an acid and as a base.

In recent years, stem cells have been the subject of increasing scientific interest because of their importance in numerous biomedical applications. Stem cells have the capacity to self-renew and to give rise to cells of various lineages. Therefore, they represent an important paradigm of cell-based therapy for a variety of diseases. There are two main types of stem cells- embryonic and non-embryonic. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst and can differentiate into cells of all three germ layers. Stem cell research has been highly controversial due to the ethical issues concerned with the culture and the use of stem cells derived from embryo. In case of non-embryonic stem cells, especially adult stem cells, which are already somewhat specialized, have limited differentiation potential. The property of plastic

adherence itself is not sufficient to allow for the purification of MSCs, at least in the case of cells from mouse marrow. Bone marrow stroma contains a unique cell population, referred to as marrow stromal cells (MSCs), capable of differentiating along multiple mesenchymal cell lineages. A standard culture system has been developed to isolate MSCs from whole marrow by utilizing their plastic adherence property, wherein the cells grow as colonel populations derived from a single precursor termed the colony-forming-unit fibroblast (CFU-F).

Mesenchymal Stem Cells have seen an unprecedented level of interest in the last decade, primarily due to their comparatively ease of isolation, and the ability to propagate these cells in culture and capacity of renewing themselves; so they can be continuously cultured in an undifferentiated state. Therefore stem cells are an important new tool for developing exclusive, *in vitro* model systems to test drugs and chemicals and have a potential to predict or anticipate toxicity in organisms. Better characterization of MSC, in 2006, was defined by the International Society of Cellular Therapy by the following three criteria

(1) MSCs must be plastic adherent under standard tissue culture conditions;

(2) MSCs must express certain cell surface markers such as CD73, CD90, and CD105, and lack expression of other markers including CD45, CD34, CD14, or CD11b, CD79alpha or CD19 and HLA-DR surface molecules;

(3) MSCs must have the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts under in vitro conditions.

. *In vitro* toxicity assays provides a means of discerning relative toxic potencies of the chemical exposed under controlled dosimetry conditions and are especially useful for rating a chemical. The potential toxic effect increases as the exposure increases. The toxic potency of a chemical is thus ultimately defined by the dose of the chemical that will produce a specific response in a system. Exposure of an

organism to various physical, chemical, biological agents, and even psychological stress will eventually be biologically translated into some response at the cell level. Main potential consequences to the chemical exposure will be cytotoxic and genotoxic.

Cell culture systems have an advantage that they allow a closer look at cellular processes and the mechanisms can be investigated in detail. The main conventional *in vivo* methods are to find out if cells die as a result of their long-term exposure to chemicals. But to assess the actual toxic effect of a compound, it is has to consider the concentrations that have not led to the death of the cells and to have a deeper look into the effects of chemicals on neighboring cells. For this several *in vitro* assays have been developed which include cell culture assays for cytotoxicity (which indicates altered metabolism, decreased growth, lytic or apoptotic cell death), proliferation, genotoxicity, and altered gene expression.

1.2 REVIEW OF LITERATURE

Stem cells are one of the most fascinating areas of biology today. These cells have the remarkable potential to develop into many different cell types in the body. They serving as a sort of repair system for the body, they can theoretically divide without limit to replenish other cells as long as the person or animal is still alive. The concept of stem cells originated at the end of the 19th century as a theoretical postulate to account for the ability of certain tissues (blood, skin, etc.) to self-renew for the lifetime of an organism even though they are comprised of short-lived cells. Many years later, identification of stem cells as discrete cellular entities followed from the development of methods for prospective isolation of stem cell and design to test their potency after transplantation *in vivo*.

The reasons behind the inclusion of BMSCs are simply, that they are currently the most prolific source of potential therapeutic strategies for human disease and numerous clinical trials. BMSCs were isolated from human BM and the first experimental evidence for the existence of a stem cell population in this tissue compartment other than the HSCs arose in the 1960s. Such cells were believed to be a component of the BM stroma which was needed to support and nurture the hematopoietic functions of the BM but they also have the ability to differentiate into other cells types. Embryonic stem cells are those cells which come from the inner cell mass of the blastocyst and from which the entire body arises. They can divide nearly endlessly and differentiate into any cell type

Bone marrow mesenchymal stem cells (BMSCs) are the multipotent adult cellular population endowed with a broad differentiation potential. BMSCs were discovered in the late 1970s by a group led by a Russian-born scientist Alexander Friedenstein, who showed that BM contains a population of plastic adherent, highly proliferative cells, that were able to form colony of fibroblasts (hence the name colony-forming unit-fibroblasts, CFU-F) [Friedenstein *et al.,* 1970]. Following implantation in diffusion chambers, CFU-Fs spontaneously formed bone, cartilage, and fibrous tissue *in vivo* [Friedenstein *et al.*, 1987]. Their multipotentiality toward other mesenchymal lineages led Arnold Caplan to coin the term "mesenchymal stem cells" [Caplan *et al.*, 1991], in analogy to "hematopoietic stem cells" (HSC), which were the best described adult stem cell type at the time. BMSCs cells can be kept undifferentiated in cultures or be differentiated to tissues of all 3 germ layers, *in vivo* and *in vitro.* BMSCs are characterized *in vitro* by their fibroblast- like morphology, adherence to plastic surface and extensive proliferative ability in an uncommitted state while retaining the potential to differentiate along various lineages of mesenchymal origin, including adipocyte, osteoblast, chondrocyte and adventitial reticular cell (BM stroma) lineages, in response to appropriate stimuli [Caplan *et al.*,1991, Bianco

et al., 2008]. BMSCs are pluripotent adult stem cells that can not only differentiate into osteoblasts, chondrocytes, adipocytes [Pittenger *et al.*, 1999] but also into excitable cells such as neurons [Bianco *et al.* 2008], skeletal muscle cells [Ferrari *et* al., 1998], and cardiomyocytes [Toma *et al.*, 2002]. BMSCs and their progeny also produce a wide array of cytokines, chemokines, and adhesion molecules that regulate hematopoiesis [Deryugina *et al.*, 1993, Nagao *et* al., 1995]. More recently, the cells have been shown to suppress T-cell immunoreactivity in response to allo-antigens [Rasmusson *et al.*, 2005,Phinney *et al.*,2005, Pittenger *et* al.,2004, Ortiz *et al.*,2003] and to benefit the treatment of neurological disorders [Phinney *et al.*2005], cardiac disease [Bianco *et* al., 2003], and pulmonary fibrosis [Ortiz *et al.*,2003].

The original naming of this class of stem cells as mesenchymal was based on the hypothesis that multiple tissues beyond skeletal lineages could be generated by postnatal MSCs, including skeletal muscle, myocardium, smooth muscle, tendon, etc. [Caplan *et al.*,2005]. However, the nonskeletal potential of single MSCs has not been formally proven in vivo, and the point remains controversial. Furthermore, whereas the original notion of MSCs specifically referred to cells in BM (bone marrow stromal cells, BMSCs), the current concept has been extended to include cells from additional sources (such as synovium, adipose tissue, dental pulp, etc.) and indeed, from almost every postnatal connective tissue. Classically, a subset of BMSCs is designated as colonogenic if it is able to generate colonies of fibroblast-like cells from single cells when plated in culture. Importantly, colony growth can be observed when cells are plated at higher, nonclonal density, but in this case, colonies cannot be assumed to be clonal, and enumeration or analysis of colonies formed under nonclonal conditions is experimentally meaningless. As assessed by current CFU-F assays, clonogenicity of BMSCs reflects the ability of a cell to grow in a density-insensitive fashion. As long as experimentation requires the use of cultured cells, sorting clonogenic progenitors by surface phenotype or "sorting" them by plastic adherence has the same practical

meaning. Consequently, the investigative value of isolation procedures based on surface phenotype will only unfold after *in vivo* assays are developed for the use of uncultured clonogenic progenitors. Adherent cells capable of density-independent growth are found in a number of nonhematopoietic tissues, such as periosteum and dental pulp, and probably in all connective tissues, and are also called CFU-Fs in the literature. Importantly, a primary culture of BMSCs can be established at clonal or nonclonal density (in most of the current literature, the latter is the case). In the first instance, the entire culture represents the progeny of CFU-Fs. In the second instance, the primary culture includes cells derived from nonclonogenic, adherent cells with limited but demonstrable potential for growth. Thus, primary cultures established at clonal or nonclonal density are remarkably different, but neither type of culture should be called a culture of stem cells, mesenchymal or otherwise qualified. Expansion of monoclonal or multiclonal primary cultures can yield populations that are homogeneous in the expression of certain markers, but not others. Functionally, within clonal cultures, the initially multipotent cells do self-renew, and may even stochastically expand, to some extent. However, simultaneously and within the same culture, some of the progeny of the culture-initiating cells differentiate or even senesce. Thus, any culture of non-transformed mammalian cells is heterogeneous due to inherent kinetics, as the expansion of stem cells within the culture is neither the sole nor the predominant event. The stochastic frequency of this event with respect to commitment or senescence in culture is as yet undefined; consequently, the expansion of stem cells cannot be measured or simply inferred from growth of the whole culture. In addition, many modifications of the original simple culture conditions have been proposed to improve the expansion of MSCs in culture. No matter what the source of the stromal population being examined, multipotency of MSCs is commonly believed to be assessable by in vitro differentiation assays. However, these assays correlate poorly with results of in vivo differentiation assays, even when conducted in parallel on the same cell strain .Furthermore,

multipotency (a property of a single cell) cannot be determined based on assays conducted on nonclonal cell strains in culture.

A primary culture of BMSCs can be established at clonal or nonclonal density (in most of the current literature, the latter is the case). In the first instance, the entire culture represents the progeny of CFU-Fs. In the second instance, the primary culture includes cells derived from nonclonogenic, adherent cells with limited but demonstrable potential for growth. Thus, primary cultures established at clonal or nonclonal density are remarkably different, but neither type of culture should be called a culture of stem cells, mesenchymal or otherwise qualified. In addition to the significant misconceptions of multipotency and of *in vitro* assays to probe it, even greater ambiguities persist concerning the generally assumed self-renewal of stem cells within BM stroma or within any other connective tissue. Evidence for self-renewal of progenitors within the BMSC population has only very recently started to emerge and does indeed support the concept that such cells include a bona fide stem cell.

In BM, MSCs serve two functions one is the classically recognized function of providing a supportive microenvironment for hematopoiesis. The other is related to the development, stabilization, and maintenance of the sinusoidal network, consistent with their sub endothelial localization. BMSCs function as stem cells in their own right and as cells that provide the microenvironment for other stem cells. While stem cells offer seemingly limitless possibilities for regenerative medicine, they have already delivered new assays to predict embryo-fetal developmental toxicity *in vitro*. In addition to providing a model of cells undergoing differentiation and proliferation, stem cells will play a major role by giving rise to many of the differentiated cell types on which this new vision depends. These will not be pure populations of single cell types but mixtures of cells much more representative of tissues *in vitro*. Moving from cells alone in a

culture dish toward the more physiological condition of multiple cell types being able to interact to maintain homeostasis in the face of a disequilibrating force (like a toxic exposure) will lead us toward more useful and correct predictions of *in vivo* toxicities.

As illustrated below, scientists have reported that adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside.

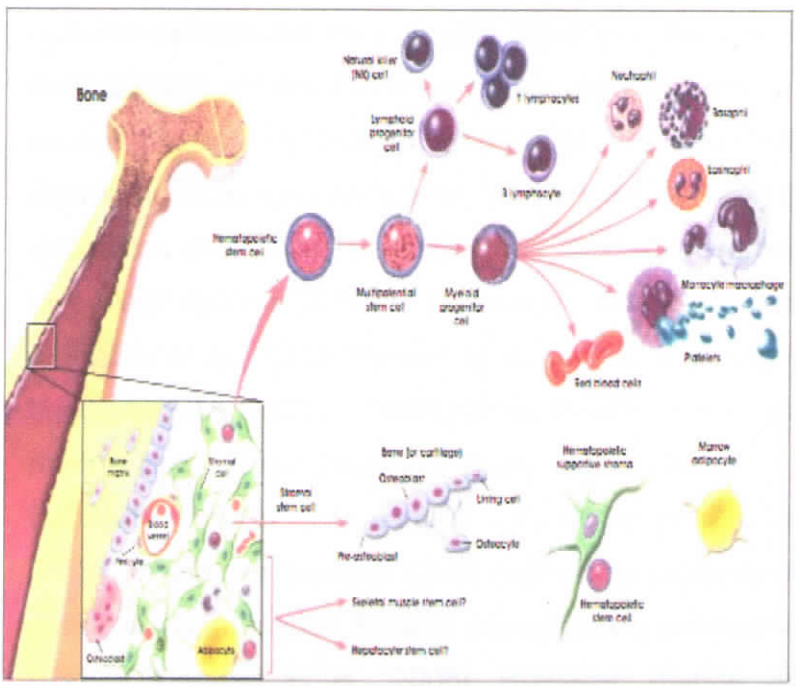


Fig:1 Hematopoietic and stromal stem cell differentiation.

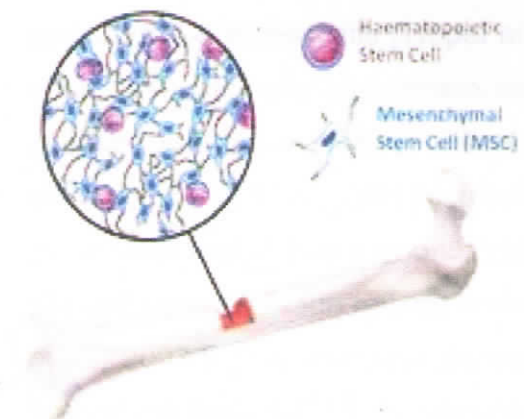


Fig: 2 MSCs and HSCs in bone marrow.

BMSCs are typically enriched from bone marrow by attachment to tissue culture plastic, but studies have shown that these adherent populations are phenotypically and functionally heterogeneous [Kuznetsov *et al.*, 2000, Muraglia *et al.*, 1997]. However, it is unclear if this heterogeneity contributes to or detracts from the potential clinical utility of BMSCs. A large number of studies aimed at evaluating the therapeutic potential of BMSCs using murine models. MSC cultures established from mouse bone marrow are replete with hematopoietic cells but they will not adhere to plastic, stromal cells or the matrix molecules they secrete [Kerk *et al.*,1985, Bearpark *et al.*,1989] and will not persist in the cultures after serial passage [Witte *et al.*,1987;Phinney *et al.*,1999]. And hence long-term expansion of adherent populations *in vitro* has been purported as a novel method to isolate murine BMSCs in several recent studies [Al-Khaldi *et al.*, 2003; Meirelles *et al.*, 2003; Peister *et al.*, 2004]. Recently it has been shown that in addition to the BMSCs, 'MSC like' cells with similar features reside in virtually all murine and human [Beltrami *et al.*, 2007; Covas *et al.*,2008] organs. BMSCs cultured *in vitro* express a number of cell surface markers, which serve to identify BMSCs; however, none of these markers is solely specific to BMSCs. There is a consensus that human BMSCs are uniformly positive for CD44, CD73, CD90 and CD105 [Dominic *et al.*, 2006]. However, murine BMSCs express stem cell antigen-1 (Sca-1) and CD44, but they vary in the frequency of the CD73-, CD90- and CD105-positive cells [Tropel *et al.*, 2003]. Murine BMSCs of different tissue origin have primarily been characterized regarding their phenotype and in some studies, by their capacity to differentiate into mesodermal cell types [Peister *et al.*, 2004; Sung *et al.*, 2008]. However, one of the most prominent MSC function, the *in vitro* and *in vivo* immunosuppressive activity, has been analyzed solely in BM and adipose tissue but not in stromal cells isolated from other organs [Djouad *et al.*, 2003.]. This immunosuppressive activity has recently been handled with special interest due to its usefulness in cell-based therapies [Phinney *et al.*, 2007; Nauta *et al.*, 2008; Jones *et al.*, 2007].

BMSCs undoubtedly play a critical role in the marrow microenvironment. BMSCs are also thought to be of great value for cell-based therapies. This focuses on the properties of BMSCs that engender their utility as therapeutic cells and specifically on BMSCs as treatment for GVHD and as targeting vehicles for antitumor therapies. Due to the ever-increasing number of studies on the clinical applications of mesenchymal stem cells in regenerative medicine, these cells have become attractive targets in clinical transplantation. However, the safety and efficacy of BMSCs for their clinical application in cell therapy is currently a matter of strong discussion. Immunodepleted murine BMSCs lacking expression of hematopoietic and endothelial markers differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro*, and osteocytes *in vivo.* They also require hematopoietic cells for growth *in vitro*, and their capacity for multilineage mesenchymal differentiation is inhibited by fibroblast growth factor 2 (FGF2) [Baddoo *et al.*, 2003]. Consequently, immunodepletion produces a cell population that, based on phenotype and function, more closely recapitulates properties of the bona fide MSC.

The recent production of induced pluripotent stem (iPS) cells adds another layer of luster to the appeal. These cells can come from a terminally differentiated cell in the body, and by transduction with a few transcription factors (Oct4, Nanog, Klf4, and Sox2), they can be returned to their undifferentiated pluripotent state [Baker *et al.*, 2007]. The big appeal of these cells are that they are derived from an adult cell and do not require the destruction of an embryo. Because, they could in theory, be made from anyone's cells, they avoid immune rejection if the starting cells were taken from the intended ultimate recipient, and they could allow the creation of cell lines from individuals with diverse genetic backgrounds for study of specific diseases.

Imidazoles are an important class of heterocycles and include many substances of both biological and chemical interest. Medicinal properties of imidazole include

anticancer activity , β-lactamase inhibitors, 20-HETE synthase inhibitors, carboxypeptidase's inhibitors, hemeoxygenase inhibitors, antiaging agents, anticoagulants, anti-inflammatory, antibacterial, antifungal, antiviral, antitubercular, antidiabetic and antimalarial [Baddoo *et al.*,2003 ; Grimmett *et al.*,1997.;Pozharskii *et al.*,1997.;Congi *et al.*, 2008;Nakamur *et al.*, 2004;Venkatesan *et al.*, 2008; Nantermet,et al.,2004]. This group presents in azoles antifungal which inhibit the accumulation of methylated sterols destroy the composition of the lipid bilayer of membranes. Some imidazole drugs, at high concentrations, could exert direct inhibitory action on membranes, without interference with sterols and sterol esters [Emami *et al.*, 2008; Ujjinamatada *et al.*, 2007]. Imidazole itself is stable to auto oxidation. Its derivative is reported to have, analgesic and anti-inflammatory activity 'cardiovascular activity, anti-neoplastic activity, anti- fungal activity, enzyme inhibition activity, antianthelmintic activity, anti-filarial agent, anti- viral activity and anti- ulcer activity.

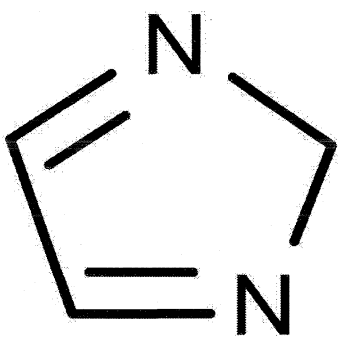


Fig: 3 Chemical structure of imidazole, a simple aromatic ring

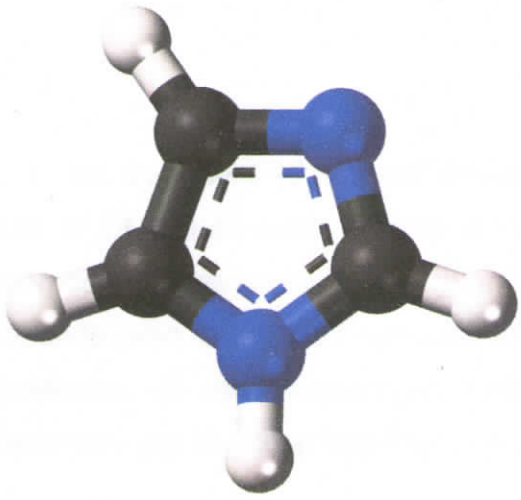


Fig: 4 Ball-and-stick model of the imidazole molecule, a nitrogen heterocycle and a simple aromatic ring.

On the basis of various literature surveys Imidazole derivatives shows various pharmacological activities: Anti fungal and Anti-bacterial activity, Anti inflammatory activity and analgesic activity, Anti tubercular activity, Anti depressant activity, Anti cancer activity, Anti viral activity, Antileishmanial activity and so on. Imidazole represents antifungal agents which inhibit the accumulation of methylated sterols and destroy the composition of the lipid bilayer of membranes. Some imidazole drugs, at high concentrations, could exert direct inhibitory action on membranes, without interference with sterols and sterol esters [Meirelles *et al.*, 2003]. Infectious microbial disease causes worldwide problem, because microbes have resisted prophylaxis or therapy longer than any other form of life. In recent decades, problems of multidrug-resistant microorganisms have reached an alarming level in many countries around the world. Resistance of anti-microbial agents such as β-lactam antibiotics, macrolides, quinolones and vancomycin etc and different species of bacteria causes increased important global problems [Sun *et al.*,2003]. Imidazole and its derivatives are reported to be physiologically and pharmacologically active and

find applications in the treatment of several diseases. Imidazole is a much stronger denaturant of DNA than urea, at least at lower concentrations. Some Imidazole containing compounds like clonidine, guanfacine and newly synthesize lofexidine hydrochloride also act as α2-agonists and clinically useful for the treatment of hypertension. α2 –adrenergic agonist also exhibit activity in human platelets and peripherally act in ocular hypertension (glaucoma) [Beltrsmi *et al*; 2007].

Imidazole derivatives are structural isomers of naturally occurring nucleotides which allows them to interact easily with the biopolymers of the living system, which is responsible for their numerous biological activities and functions. DNA intercalating agents, containing a planar chromophore with two to four fused aromatic rings, represent a large family of anti-tumor agents. [Li *et al* ;2007] synthesized a novel series of pentacyclic naphthalimides fused to an imidazole ring containing an unfused aryl or heteroaryl ring and evaluated for their *in vitro* cytotoxicity against P388 (murine leukemia), A-549 (human lung cancer), SMMC-7721 (human hepatoma), HeLa (human cervical carcinoma), and HL-60 (human acute promyelocytic leukemia) cell lines. B-Lactamases are serine and metallo-dependent enzymes produced by the bacteria in defense against all classes of β-lactam antibiotics, such as penicillins, cephalosporin's, carbapenems and monobactams. In order to overcome this resistance, several β-lactamase inhibitors such as clavulinic acid, sulbactam, and tazobactam are widely used in the clinics in combination with the b-lactam antibiotics. Carboxypeptidase A is one of the most studied zinc containing proteolytic enzymes and serves as a prototypical enzyme for metalloproteases that play important roles in the biological system. In view of the above, synthesized competitive inhibitors of carboxypeptidase A, 2-(4-imidazolyl) hydrocinnamic acid and its congeners that bear an imidazole ring as zinc-ligating functionality are being evaluated for their CPA inhibitory activity. From the study, it was reported that compound 2-benzyl-

3-(1H-imidazol-4- yl) propionic acid may potentially be explored as therapeutic agent. Thrombin-activatable fibrinolysis inhibitor (TAFI) was recently identified as an inhibitor of fibrinolysis. Imidazole acetic acid TAFI inhibitors was synthesized and found that 3-(3-aminocyclopentyl)- 2-[1-(3,3-dimethyl-butyl)-1H-imidazol-4-yl] propionic acid was the most active one.

Many methods have been developed to measure cell proliferation including those based on direct counting of viable cells, measurement of metabolic activity and cellular DNA content.. The most common assays for estimation of cell viability are based on cell membrane integrity and among them dye exclusion assay with trypan blue is widely used in routine laboratory work[Strober.2001].Trypan Blue is widely used for staining dead cells. Blue stained dead cells and the percentage of viable cells are calculated as ratio of viable (unstained) cells to the total number of enumerated cells (dead and viable cells). Another cytotoxic assay commonly used in laboratory purpose is MTT assay. It was first described by Mosmann [Mosmann *et al.*,1983]. It is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow soluble substrate 3(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cells lines .

.Measurement of mitochondrial metabolic rate using MTT and MTS to indirectly reflect viable cell numbers has been widely applied .However, metabolic activity may be changed by different conditions or chemical treatments which can cause considerable variation in results reported from these assays.

Increased production of reactive oxygen species outstripping endogenous anti-oxidant defense systems has been referred to as oxidant stress, which in turn

contributes to pathogenesis of many cardiovascular diseases including hypertension, atherosclerosis, retinosis, heart failure, and diabetic vascular complications. In mammalian cells, potential enzymatic sources of reactive oxygen species include the mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxygenase and cycloxygenase, the cytochrome P450s, xanthine oxidase, NAD(P)H oxidises, uncoupled nitric oxide synthase (NOS), peroxidases and other hemoproteins. Among biologically relevant and abundant reactive oxygen species, superoxide and its dismutation product hydrogen peroxide, appears most important in vascular signaling .The chemical basis of Dichlorofluorescein Fluorescent Assay is that upon entry into cells, cleaved by intracellular esterases to form DCFH, which is then oxidized by peroxides to form highly fluorescent DCF. The presence of oxidative stress may be tested in one of three ways: (1) direct measurement of the ROS; (2) measurement of the resulting damage to biomolecules; and (3) detection of antioxidant levels. Directly measuring ROS might seem the preferred method, but many reactive oxygen species are extremely unstable and difficult to measure directly. Because of this, many scientists prefer to measure the damage on proteins, DNA, RNA, lipids, or other biomolecules. While this is an indirect approach, many markers of damage are extremely stable and therefore provide a more reliable method to measure oxidative stress. Another approach is to measure the levels of antioxidant enzymes and other redox molecules which serve to counterbalance ROS generated in the cell. Assays are available to measure the activity of specific antioxidant enzymes, such as catalase and superoxide dismutase.

Cytogenetic abnormalities are a characteristic attribute of cells abnormality. The first chromosomal abnormality associated with cancer using cytogenetics was discovered in 1960. New cytogenetic techniques, namely quinacrine fluorescence and G-banding, to examine patients' karyotype, were developed.

From these techniques, it was discovered that the Philadelphia chromosome actually formed from a specific translocation of DNA between chromosomes 9 and 22.This translocation results in the formation of an abnormal protein that functions as a kinase molecule, which is constitutively, or always active and can, in turn, activate cell cycle controlling proteins and enzymes . This interferes with normal cell cycle regulation and allows cells that express the aberrant protein to divide more rapidly. Chromosomal changes are the signature of gene deregulation in cancer and lead to instability of the genome. Chromosomal changes are highly variable in different cancers, and the resultant phenotypic effects are equally variable.

Even under the best of circumstances, DNA is constantly subjected to chemical modifications. There are several types of DNA damage, such as SSB (single strand break), DSB (double strand break), CPDs (cyclobutane pyrimidine dimers), 6-4PPs (6-4 photoproducts). Sequences that facilitate bending and unwinding are favorable sites for damage formation e.g. CPDs are produced at higher rates in single-stranded DNA and at flexible ends of poly d(A)-d(T) tracts, but not in their rigid centre .

Halo assay is a technique which was first described by Vinograd (Vinograd *et al.* 1965) and refined by Roti and Wright (Roti and Wright 1987). In this assay , a fluorescent dye, intercalates into the DNA helix and causes the change in super coiling status of the DNA. Thus, DNA can be seen as a fluorescent halo that changes diameter with PI concentration. With this technique, cells are lysed and individual nucleoids are visualized as 'halos' and thereafter, halo area can be measured by an image analysis system which determines the chromatin fragility. It is used to detect the alterations in DNA organization in individual cell . For the assessment of single- strand breaks at the single cell level, this assay was improved as alkaline-halo assay. In this modified assay, the cells are first

embedded in melted agarose and spread on the microscope slides. Thereafter, it is incubated in a high-salt alkaline lysis solution followed by another incubation in a hypotonic alkaline solution and finally, stained with Ethidium bromide (EtBr). Under these conditions, single-stranded DNA fragments diffuse radically from the nuclear cage. Fast halo assay (FHA) is a technique similar to alkaline-halo assay (AHA) but there is some modification such as simplification of the lysis, denaturation and staining procedures

1.3 HYPOTHESIS

The toxicological evaluation is a real life practical application to understand and prevent life threatening crises associated with accidental exposures, overdoses, deliberate poisoning, unintended consequences of mixtures of toxins and toxicants. Multiple end points of toxicity, at the molecular, biochemical, cellular, physiological, pathologies at the whole organism or ecosystem levels can be assessed by using specific concepts and techniques like *in vitro* or *in vivo* studies. These assessment help in identifying specific mechanisms of toxicities such as mutagenesis, cytotoxicity, and altered gene expression that might be responsible for the pathogenesis of diseases. Exposure of the human organism to chemical, physical, biological agents, and even psychological stress will eventually leads to certain response at the cellular level. In toxicity testing, stem cells can be use for assessing the potential of any toxin or toxicant which affect stem cells. It might be wise to examine some theoretical and practical matters related to current state of understanding basic stem cell biology and their roles in normal development and any stem cell related disease. However, at the moment of trying to identify the realistic potentials about the stem cells, the attempts are being made to apply stem cells for wide diverse applications, such as for toxicity testing. Because of ease of isolation and the capability of mesenchymal stem cells to continuously cultured in undifferentiated state, this primary cells can be

used for assessing in vitro toxicity testing. By using a pure chemical imidazole, the toxicity can be studied in mesenchymal stem cells. The three potential consequences to chemical exposure of any toxicant will be mutation, caused by either an error in DNA repair or by an error of DNA replication (genotoxicity), cell death by necrosis or apoptosis (cytotoxicity), and altered gene expression at the transcriptional, translational, or posttranslational levels (epigenetic toxicity). By evaluating the toxicity of chemical with respect to in vivo studies, its similarity can leads to replace an organism with MSCs. Thereby the use of organism for testing can be decreased to certain level. The toxic effect of compound on the cell can be detected by their morphological changes, cell viability, adherent property, cell stress and changes in gene level can be assess by using chromosomal aberrations and DNA damage detection .MSCs are unique cell population capable of differentiating along multiple mesenchymal cell lineages under particular conditions and it can be also exist in undifferentiated state for long time and hence it can be used as an alternate model for toxicity screening.

OBJECTIVE

The main objective of the present study is to:

(1) Use of bone marrow derived mesenchymal stem cells as an alternate model for assessing *in vitro* toxicity testing

(2) Analyze the toxic effects of pure imidazole in bone marrow derived mesenchymal stem cells.

CHAPTER-2

MATERIALS AND METHODS

2.1 MATERIALS

Sl No:	Materials
1	Pure imidazole
2	BSA
3	DMEM-HG (GIBCO)
4	FBS (GIBCO)
5	Antibiotic-antimycotic (GIBCO)
6	PBS
7	MTT Reagent
8	DMSO
9	DCFDA
10	0.25%Trypsin-EDTA
11	$HNaCO_3$
12	NaOH
13	KCl
14	Colchicines
15	Giemsa stain

16	Sorrenson phosphate buffer
17	LM agarose
18	1X TAE Buffer
19	Methanol: Acetic acid
20	Genomic DNA isolation kit
21	DAPI
22	0.4% trypan blue
23	MItomycin C

2.2. EQUIPMENTS

Sl No	Equipment used
1	Microplate reader
2	Fluroscent spectrophotometer
3	Metafer (ZEISS)
4	Microscope (LEICA)
5	Centrifuge (HERMLE)
6	Refrigerated centrifuge (EPPENDORF)
7	CO_2Incubator (ESCO)
8	Deep freezer (SANYO)
9	Fluorescent microscope (ZEISS)
10	Electrophoretic apparatus
11	Biophotometer
12	Thermo mixer
13	Vortex mixer
14	Microwave oven

2.3 METHODS

2.3.1 ISOLATION AND CULTURE OF MESENCHYMAL STEM CELLS FROM MURINE BONE MARROW

To isolate bone marrow, Swiss albino mice (6–8 weeks old) were sacrificed by cervical dislocation and wiped with 70% ethanol. An incision was made around the perimeter of the hind limbs, where they attached to the trunk. The skin was removed by pulling toward the foot, which is cut at the anklebone. Each hind limb was bisected by cutting through the knee joint. To clean the bone, the muscles and connective tissue from both the tibia and the femur were removed by scraping the diaphysis of the bone and then pulling the tissue toward the ends of the bone. BMs were harvested under proper sterile conditions. The ends of the tibia and femur were cut just below the end of the marrow cavity using a sharp scalpel blade. A 24-gauge needle attached to a 1ml syringe containing complete media was inserted into the spongy bone, exposed by the removal of growth plate. Flushed the marrow plug out of the cut end of the bone and were collected in a 15ml tube. Bone marrow cells were cultured in 25cm² flasks with 5 ml complete medium. The cells at 37°C were incubated with 5% CO2 in an incubator. After 6 h, the non-adherent cells were removed by changing the medium and replacing with fresh complete medium. After an additional 12 h of culture, the medium was replaced with 5 ml of fresh medium. Thereafter, the adherent cells (passage 0) were washed with phosphate buffered saline, and added fresh medium every 3 to 4 days. The initial adherent spindle shaped cells appeared as individual cells and on 4 to 8 day, the culture became more confluent and reached 65–70% confluence within 2 weeks. At this stage, the cultures typically exhibit two characteristics: first, plates may contain distinct colonies of fibroblastic cells that vary in size; and second may contain very small numbers of hematopoietic cells interspersed between or on the colonies. After 2 weeks of initial culture, the cells were washed with phosphate-buffered saline for

passaging. Cells were detached by adding 1ml of 0.25% trypsin EDTA for 3 min at room temperature. The trypsin was neutralized by adding 5ml of complete medium, and all lifted cells were sub-cultured in another 25-cm^2 flask. The cells obtained after the third passage were used for further studies.

2.3.2 CHARACTERIZATION OF BMSCs

Cells are generally cultured in basal medium such as Dulbecco's modified Eagle's medium (high glucose) in the presence of 15% fetal bovine serum (FBS). Primary cultures are usually maintained for 12–16 days, during which time the nonadherent hematopoietic cell fraction is depleted. The main properties used for the characterization of MSCs are their adherent property, differentiation potential and the expression of cell surface markers.

IMMUNOPHENOTYPING FOR BMSCs

Immunophenotyping is the analysis of heterogeneous populations of cells for the purpose of characterizing the presence and proportions of the various populations of interest It is mainly used to study protein expressed by the cell. MSCs cultured in vitro express a number of cell surface markers, which serve to identify MSCs; however, murine MSCs express stem cell antigen-1 (Sca-1), CD90 and CD44. Here cells were characterized by immunophenotyping with cell surface CD markers which are specific for MSC. CD marker used here for characterization was CD90Appropriate cells were seeded on cover slip, removed the media and rinsed with PBS. For fixation. Cells were fixed in 3-4% Para formaldehyde in PBS for 15min at room temperature. After fixation, the cells were washed twice with PBS and incubated in 1%BSA in PBS for 30 min and excess BSA was removed by PBS wash. The cells were incubated in a humidified chamber in presence of primary antibody (1:100 dilutions in 1%BSA) at RT for 2 to 3h. Excess antibodies were washed with PBS and the cells were incubated

with secondary antibody –Rabbit anti-mouse IgG(1:400 dilutions in 1% BSA) for 1h at room temperature in dark. Again washed with PBS for three times in dark. Finally the cells were incubated with 4',6-Diamidino-2-Phenylindole (DAPI-1µg/ml) for 5-10min. After incubation, washed twice with PBS and observed under fluorescent microscope.

CYTOTOXICITY ASSAYS

Cytotoxicity assays are widely used to investigate the toxicity of compounds on cellular systems, mainly for drug screening and cytotoxicity tests of chemicals. These assays provide a rapid, sensitive, and validated approach to quantify harmful-dose ranges of compounds, and to analyze the biological effects of toxicity on living cellular systems. Cytotoxicity assays are mainly used based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Most commonly used cytotoxicity assays such as trypan blue exclusion assay and MTT assay were done here for cell counting and cytotoxic evaluation.

TRYPAN BLUE EXCLUSION ASSAY

Trypan blue is a vital dye particularly used for dead cell detections. Calculation of cell viability and the total number of viable cells are widely used methods for cell treatment with toxins, drugs, and cytokines and for estimating the effects of apoptosis triggering molecules. This dye pass through the cell membrane, in a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Cell counting is commonly done using hemocytometer and classic light microscope. However, Trypan Blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions.

The trypsinized cells are mixed with trypan blue in 1:1 ratio and count the cells using hemocytometer. Count the cells under microscope in four 1X1mm

square of one chamber and determine the average number of cells per square. When observed under the microscope non viable cells appear stained where as the viable cells are excluded from the stain. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. So all the cells, that exclude the dye are viable.

2.3.3 MTT ASSAY

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay which measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase by living cells, which determines mitochondrial activity. This assay is broadly used to measure the *in vitro* cytotoxic effects of chemicals on cells. The MTT enters the cells and passes into mitochondria where it is reduced to an insoluble, dark purple colored formazan product. The cells are then solubilised with an organic solvent and the released,solubilised formazan reagent is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of viability of the cells.

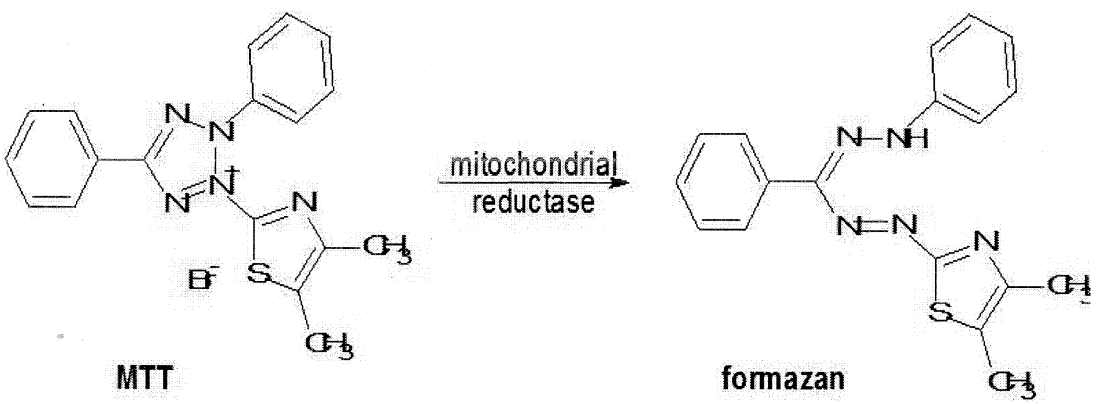


Fig:5 Schematic representation of MTT reaction (Mosmann *et al.*,1983)

Cells were trypsinized and approximate density of cells were seeded on 96 well plate After the attachment of cells, different concentration of pure imidazole (0.5mg/ml,1mg/ml,2.5mg/ml,5mg/ml) were treated and incubated at 37°C for 24h. Chemical free media used for negative control and phenol were taken as positive control. After 24h incubation, the treated media was removed and 100µl MTT reagent (50µg/well) was added and incubated for 4h in dark by covering with aluminum foil. After incubation, the reagent was removed and 200ul DMSO was added to all wells and incubated for 20min at 37°C. After incubation, read by using micro plate reader at the absorbance of 540nm.

Percentage viability = (OD of test /OD of control)*100

2.3.4 OXIDATIVE STRESS- ROS DETECTION

Reactive oxygen species include a number of molecules that damage DNA and RNA and oxidize proteins and lipids (lipid peroxidation). These reactive molecules contain an oxygen and include H_2O_2 (hydrogen peroxide), NO (nitric oxide), O_2^- (oxide anion), peroxynitrite ($ONOO^-$), hydrochlorous acid (HOCl), and hydroxyl radical (OH^-).

ROS play important roles in many cellular signaling pathways (proliferation, cell activation, migration, etc). ROS can be detrimental (it is then referred to as "oxidative and nitrosative stress") when produced in high amounts in the intracellular compartments. Cells generally respond to ROS by up regulating antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxides (GPx) and glutathione (GSH) that protects them by converting

dangerous free radicals to harmless molecules (i.e. water). Vitamins C and E have also been described as ROS scavengers (antioxidants).

ROS Production by Dichlorodihydrofluorescein Diacetate

The cell permeant 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), also known as dichlorofluorescin diacetate, is commonly used to detect the generation of reactive oxygen intermediates in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent DCFH-DA is converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF).

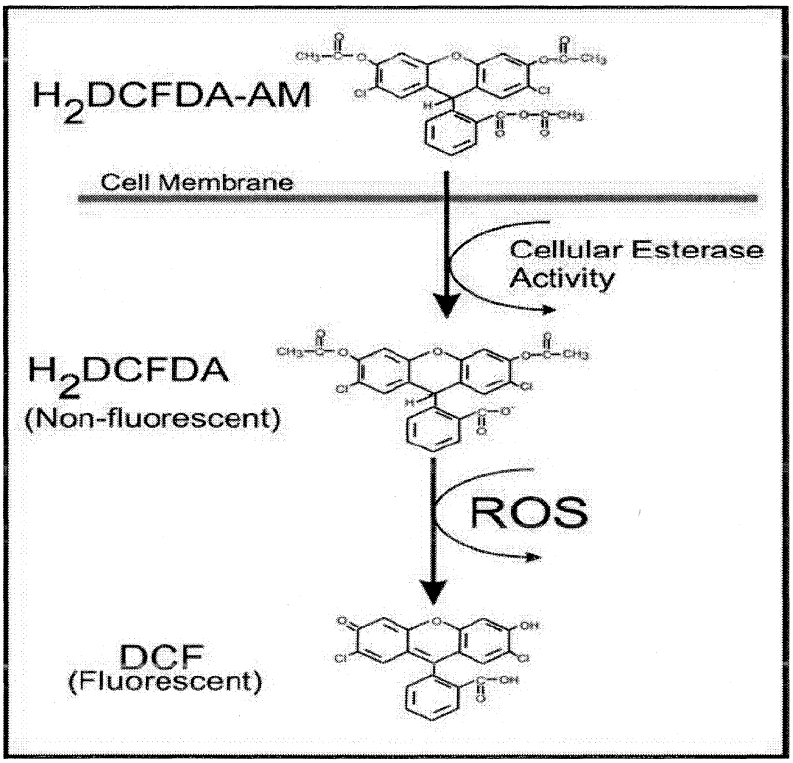


Fig: 6 Formation of fluorescent Compound DCF by ROS. (Hoffman *et al.*,2008)

Cells were trypsinized and seeded on 96 well plates. After the attachment of cells they were incubated with 200µl of 2 µM DCFH-DA for 1h in a 96-well plate. After

incubation, DCFH-DA containing medium was removed, the cells were washed with PBS and the treated with different concentrations of pure imidazole (0.5mg/ml, 1mg/ml, 2.5mg/ml, 5mg/ml) in exposure media for 4 h. At the end of exposure, dichloro-fluorescein fluorescence was determined by using fluorescent spectroscopy at excitation wavelength 450 nm and emission wavelength 535 nm

2.3.5 CHROMOSOMAL ABERRATION

Visible changes to chromosome structure and morphology have played a very important part as indicators of genetic damage in both clinical and cancer studies. Chromosomal aberration can result from either variation in the chromosome number or from structural changes. These events may occur spontaneously or can be induced by environmental agents such as chemical, radiation and UV light. Most commonly, observation is made at metaphase, using staining with dyes which give high-contrast chromatin staining and negligible cytoplasmic coloration. CA is the one of the most important biological consequences of genotoxic agents. Chromosomal mutations produce changes in whole chromosomes or in the number of chromosomes present. It may be Deletion – loss of part of a chromosome, Duplication – extra copies of a part of a chromosome, Inversion –reverse the direction of a part of a chromosome, Translocation – part of a chromosome breaks off and attaches to another chromosome. All may result when there is breakage of chromosome and rejoining or loss of chromosome fragments. The diploid chromosome number in mice is 40 (19 autosomes and X and Y sex chromosome). The autosomes and X chromosomes are telocentric whereas Y chromosome is acrocentric.

Different concentrations of pure imidazole (1mg/ml, 2.5mg/ml, 5mg/ml) were added to 80-90% confluent flasks and incubated for 24h. 12.5µl colchicines (0.1µg/ml) were added to all flasks before 1½h processing time. Chemical free media cultured cells were used as negative control. For processing, trypsinized

the treated flasks and centrifuge cell suspensions at 3000rpm for 10min. Then dispense the pellet at 100ul PBS and add 2ml 0.56% KCl for 10min .at room temperature. Add 5 drops of methanol: acetic acid (3:1) and centrifuge for 5min. After centrifugation remove the supernatant and 1ml methanol:acetic acid and again centrifuge at 3000rpm for 10min. Repeat the procedure twice with methanol :acetic acid. After processing the cells, gently mix the cells with a glass pipette and drop the cells evenly spaced along a cold slide. Tilt the slide and drain off excess fixative and dry the slides. The prepared smear is then stained using Giemsa stain.

2.3.6 DNA LADDER ASSAY

DNA fragmentation is a key feature of apoptosis, which is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into inter nucleosomal fragments. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. It occurs in response to various apoptotic stimuli in a wide variety of cell types. Apoptotic DNA fragmentation is often analyzed using agarose gel electrophoresis to demonstrate a 'ladder' pattern at ~180-bp intervals. Necrosis is usually characterized by random DNA fragmentation which forms a "smear" on agarose gels. Different concentrations of pure imidazole treated cells DNA were isolated using genomic DNA isolation kit.

DNA Isolation

BMSCs were treated with different concentrations of pure imidazole (0.5mg/ml, 1mg/ml, 2.5mg/ml, and 5mg/ml) for 24h. Chemical free media cells were taken as negative control and mitomycin treated cells were taken as positive control. All cells were trypsinized and centrifuged at 2750 rpm for 10min, discarded the medium and added 500ul PBS. Cells were centrifuged at 2500rpm for 10min. Then cells were resuspended in 200ul resuspension solution and then 20ul of RNase A solution was added and incubated for 2min at room temperature., To

that mixture 20ul proteinase K solution was added, followed by 200ul of lysis solution C. Vortex the sample about 5sec and incubate at 70°C for 10min.

Column preparation- 500ul of column preparation solution was added to the pre assembled binding column and centrifuged at 12,000xg for 1min. The flow through liquid was discarded and the treated binding column was used for DNA isolation.

The incubated samples were taken and 200ul of ethanol was added to that lysate and mixed thoroughly by vortexing for 10sec. Then transferred the entire content of the tube into the treated binding column and centrifuged at 6,500xg for 1min. the collection tube containing flow through liquid was discarded and the binding column was placed in a new 2ml collection tube. The same step was repeated again. Another 500µl of wash solution was added and centrifuged for 3 min at 14,000xg to dry the column and the binding column was placed in a new tube. Then 200µl elution solution was directly added into the center of the binding column and incubated it in room temperature for 5mins and then centrifuged for 1min at 6,500xg. The elute containing pure genomic DNA was estimated using biophotometer. The eluted DNA samples were then taken for DNA ladder assay.

AGAROSE GEL ELECTROPHORESIS

Of the various types of electrophoresis, agarose gel electrophoresis is one of the most common and widely used methods. It is a powerful separation method frequently used to analyze DNA fragments and it is also a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs

Gel casting- The gel is made by dissolving agarose powder (1%) in boiled 1X TAE buffer solution and Ethidium bromide is added to that preparation. The solution is then cooled to approximately 55°C and poured into a casting tray

which serves as a mold. A comb is placed across the end of the casting tray to form wells when the gel solution solidifies.
DNA was extracted using a genomic DNA isolation Kit. Then 10 µL DNA samples were mixed with 4 µL loading dye and analyzed by a 1% agarose gel pre-stained with 1 mmol EtBr. Gel electrophoresis included a DNA marker for comparison

After the gel solidified, the gel was submerged in electrophoretic chamber containing a positive electrode at one end, and a negative electrode at the other. Samples are prepared for electrophoresis by mixing them with loading buffer. This makes the samples sink through the buffer and remain in the wells. These samples are delivered to the sample wells with a micropipette or transfer pipette. Charged molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). Within a range, the higher the applied voltage, the faster the samples migrate. The buffer serves as a conductor of electricity and to control the pH, which is important to the charge and stability of biological molecules. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis.

2.3.7 HALO ASSAY

The fast-halo assay (FHA) is a very recent method to detect DNA-strand breakage induced either by various genotoxic agents or apoptosis inducing agents, and also to study the repair of primary DNA breaks at the single-cell level. In FHA, damaged DNA is separated from intact ones by means of solvent gradient and stained with ethidium bromide to visualize under a fluorescence microscope. The level of DNA breaks is then determined with an image analysis software. FHA is as sensitive, reliable, and flexible as the well-established comet assay, but it has the advantage of being, as compared to any other existing method, the most rapid and less expensive one.

Gel slide preparation for halo assay

The slides were washed with alcohol and dried at room temperature. Pour 1% low melting (LM) agarose into the beaker. Slide were held firmly using tweezers and dipped into the beaker. The slides were slowly removed and the undersides were wiped and kept in a flat surface overnight for drying.

Halo assay procedure.

The cells were treated with different concentrations of pure imidazole (1mg/ml,5mg/ml) for 24 h. Chemical free media used cells as negative control and 1mM hydrogen peroxide used as positive control. After 24h all cells were trypsinized and centrifuged at 2750 rpm for 10 min. Pellets were resuspended in 200µl PBS and kept it in cold environment.

Sample preparation

30µl of cell sample and 80µl of LM agarose at 40°C were mixed and from the prepared sample 40µl of cell samples were spread on agarose coated gel slides and was rapidly covered with a cover slip. Before agarose hardened, cover slip was slightly pressed and kept on an aluminum foil resting on ice until agarose layer hardens. Cover slip was gently removed from the slide. The remaining process was done under denaturing conditions. Kept the slides in 40ml NaOH (300mM) for 15mints in staining jar , then 100X EtBr was added to that solution and incubated it for 5min. The staining jar was gently shaken and the slide was passed into second jar contain distilled water for 15min. After distaining, the slides were analyzed by using fluorescent microscope.

COMPLETE MEDIA PREPARATION

DMEM HG-15% FBS

Autoclaved 1L distilled water and 1Lreagent bottle were used for media preparation. DMEM-HG powder was dissolved in 1L distilled water in sterile environment, then added weighed 3.7g $HNaCO_3$ and mixed well. 150ml FBS (15% FBS) was added to the media by syringe filtration and then 10ml antibiotic – antimycotic (1%) was added to the media through filtration. The prepared complete media was stored at 4°C.

REAGENT PREPARATION

Reagents for Halo assay

Lysis denaturing extracting solution (300mMNaOH) : 3g NaOH in 250ml distilled water

Ethidium bromide (100X) -2.5mg dissolved in 1ml distilled water.

Reagents for DNA isolation (genomic DNA isolation kit)

Proteinase K solution : dissolved the 5mg powder in one bottle of proteinase K(isolation kit) in 0.25ml water .

Wash solution: dilute the 3ml wash solution concentrate (isolation kit) with 10ml 100% ethanol.

CHAPTER 3

RESULTS & DISCUSSION

3.1 RESULTS

MORPHOLOGY OF ISOLATED MESENCHYMAL STEM CELLS FROM MURINE BONE MARROW

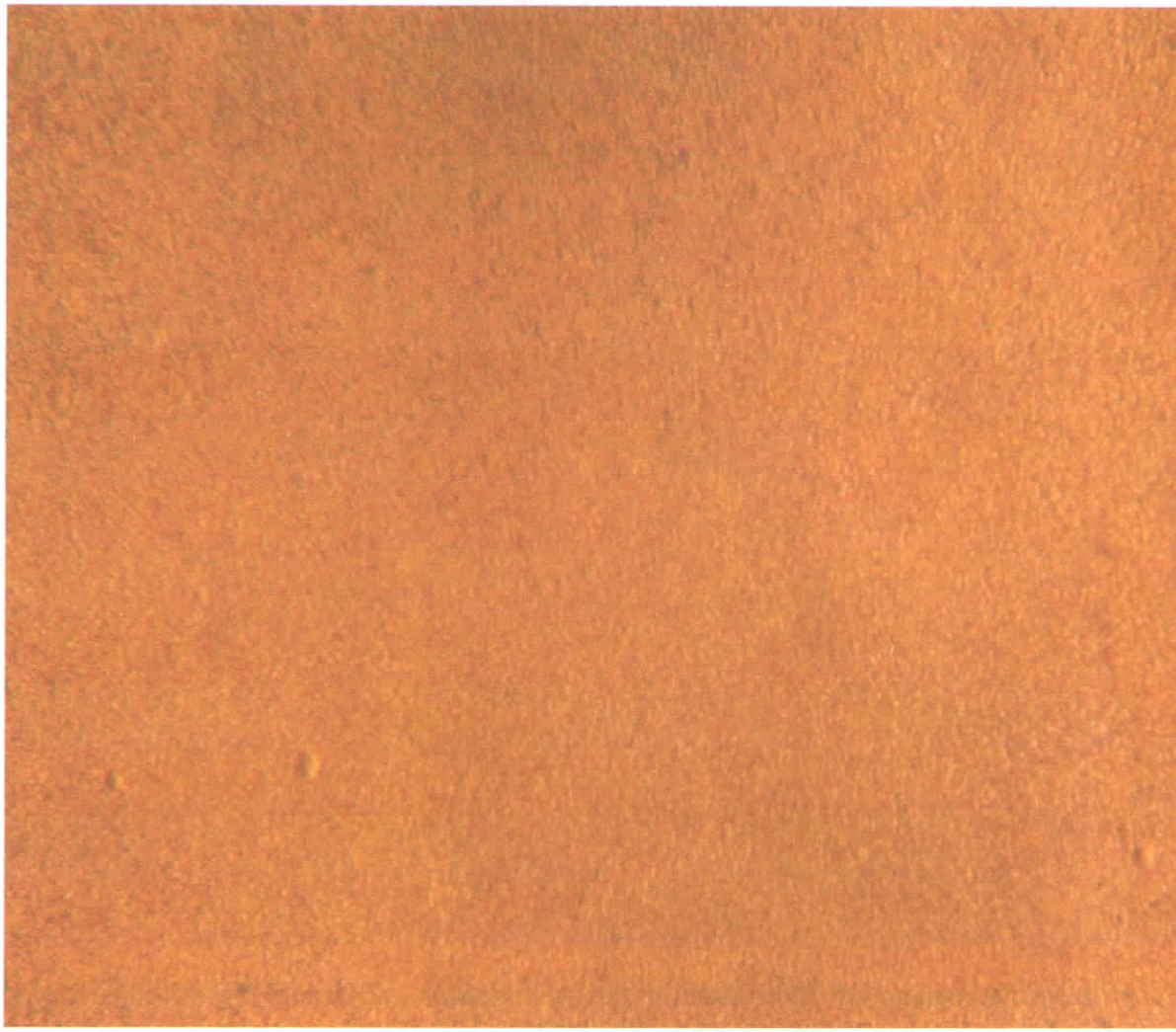


Fig: 7 Isolated cells with both HSCs and MSCs (P0 Passage) having round shape in which all cells are floated on medium. 10X (Masoud *et al.*, 2009)

Fig:8 BMSCs (P3 passage) established in culture with fibroblast shaped morphology after two passages 10X (Masoud *et al.*, 2009)

CHARACTERIZATION OF BMSCs

1) By adherent property and morphology

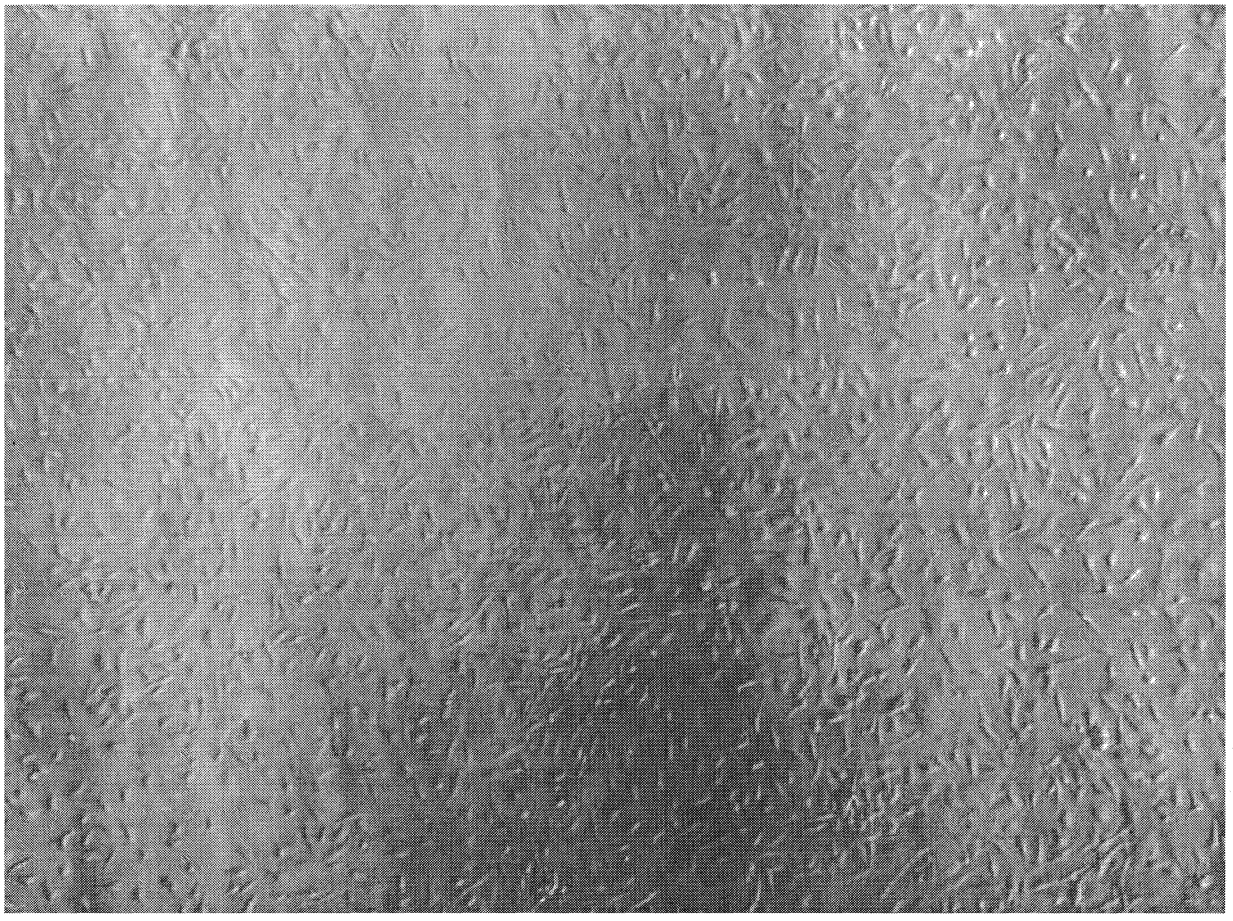


Fig: 9 Spindle shaped morphology of BMSCs 10X (passage 3) after two passages. (Masoud *et al.*, 2009)

Under normal tissue culture conditions, MSCs attached to the plastic surfaces which having fibroblast like morphology. They were characterized in vitro mainly by their fibroblast like morphology, adherence to plastic surface. The cells obtained in third passage were used for further studies. These cells retained there morphology in culture system in undifferentiated state.

CHARACTERIZATION OF BMSCs

1) By adherent property and morphology

Fig: 9 Spindle shaped morphology of BMSCs 10X (passage 3) after two passages. (Masoud *et al.*, 2009)

Under normal tissue culture conditions, MSCs attached to the plastic surfaces which having fibroblast like morphology. They were characterized in vitro mainly by their fibroblast like morphology, adherence to plastic surface. The cells obtained in third passage were used for further studies. These cells retained there morphology in culture system in undifferentiated state.

2) immunophenotyping for BMSCs

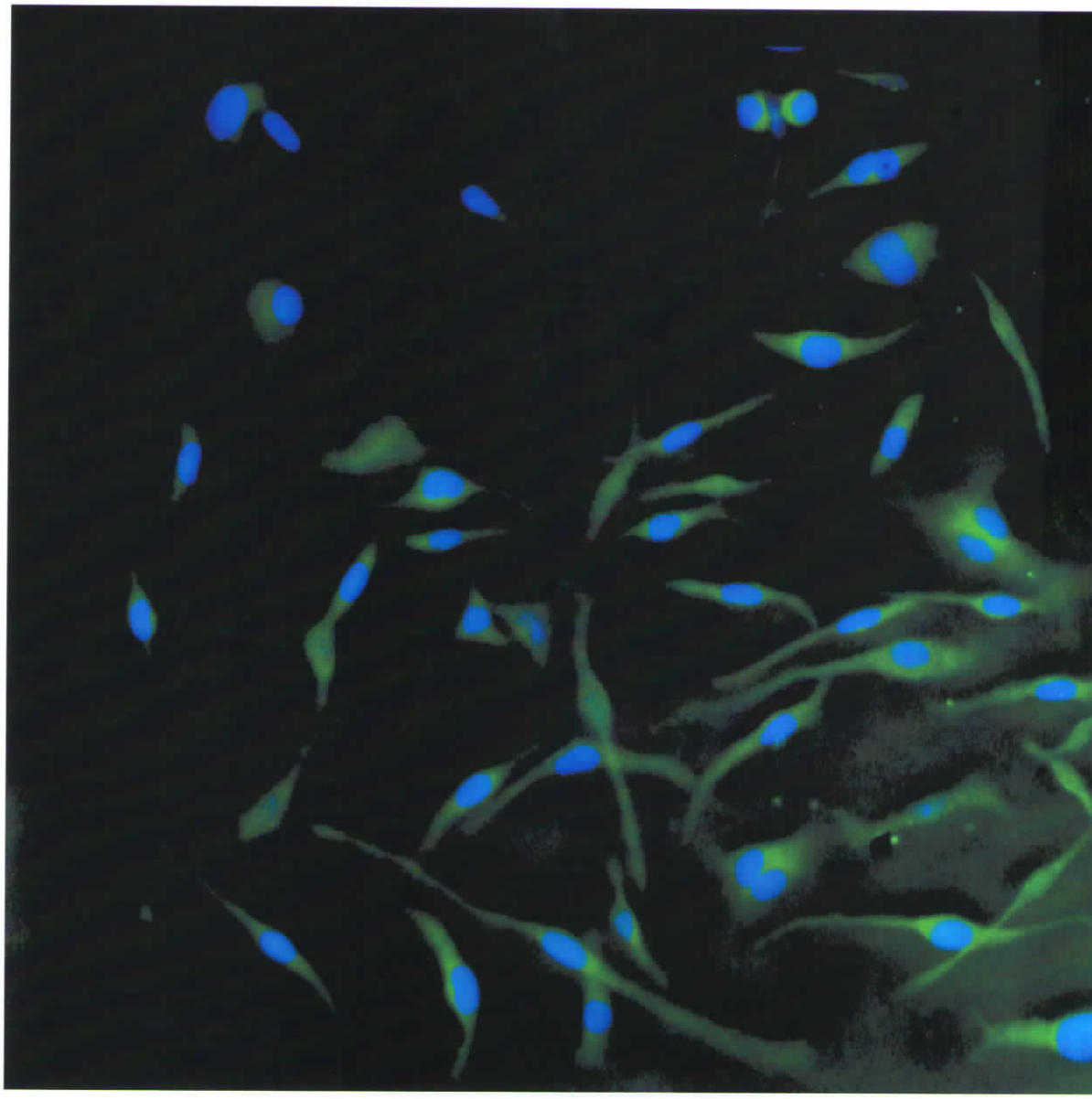
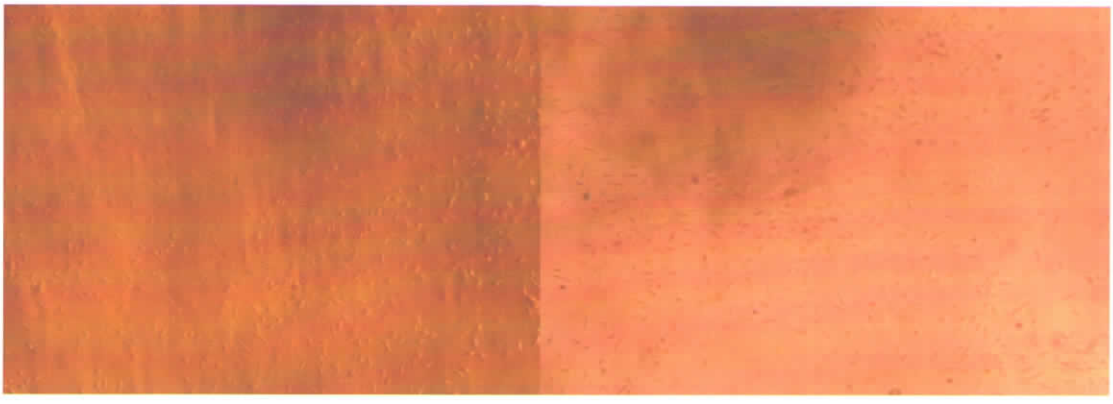


Fig:10 Expression of CD90 surface marker in BMSCs 40X (Chen *et al.*, 2010)

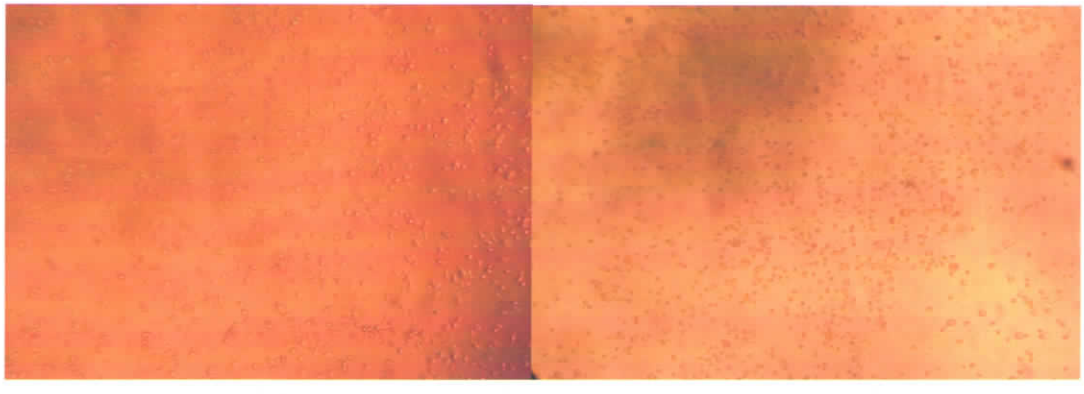
CD 90 expression analysis was used for the determination of mesenchymal stem cells immunophenotyping. These cells expressed this particular surface marker and which is used for the characterization of BMSCs in culture.

CYTOTOXICITY BY MORPHOLOGICAL ALTERATIONS



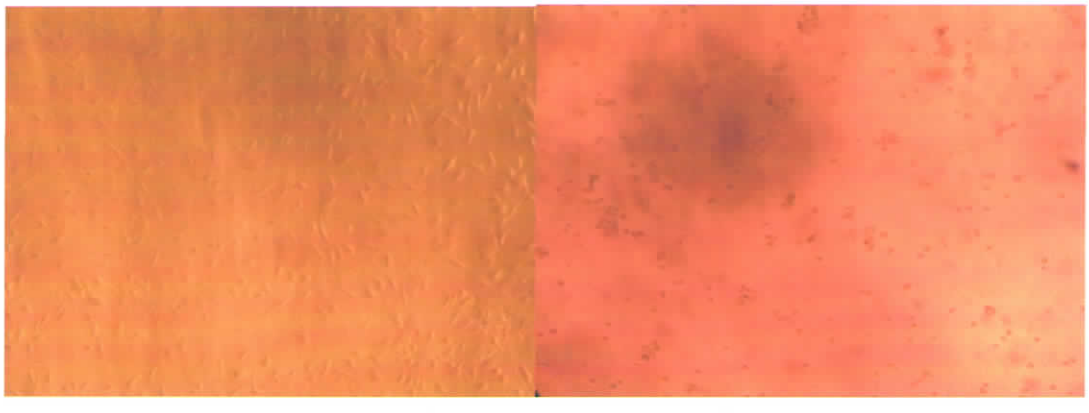
0.5mg/ml imdazole treated cells

1mg/ml imdazole treated cells



2.5mg/ml imidazole treated cells

5mg/ml imidazole treated cell



Negative control (chemical free)

positive control (phenol

Fig: 11 Morphological changes induced by different concentration of pure imidazole (0.5mg/ml, 1mg/ml,2.5mg/ml,5mg/ml)after 24h incubation(10X)

BMSCs are plastic adherent cells with spindle shaped morphology. Different concentrations of Pure imidazole (0.5mg/ml, 1mg/ml,2.5mg/ml,5mg/ml) treatment shows the morphological changes in the cells after 24h incubation. There is no specific change in low chemical dosage and in higher concentration the cells became round and detached from the adherent surface.

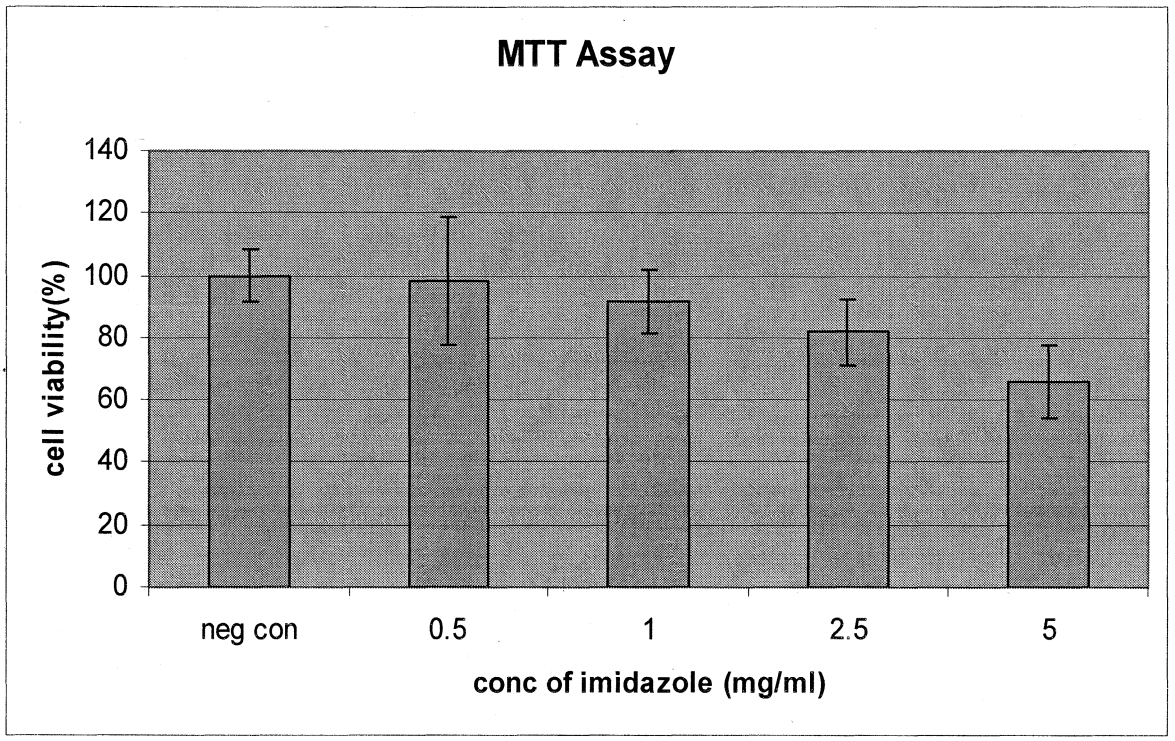


Fig:12 Dose response of cell viability was detected by MTT assay(Mosmann *et al.*,1983)

Effect of imidazole on mitochondrial function in MSCs was detected by using cell viability assay. Cells were exposed with different concentrations of pure imidazole for 24 h. At the end of the incubation period, mitochondrial function was determined by the MTT reduction assay. The OD value of control cells (chemical free) was taken as 100% and then calculated as the percentage of reduction of OD in chemical exposed cells. Control cells with chemical free media were run in parallel to treatment groups. Cells exposed with different

concentration of imidazole in higher dosage (5mg/ml) showed that the cell viability is less than 70% in *in vitro* culture.

OXIDATIVE STRESS- ROS PRODUCTION (DCFDA)

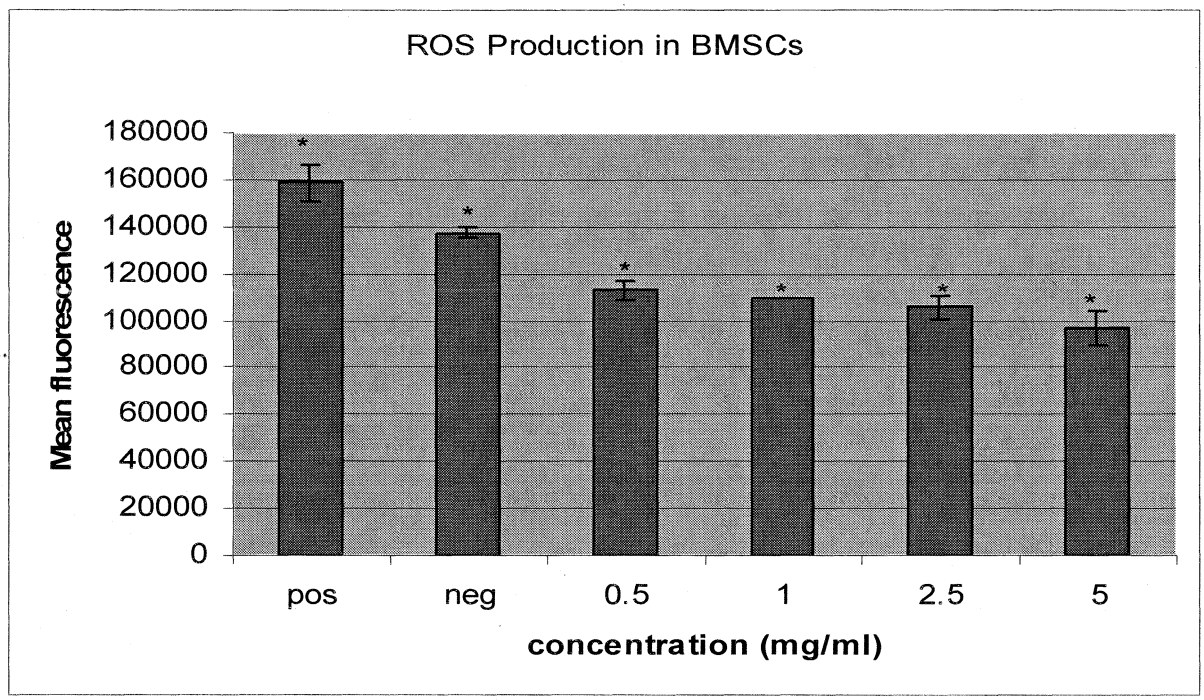


Fig: 13 Dose response of ROS production by DCFDA (Hoffman *et al.*,2008)

ROS are unstable molecules which are naturally occurring in metabolic reactions. Higher production of ROS can cause cellular damage. Data showed that a decrease in fluorescence intensity (ROS production) relative to positive control. Control cells cultured in imidazole free media were run in parallel to treatment groups. The results show that pure imidazole treated cells shows a dose dependent gradual decrease in ROS production compare to positive control. May be because of antioxidant property of imidazole, most of all imidazole derivatives are having anti oxidant property. In low dose treated cells shows high ROS

production and then its gradually decreased. Results were expressed as mean ± SD. The Student *t* test was performed to compare mean values. Probability of null hypothesis less than 5% ($P \leq .05$) was considered statistically significant.

CHROMOSOMAL ABERRATION

Visible changes to chromosome structure and morphology have played a very important part as indicators of genetic damage. The diploid chromosome number in mice is 40 (19 autosomes and X and Y sex chromosome). Whereas autosomes are telocentric (centromere at one end of the chromosome), Y chromosome is acrocentric 9 centromere near one end of the chromosome).Due to low proliferation of BMSCs, the number of metaphases are low in both treated and negative control cell samples. Total 50 metaphases were counted from each sample

Table :1 chromosomal aberrations in samples(negative control and 5mg/ml pure imidazole treated cells)

SAMPLES	CHROMOSOMAL ABERRATIONS
Negative control	1gap-0.1%
1mg/ml imidazole treated sample	6 Chromatid gaps – 0.3% 8 chromatid breaks- 0.4% 2 chromatid fragments-0.1%

Different concentrations of pure imidazole (1mg/ml,2.5mg/ml,5mg/ml) were treated for 24h. A chemical free media cells were used as negative control. In higher doses (5mg/ml and 2.5mg/ml), the cells changed its morphology from spindle to round and is detached from the adherent surface. By comparing to negative control 5mg/ml treated cells shows some chromatid aberrations. Total 0.8% of aberrations were present in chemical exposed sample.

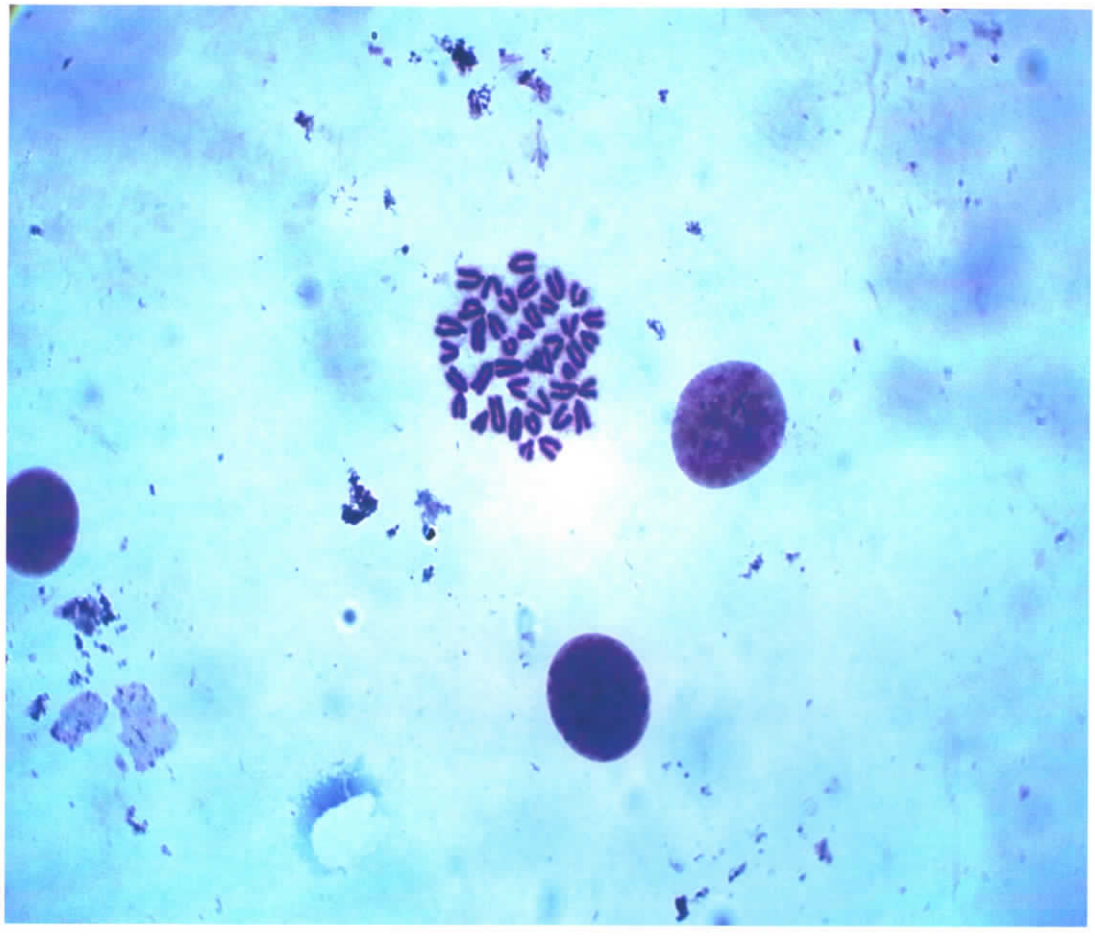


Fig :14 No aberration in untreated cell metaphase. Chemical free media was used as negative control. (100X)

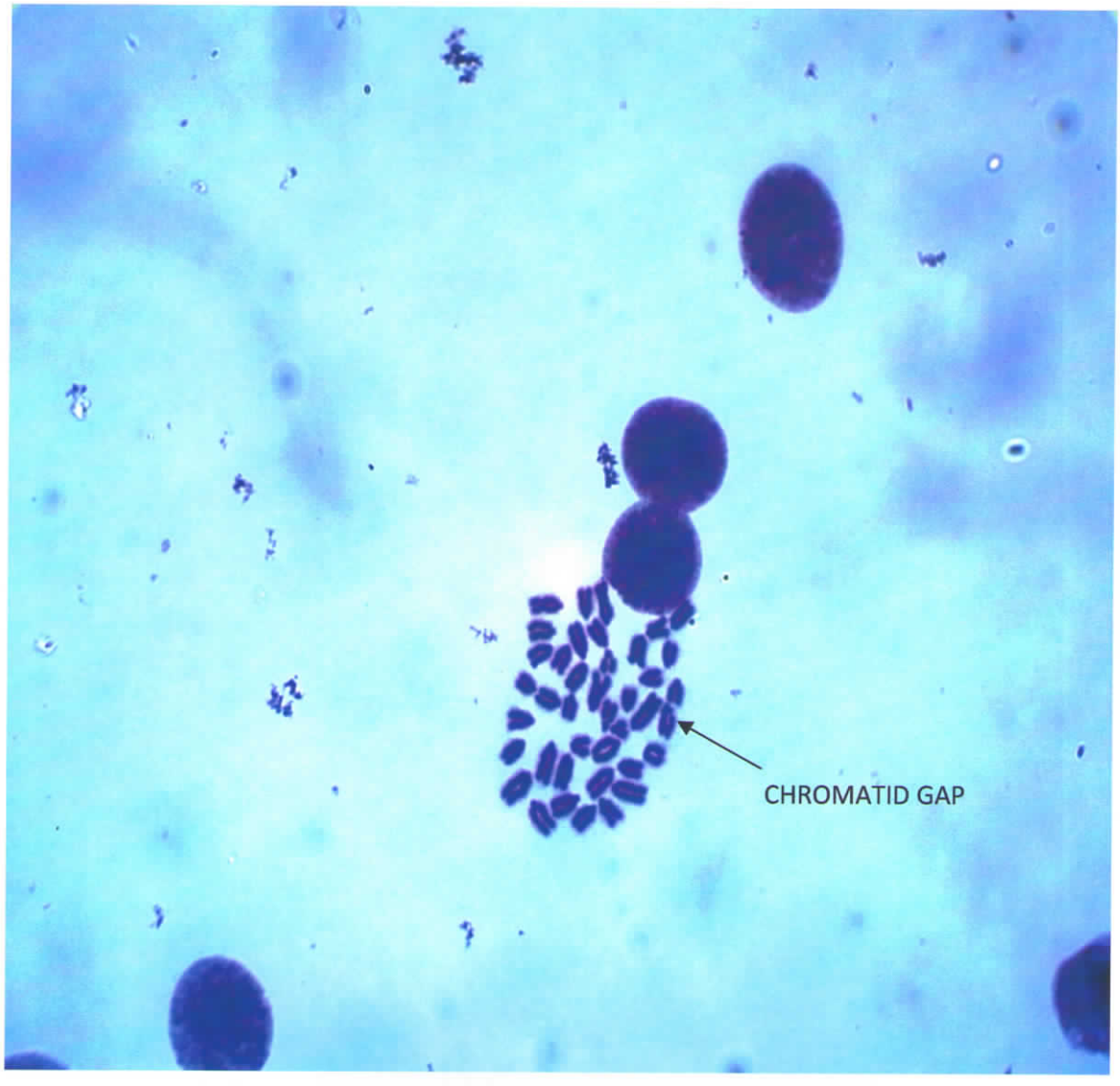


Fig:15 chromosomal aberration in 1mg/ml imidazole treated cell metaphase (100X)

Higher concentration treated cells chromosomal aberration cannot be evaluated. In 1mg/ml dosage, chromatid breaks, gaps and fragments are more compared to negative control.

DNA LADDER ASSAY

DNA was extracted from the treated and non treated cell by using genomic DNA isolation kit. Mitomycin C was used as positive control (PC), chemical free media used as negative control. DNA fragmentation After treatment with pure imidaozle for 24 h, DNA electrophoresis of BMSC cells showed a typical DNA smear.

DNA ladder assay showed that 24 h after treatment with chemical, a smear was seen in all treated samples based on their concentration of chemical. In positive control used as Mitomycin C resulted in clear smear formation.

Table:2 Concentration of DNA in each sample was estimated using biophotometer

Samples	Conc. of DNA (µg/ml)
Negative control(NC)	2.3
Positive control (PC)	4.2
0.5mg/ml treated cells	3.5
1mg/ml treated cells	3.1
2.5mg/ml treated ells	3.5
5mg/ml treated cells	3.8

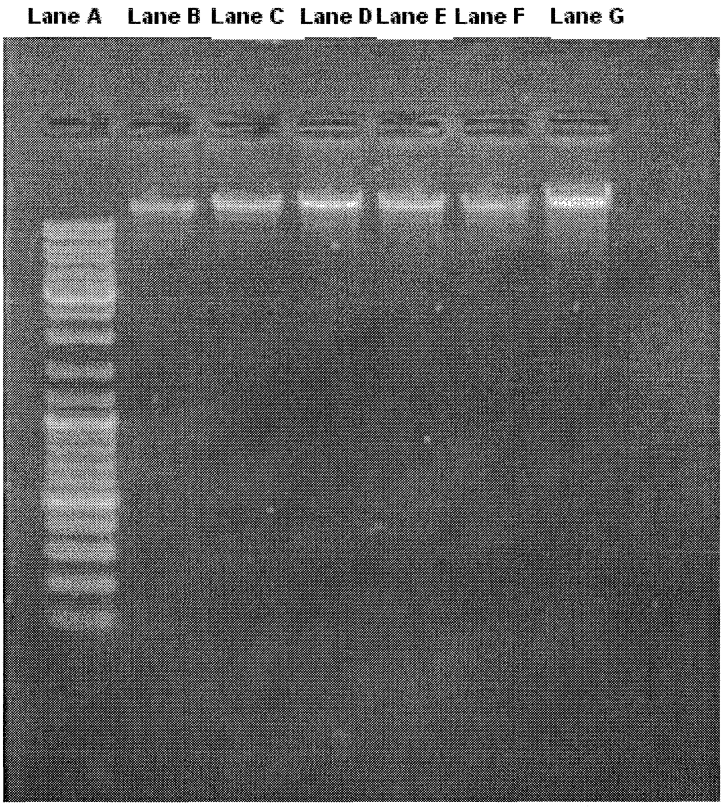


Fig:16 Agarose gel electrophoresis of DNA extracted from BMSCs treated with Pure imidazole for 24 h. Lane A: DNA marker; Lane B: control Lane C: treatment with imidazole 0.5mg/ml; Lane D: treatment with 1mg/ml; Lane E: 2.5mg/ml; Lane F:5mg/ml; Lane G:PC

The treated samples DNA shows a smearing band which represents the presence of DNA damage. By visualizing the smear, a dose dependent damage occurred in all treated samples. Damage is mainly obtained by double strand breakage.

HALO ASSAY

Fast Halo Assay is used for the Assessment of DNA Damage at the Single-Cell Level. The damaged DNA is separated from intact one by means of solvent gradient, stained with Ethidium bromide and visualized under a fluorescence microscope. By analyzing the halo, the level of DNA breakage can be calculated. Here the cell membrane are lysed using lyses buffer and the stained with ethidium bromide. This result reveals that a dose dependent DNA damage occurred in exposed samples.

The halo assay photographs of different concentrations (1mg/ml, 5mg/ml)showed that a halo around the cell nucleus which represent the DNA damage in the cells because of the toxic effect of pure imidazole.

Fig:17 Halo assay data in Negative control(chemical free media used cell) (40X)

Fig:18 Presence of Halo in 1mg/ml imidazole treated cell (40X)

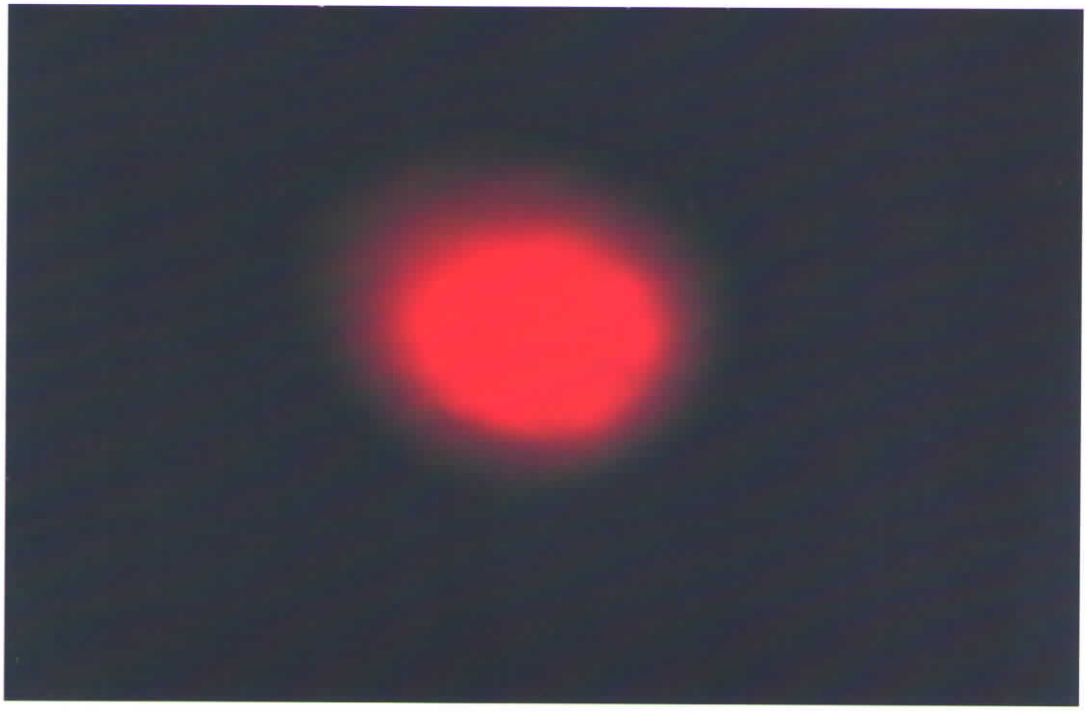


Fig:19 presence of Halo in 5mg/ml imidazole treated cell(40X)

By analyzing halo around the nucleus is used for determination of DNA damage. The diameter of halo formed around the cell nucleus is used for assessing DNA damage in each sample. Here comparing the visualizing image of halo assay by the presence of halo around the nucleus in treated samples and negative control. The cells treated with imidazole shows a halo around the nucleus by comparing to negative control. This damage may be double stranded or single stranded.

3.2 DISCUSSION

In vitro culture system provides a useful application for toxicity screening in cellular level. Mesenchymal stem cells (MSCs) are the spindle shaped plastic-adherent cells isolated from BM, with multipotent differentiation capacity in vitro. MSCs are relatively easy to isolate and purify, and currently have means to identify the cells by using there morphology, adherent property, differentiation potential and cell surface markers. Pure imidazole is a organic compound mainly using in medications. Its mainly using in the field of clinical chemistry for drug discovery. Its derivatives shows various pharmacological activities like Anti fungal and Anti-bacterial activity,Anti inflammatory activity and analgesic activity, Anti tubercular activity, Anti depressant activity, Anti cancer activity, Anti viral activity, Antileishmanial activity. Chemical toxicity testing became a subject to know the toxic effects of chemical to living organism.

Toxicological evaluation of pure imidazole on BMSCs reveals that they having toxic potential, such as cytotoxic as well as genotoxic. Cell viability assay shows the effect of chemical on cells by morphological changes and by mitochondrial activity. Cell morphology is an important sign of all healthy cells. BMSCs are spindle morphology in which it changes its shape based on the dosage of chemical. Another important factor for cytotoxiity or genotoxicity is oxidative stress. ROS production is a naturally occurring metabolic event; if its production increases inside a cell it can cause DNA damage. Imidazole treated cells shows that the high dose chemical having low ROS production and vice versa. May be pure imidaozle have an antioxidant property to reduce oxidative stress.

Pure imdazole exposed BMSCs cell culture system shows a genotoxic effect on cells. It is assessed by chromosomal aberration and DNA damage. Chromosomal aberration is principally due to double strand breakage. The higher

dosage imidazole treated cells resulted in chromosomal aberrations such as chromatid gap, breaks and fragments. DSB may lead to broken chromosomes. DSB may lead to mutations, chromosome rearrangements. CA are a small fraction of a huge amount of changes in chromosomal DNA and reflect an enormous plasticity of the genome .By running agarose gel electrophoresis with isolated chemical treated DNA resulted a damage in DNA by giving a smear. Through DNA ladder assay, it can be detect only double stranded breakage, single strand breaks measure by means of halo assay. Halo assay can show both double stranded and single stranded breakage. Imidazole treated cells shows a dose dependent halo formation.

All these in vitro assays used to detect primary toxicological effects of chemical pure imidazole on cells. BMSCs are multipotent cells which differentiated into multiple lineages and these cells having an important role in the development of an organism. Here BMSCs as a model for in vitro chemical toxicological screening. These assays in *in vitro* system can replace an organism to certain extent.

SUMMARY AND CONCLUSION

In the field of toxicology, the current concepts, techniques, and strategies for assessing the toxicity of chemicals is simply inadequate for an accurate prediction to the human situation. In stem cells, by understanding the basic biological characteristics of the cells and the roles they play in normal development, proliferation, differentiation, apoptosis, and senescence, could help to develop in vitro screening assays .That could assist in their use to screen for new drugs and assess toxicities of chemicals. The potential of obtaining new mechanistic insights to the roles of stem cells in various chemical-induced toxicities such as cancer, and in the aging process, is possible. stem cells have been considered to be used for basic mechanistic understanding of its cell behavior (cell proliferation in a symmetrical or asymmetrical fashion, differentiation, apoptosis, immortality, senescence, etc.), regenerative medicine , drug discovery, toxicity testing of pharmaceuticals and stem cell therapy, genetic therapy, and the role of stem cells in stem cell derived diseases and in the aging process

By screening for toxic agents that might affect symmetrical or asymmetrical division of stem cells could provide valuable insights to the signaling pathways controlling a cell. This could be a very important area of toxicology, in that to alter the number of stem cells during development might affect any stem cell based developmental or disease process later in life. This raises an important role for considering the use of stem cells for toxicity testing. Pure imidazole toxicity was tested in BMSC by using different methods. Imidazole drugs have broad applications in many areas of clinical medicine. Mesenchymal stem cells were isolated from murine bone marrow (femur and tibia).These cells were characterized by using their morphology, adherent property and by using expression of specific surface markers. MTT assay in MSCs by using pure imidazole reported that the chemical is cytotoxic at 5mg/ml dosage in *invitro*

culture system. Oxidative stress data revealed that pure imidazole having an antioxidant property. Higher dosage of chemical exposed cells shows low ROS production.

Genotoxic effects of pure imidazole shows chromosomal aberrations in treated cells compared to normal untreated cells. In higher dosage (5mg/ml & 2.5mg/ml) the cells became round and detached, so chromosomal aberration detection was not possible in that concentration. In 1mg/ml pure imidazole shows numerous chromatid gaps, breaks and fragments. These aberrations are mainly due to double stranded breakage. It is confirmed in DNA ladder assay, DNA fragments are obtained in exposed samples by running agarose gel electrophoresis. Halo assay was also used to detect DNA damage by visualizing a halo around the cell nucleus. Through these data, its confirmed that pure imidazole having both cytotoxic and genotoxic effects on cell system.

In vitro chemical toxicity evaluation shows the action of pure imidazole in cell culture system. It is possible to profile a chemical based on their effect on cellular level by using different methods evaluating both cytotoxicity and genotoxicity. From this study it can be concluded that BMSCs can be use as an alternate model for in vitro toxicity testing.

BIBILOGRAPHY

Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;105:1815–1822.

Al-Khaldi A, Eliopoulos N, Martineau D et al. Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response *in vivo*. *Gene Ther.* 2003; 10:621–629.

Arnold I. Caplan. Review: Mesenchymal Stem Cells: Cell–Based Reconstructive Therapy in OrthopedicsTissue Engineering.. 2005; 11:1198

Baddoo M, Hill K, Wilkinson R et al. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem 2003;89:1235–1249.

Baker, Monya "Adult cells reprogrammed to pluripotency, without tumors".Nature Reports Stem Cells.2007;10:1038.

Bearpark AD, Gordon MY. Adhesive properties distinguish sub-populations of hematopoietic stem cells with different spleen colony-forming and marrow repopulating capacities. Bone Marrow Transplant 1989;4: 625–628.

Beltrami, A. P., Cesselli, D., Bergamin, Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood. 2007;110:34-38

Bianco P, Robey P. G, Simmons P. J. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell .2008;2: 313

Caplan A. I., "Mesenchymal stem cells," *Journal of Orthopaedic Research*. 1991; 9: 641–650.

Chen ' Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. J Cell *Biol* 2010; 188:*595-609*

Congiu C.,. Cocco M. T, Onnis V. 'Design, synthesis, and in vitro antitumor activity of new 1,4-diarylimidazole-2-ones and their 2-thione analogues'Bioorganic & Medicinal Chemistry Letters., 2008;18: 989–993

Covas, D. T., Panepucci, R. A., Fontes, A. M. et al. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp. Hematol. . 2008 ; 36:642

Deryugina EI, Muller-Sieburg CE. Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development. Crit Rev Immun .1993;13:115–150.

Djouad F,Plence, P, Bony C. et al.. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood. 2003 ;102:3837.

Dominici, M., Le Blanc, K., Mueller, IMinimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy .2006 ; 8:315.

Emami S, Foroumadi A., Falahati M., Lotfali E., Rajabalian S., Ahmed Ebrahimi S,.Farahyarc S, Shafiee A., 2-Hydroxyphenacyl azoles and related azolium derivatives as antifungal agents Bioorganic & Medicinal Chemistry Letters ., 2008; 18: 141–146.

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science.1998; 279: 1528–1530.

Friedenstein A J, Chailakhjan R K, Lalykina K S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970;3: 393-403.

Friedenstein A.J.,.Chailakhyan R.K, Gerasimov U.V.. "Bonemarrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers," *Cell and Tissue Kinetics*.1987;20: 263–272.

Grimmett, M. Ross. Imidazole and Benzimidazole Synthesis. Academic Press, 1997

Hoffman, A, L.M. Spetner, and M. Burke Ramifications of a Redox Switch within a Normal Cell; its Absence in a Cancer cell. Free Radical Biology and Medicine 2008; 45:265-268.

Jones, B. J. and McTaggart, S. J.. Immunosuppression by mesenchymal stromal cells: from culture to clinic. *Exp. Hematol.* 2008; 36:733.

Kerk DK, Henry EA, Eaves AC et al. Two classes of primitive pluripotent hematopoietic progenitors: separation by adherence. *J Cell Physiology* 1985; 125:127–134.

Kuznetsov S A, Krebsbach PH, Satomura K . Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Miner Res* 1997;12:1335–1347.

Le Blanc K, Tammik L, Sundberg B et al. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* . 2003;57:11–20.

Masoud Soleimani & Samad Nadri 'A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow nature; 2009;10:1038/

Meirelles LS, Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion and characterization. *Br J Haematol.* 2003;123:702–711.

Mosmann, Tim. "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays" *Journal of Immunological Methods*1983 ;65 :55–63.

Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J Cell Sci* .2000; 113:1161–1166

Nagao T. Significance of bone marrow stromal cells in hematopoiesis and hematological disorders. *J Exp Clin Med.*1995; 20:121–130.

Nakamura T., Kakinuma H.,. Umemiya H,. Amada H, Miyata N., Taniguchi K.,. Bandoand K Sato M., 'Imidazole derivatives as new potent and selective 20-HETE synthase inhibitors' Bioorganic & Medicinal Chemistry Letters., 2004;14: 333–336

Nantermet P.G., Barrow J.C., Lindsley S.R., Young M., Mao S., Carroll S., Bailey C.,.Bosserman M, Colussi D., McMasters D.R.,. Vacca J.P,. Selnick H.G, *Bioorg.'* Imidazole acetic acid TAFIa inhibitors: SAR studies centered around the basic P_1' group'Med. Chem. Lett., 2004, 14, 2141–2145

Nauta A. J, Fibbe, W. E.. Immunomodulatory properties of mesenchymal stromal cells. *Blood.* 2007;110:3499.

Ortiz LA, Gambelli F, McBride C. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A.* 2003; 100 :8407– 8411.

Peister A, Mellad J A, Larson B L et al. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood.* 2004;103:1662–1668

Peister A, Mellad JA, Larson BL et al. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood.*2004;103:1662–1668.

Phinney D. G, Prockop, D. J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair–current views. *Stem Cells.*2007; 25:2896.

Phinney DG, Isakova IA. Plasticity and therapeutic potential of mesenchymal stem cells in the nervous system. *Curr Pharm Des* .2005;11:1255–1265.

Phinney DG, Kopen G, Isaacson RL et al. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variation in yield, growth, and differentiation. *J Cell Biochem* 1999; 72:570– 585

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999284: 143–147,

Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res.* 2004; 95:9 –20.

Pozharskii, A. F.; Soldatenkov, A. T.; Katritzky, A. R., Heterocycles in life and society. An introduction to heterocyclic chemistry and biochemistry and the role of heterocycles in science, technology, medicine and agriculture. , 1997. 0-471-96034-9

Rasmusson I, Ringden O, Sundberg B. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005; 305 :33– 41.

Roti J.L and. Wright W.D Visualization of DNA Loops in Nucleoids From HeLa Cells: Assays for DNA Damage and Repair 1987 62.645-661

Sun S, Guo Z, Xiao X et al. Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. *Stem Cells* 2003; 21:527– 535.

Sung, J. H, Yang, H. M., Park, J. B. et al.. Isolation and characterization of mouse mesenchymal stem cells. *Transplant. Proc.*2008; 40:2649.

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.*2002; 105: 93–98.

Tropel P, Noe I D, Platet N , Legrand P, Benabid A. L, Berger, F.. Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Exp. Cell Res*;2004:295:395.

Ujjinamatada R.K, Baier A.,. Borowski P,. Hosmane R.S, 'An analogue of AICAR with dual inhibitory activity against WNV and HCV NTPase /helicase: synthesis and in vitro screening of 4-carbamoyl -5-(4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl)imidazole-1-β-D-ribofuranoside' *Bioorg. Med. Chem. Lett.*, 2007;17: 2285–2288.

Venkatesan A.M., Agarwal A., Abe T.,. Ushirogochi H.O,. Santos D,. Li Z,. Francisco G,. Lin Y.I,. Peterson P.J, Yang Y.,. Weiss W.J, Shales D.M.,. Mansour T.S '5,5,6-Fused tricycles bearing imidazole and pyrazole 6-methylidene penems as broad-spectrum inhibitors of β-lactamases, *Bioorg. Med. Chem.*,2008; 16: 1890–1902

Vinograd , J., Lebowitz.J.,Radloff, R.,Watson,R. & Lapis,P'The twisted circular form of polyoma viral DNA'. Proc. natn. Acad. Sci. U.SA. 1965 53, 1104-1111.

Warren Strober 'Trypan Blue Exclusion Test of Cell Viability'2001;10:1002/0471142735

Witte PL, Robinson M, Henley A et al. Relationships between B-lineage lymphocytes and stromal cells in long-term bone marrow cultures. *EuropeanJ Immunol* 1987; 17:1473–1484.