A DISSERTATION ON

Effect of Cadmium on the differentiation potential of Mesenchymal Stem Cells

SUBMITTED TO THE

DEPARTMENT OF BIOENGINEERING

FACULTY OF ENGINEERING

INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT FOR THE

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

BY

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M.Tech Biotechnology (IV Semester)

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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, **Safia Baig**, a student of **M. Tech Biotechnology** (2nd Year/ 4th Semester), Integral University have completed my six months dissertation work entitled **"Effect of Cadmium on differentiation potential of Mesenchymal Stem Cells"** successfully from **CSIR- IITR** under the able guidance of **Dr. Neeraj Kumar Satija, Senior Scientist, CSIR-IITR.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

Name and Signature of Course Coordinator with Date

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Safia Baig,** a student of **M.Tech Biotechnology** (2nd Year/ 4th Semester), Integral University has completed him six months dissertation work entitled **"Effect of Cadmium on differentiation potential of Mesenchymal Stem Cells"** successfully. She has completed this work from CSIR- IITR under the guidance of Dr. Neeraj Kumar Satija, Senior Scientist, CSIR-IITR. The dissertation was a compulsory part of her **M.Tech Biotechnology** degree**.**

I wish her good luck and bright future.

Dr. Archana Vimal
Associate Professor
Department of Bioengineering
Faculty of Engineering

TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

Dr. Alvina Farooqui
Head
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ACKNOWLEDGEMENT

First of all I bow in reverence to the Almighty for blessing me with strong will power, patience and confidence, which helped me in completing the present work.

I feel deeply obliged indebted to my supervisor **Dr. Neeraj Kumar Satija, Senior Scientist CSIR-IITR**. I wish every trainee and research student were fortunate enough to have such an affectionate guide.

I would like to express my special thanks to **Prof. (Dr.) S. W. Akhtar** Hon'ble Chancellor, **Prof. Javed Musarrat** Hon'ble Vice Chancellor, **Dr. Syed Nadeem Akhtar** Hon'ble Pro-Chancellor, **Prof. (Dr.) Mohd Haris Siddiqui**, Registrar and **Prof. T. Usmani**, Dean Engineering, for giving me an opportunity to join the prestigious Integral University and providing all the necessary facilities ever since I started my work.

I express my profound and sincere thanks to the **Dr. Alvina Farooqui** Head of Department Bioengineering, PG Coordinator **Dr Roohi,** Course Coordinator **Dr. Salman Akhtar**, and Internal Advisor **Dr. Archana Vimal,** for always encouraging me for the work.

I am elated with delight to avail this wonderful opportunity to express my sincere thanks to all the faculty members of the department for their co-operation, affection, encouragement during my project pursuit.

My acknowledgement will be incomplete if I do not mention **my parents** with whose blessing I was able to achieve my goal successfully. There are no words to express my feelings towards them. I silently acknowledge my dept to them.

I also like to acknowledge **Indian Institute of Toxicology Research (CSIR-IITR)**, Integral University for providing facilities required for completion of my dissertation.

Date: Safia Baig

List of Abbreviations

Cd   : Cadmium

CD   : Cluster of Differentiation

CEBP α   : CCAAT enhancer- binding protein

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

DNA   : Dexyribonucleic acid

HLA   :   Human leukocyte antigen

MHC   :   Major histocompatibility complex

MSC   : Mesenchymal Stem Cell

PPARɣ   :   Peroxisome proliferator activated receptor

SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis

ULK   : Unc 51 like autophagy activating kinase

Vsp   : Vacuolar Protein Sorting

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Introduction

Cadmium is a heavy metal carcinogen, abundantly found in the environment. Indian rivers have been shown worrisome levels of Cadmium in them. It having a long half-life (~ 10-30years) possess the potential of being toxic to human health and the environment as well. As reported and seen in many of the previous studies that Cd has been highly contaminated into the natural water bodies, in certain occupational settings, and some specific dietary intakes and lifestyle habits (smoking). The troublesome point is the accumulation of Cd after it enters the body. Cadmium being accumulated in different parts of the body like blood, bone, kidney, etc. One such destination of our interest in Mesenchymal stem cells that are found in various places like bone marrow, adipose tissue, umbilical cord along with some other destinations.

The terms MSC and MSCs have become the preferred acronym to describe a cell and a cell population of multipotent stem/ progenitor cells commonly referred to as mesenchymal stem cells, multipotent stromal cells, mesenchymal stromal cells, and mesenchymal progenitor cells. The MSCs can differentiate to important lineages under defined conditions in vitro and in limited situations after implantation in vivo. MSCs were isolated and described about 30 years ago and now there are over 55,000 publications on MSCs readily available. Here, we have focused on human MSCs whenever possible. The MSCs have broad anti-inflammatory and immune-modulatory properties. At present, these provide the greatest focus of human MSCs in clinical testing; however, the properties of cultured MSCs in vitro suggest they can have broader applications. The medical utility of MSCs continues to be investigated in over 950 clinical trials. There has been much progress in understanding MSCs over the years, and there is a strong foundation for future scientific research and clinical applications, but also some important questions remain to be answered.

Developing further methods to understand and unlock MSC potential through intracellular and intercellular signaling, biomedical engineering, delivery methods and patient selection should all provide substantial advancements in the coming years and greater clinical opportunities. The expansive and growing field of MSC research is teaching us basic human cell biology as well as how to use this type of cell for cellular therapy in a variety of clinical settings, and while much promise is evident, careful new work is still needed.

Like all other cells Mesenchymal stem cells also undergo the process autophagy that is the housekeeping cellular process. Autophagy is a highly conserved cellular process by which

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cytoplasmic components are sequestered in autophagosomes and delivered to lysosomes for degradation. As a major intracellular degradation and recycling pathway, autophagy is crucial for maintaining cellular homeostasis as well as remodeling during normal development, and dysfunctions in autophagy have been associated with a variety of pathologies including cancer, inflammatory bowel disease and neurodegenerative disease. Stem cells are unique in their ability to self-renew and differentiate into various cells in the body, which are important in development, tissue renewal and a range of disease processes. Therefore, it is predicted that autophagy would be crucial for the quality control mechanisms and maintenance of cellular homeostasis in various stem cells given their relatively long life in the organisms. In contrast to the extensive body of knowledge available for somatic cells, the role of autophagy in the maintenance and function of stem cells is only beginning to be revealed as a result of recent studies. Macroautophagy/autophagy occurs at basal levels in all eukaryotic cells and plays an important role in maintaining bio-energetic homeostasis through the control of molecule degradation and organelle turnover. It can be induced by environmental conditions such as starvation, and is deregulated in many diseases including autoimmune diseases, neurodegenerative disorders, and cancer. Interestingly, the modulation of autophagy in mesenchymal stem cells (MSCs) represents a possible mechanism which, affecting MSC properties, may have an impact on their regenerative, therapeutic potential. Furthermore, the ability of MSCs to modulate autophagy of cells in injured tissues/organs has been recently proposed to be involved in the regeneration of damaged tissues and organs. In particular, MSCs can affect autophagy in immune cells involved in injury-induced inflammation reducing their survival, proliferation, and function and favoring the resolution of inflammation. In addition, MSCs can affect autophagy in endogenous adult or progenitor cells, promoting their survival, proliferation and differentiation supporting the restoration of functional tissue. A better elucidation of the mechanism(s) through which MSCs can modulate the autophagy of target cells and how autophagy can affect MSCs therapeutic properties is needed.

One such research gap in connecting the links is how Cadmium mediated autophagy affects adipogenesis of the Mesenchymal stem cells.

Objective –

The objective of this study are as follows –

▶ To check the cytotoxicity of Cd after acute exposure to MSCs

▶ To evaluate adipogenic differentiation potential of MSCs after exposure to Cd

▶ To check the effect of Cd on autophagy

Review of Literature

Cadmium-

Cadmium (atomic number – 48) is a heavy metal that belongs to the group IIB of the periodic table of elements. Toxicity by Cd is reported worldwide by chronic exposure via air, water, or food potentially affecting the pulmonary, urinary, reproductive, cardiovascular and muscular system, even leading to cancer. (Sandhya Iyer et al. 2020)

The heavy metal cadmium (Cd) causes environmental pollution that leads to a number of diseases, including kidney damage (So et al., 2020), bone loss (Buha et al., 2019), and cancers (Ebrahimi et al., 2020). Cadmium has leached into the environment through man made activities. The main sources of Cd in environment are more or less through industry and agriculture contamination. Cd enters the body mainly through the food chain and, to a lesser extent, through the respiratory tract. In the body, Cd is mainly accumulated in the kidney, liver, and bone. After entering the body, Cd has a realtively long half-life of approximately 10–30 years (Suwazono et al., 2009). And hence, the long-term accumulation or the chronic exposure leading to accumulation of Cd makes it potentially toxic even under low-dose exposure conditions (Sugita and Tsuchiya, 1995).(Yonggang Ma et al, 2021)

Commercially, Cd is used in a variety of things like television screens, lasers, batteries, paint pigments, cosmetics, and in galvanizing steel, which are in our day to day usage and also as a barrier in nuclear fission, and with zinc for seal- welding in lead water pipes before the 1960s.

Human exposure to Cd happens mainly by inhalation or ingestion. About 10%-50% of inhaled cadmium dust is absorbed, depending on particle size. Absorption pf Cd through skin contact is negligible. Depending on the particle size, approximately five to ten percent of ingested Cd is absorbed. Intestinal absorption is seen to be more in individuals with deficiency of iron, calcium, or zinc.

Cigarette smoking is considered to be the most significant source of human cadmium exposure. Cd levels in blood and kidney in smokers are consistently higher than nonsmokers. Inhalation caused due to industrial exposure is quite significant in occupational settings like, welding or soldering, and can be a reason for severe chemical pneumonitis.

Cadmium exposure also occurs from ingestion of certain contaminated food (e.g., crustaceans, organ meats, leafy vegetables, rice from some specific areas of Japan and China) or water (either from old water pipes sealed with Cd/Zn or industrial pollution) and can cause long-term health effects.

Data from some studies that have shown the exposure geographically are as follows

Table 1- Cadmium exposure levels

S.No.	Population	Sex	Exposure	Blood Cd levels	Sample Size	Reference
1	Sweden	Male	Non - Smoker	0.2µg/l	473	Carl Gustaf et al., 1983
2		Female	Non - Smoker	0.3µg/l		
3		Male	Smoker	1.4µg/l		
4		Female	Smoker	1.4µg/l		
5	Canada	Male	Mixed	0.35µg/l	10099	Rochelle E. Garnet et al., 2017
6		Female	Mixed	0.45µg/l		
7	Sweden	Female	Occupational	0.9±0.3µg/l	75	Palminger Hallen et al., 1995
8	India (Rajasthan)	Male	Occupational	2.48±1.2µg/l	110	Taru Goyal et al., 2020
9		Male	Unexposed	1.09±0.73µg/l	97	

After the Cd is absorbed by the body, it is transported throughout the body, by being bound to a sulfhydryl group-containing protein like metallothionein. About 30% of that gets deposited in the liver and 30% in the kidneys, with the rest being distributed throughout the body. Studies

have shown that it has a clearance half-life of twenty-five years. The half-life of cadmium in the blood has been estimated to be of 75 to 128 days, but this half-life is basically a representation of deposition in organs, not clearance from the body. (Robin A Bernhoft, 2013) Exposure to Cd can be associated with various toxic effects on kidneys, liver, lungs, bones, cardiovascular, endocrine, reproductive system, as well as genotoxicity and carcinogenicity (Danijela Dukic et al, 2020)

Consequently, blood, hair, and urine Cd levels are poor surrogates for body burden and chiefly reflect recent exposure, as is also the case with other heavy metals. An approximate estimate of the body burden of Cd will require urine provocation testing.

Cadmium toxicity has been seen in several organs. Cadmium induces tissue injury by creating oxidative stress (R.C. Patra et al, 2011), epigenetic changes in DNA expression (B Wang et al, 2012), inhibition or upregulation of transport pathways (L wan et al, 2012) particularly in the proximal S1 segment of the kidney tubule (D. A. Vesey, 2010). Other pathologic mechanisms include competitive interference with the physiologic action of Zn or Mg (J. M. Moulis et al, 2010), inhibition of heme synthesis (A. Schauder et al, 2010), and impairment of mitochondrial function potentially inducing apoptosis (G. Cannino et al, 2009). Depletion of glutathione has been observed, as has structural distortion of proteins due to Cd binding to sulfhydryl groups. (Robin A Bernhoft, 2013)

Cadmium, reportedly has also shown to induce autophagy in Mesenchymal Stem cells when treated with various concentrations (3.5 µM, 7 µM, 14 µM) for 24 hours.(Huifeng Pi et al, 2018)

Stem cells –
Stem cells are the template cells that are present throughout the body and they grow to become specialized cells with specific functions. These cells then undergo replication to generate "offspring" cells that can be either stem cells (and hence, self-renewing) or specialized cells (i.e. differentiated cells) that play a certain role - becoming blood, bone, fat, nerve, or skin cells, among various others. Stem cells, therefore, have the capacity to act as repair machinery for the substitution of damaged cells. The field in which a great amount of research interest is currently invested to determine the use of stem cells in the treatment of various diseases and

other damages is called as "regenerative medicine". Under normal conditions stem cells replicate themselves until they receive a signal to differentiate into a specific cell type. When stem cells get any such signals they first turn into progenitor cells, and then later, the final mature determined cell type. Lineage determination for a stem cell plays a vital role (and, in some cases, it is the absence rather than the presence of a signal that is the deciding factor). The ability of stem cells from one specialty to differentiate into another completely different type is known as plasticity, and embryonic stem cells are considered to be the "most plastic" of the rest.

Stem cells are technically labelled as being of a specific cell line, dependent on the characteristics and location of the original template cells from which all future offspring cells have grown (reflecting the self-renewing capability of the cells). Assuming that no contamination of the cell line occurs as a result of mutations or infections, and no differentiating triggers occur, the cell lines could potentially grow ad infinitum.

There are several types of stem cells: embryonic stem cells, fetal stem cells, adult stem cells, embryonic germ cells, and amniotic and umbilical cord stem cells. These different type of stem cells have distinct properties.

Embryonic and Fetal Stem Cells

The blastocyst, a collage of 50 to 150 cells in the intial stages of conception after fusion of the egg and sperm, is composed of three parts: the trophoblast which is the outer surface, the blastocoel which is the inner cavity, and the inner cell mass found inside the blastocoel which is composed of stem cells. These inner-lying cells are called as "embryonic" even though the term embryo technically applies after the initial two-week stage.

The next eight-week period is marked by cell growth and multiplication. Following this eight-week stage, the embryo has recognizable structures and is classified as a fetus. At this time, embryonic stem cells continue to proliferate and are considered to be pluripotent or plastic, which means that they can differentiate into almost any type of cell that makes up the body. The embryonic stem cell is believed by many scientists to be the most useful for potential medical treatments, but its use is restricted by federal legislation. Existing stem cells for medical research can be obtained from four primary sources: existing stem cell lines, aborted or miscarried fetuses, discarded embryos from fertilization treatments, or cloned embryos.

Adult Stem Cells

A small percentage of stem cells can be found in adult humans, at some specific locations, such as in the bone marrow or the subventricular zone of the brain. Until the discovery of these and other cells in the central nervous system, it was believed that the brain was the only organ that could not replicate. However, it is now clear that certain regions of the brain may have some limited capability to replace damaged or dead cells as a consequence of endogenous stem cells The adult stem cells enter a state of quiescence, until they receive an activation signal in case of cell damage. Determination of this signal that triggers the adult stem cells is very important to benefit from it.

Adult stem cells are considered to be harder to manipulate or control as compared with embryonic cells. In the majority of cases, the potential of adult stem cells to replicate also appears to be limited in contrast to embryonic stem cells, thus minimizing their utility.

34 However, these cells do have an advantage over embryonic stem cells: theoretically, they can be removed from a patient, grown in culture, and then returned to the patient.35 Therefore, they would not induce an immunological rejection response that may be seen with embryonic stem cells.(Wu DC et al, 2007) Some studies have shown that certain adult stem cells can differentiate into a various cell types, that may include neurons of the peripheral and central nervous system.(Kim S et al, 2006) However, more research and appropriate data is required to determine how useful these cells might be for their use in therapy.

Most research studies on adult stem cells are based on mesenchymal cells, i.e., cells from regions originally derived from the mesodermal layer of the embryo.

Embryonic Germ Cells

The precursors to the gametes (egg and sperm) are called as germ cells and are therefore found in adult testes and ovaries, and in the embryo that ultimately differentiate into testes or ovaries.(Giordano et all, 2007) These cells are as pluripotent as other embryonic stem cells. However, they have been seen to differentiate spontaneously, which infers that there is less control over their development than with other stem cells. (Clark AT et al, 2004)

Studies have suggested that adult stem cells can be conveniently obtained from germ cells of both sexes. (Seandel M, 2007)

Amniotic Fluid (or Placental) and Umbilical Cord Blood Stem Cells

The amniotic fluid is what surrounds and protects a growing fetus in its mother's uterus, as well as the placenta, have also been shown to have stem cells.(Prusa AR et, 2002) Amniotic fluid can be obtained from the female during the procedure of amniocentesis which is done for detection of any abnormality. The collected amniotic fluid is of no further use once the testing is complete, but now that it has been found to contain stem cells, there is potential for further research and storage of such fluid. The current studies convince that the amniotic fluid contains a mixture of embryonic and adult stem cells (Siegel N et al, 2007). Testing of these cells is very limited up to this date. It is believed that they are able to differentiate into a variety of cell types, but it is not known whether they are as pluripotent as other types of stem cells. Some authorities have suggested they could be used as a potential treatment for diabetes (Wei JP et al, 2003).

Some studies have demonstrated an additional potential source of adult multipotent stem cells: menstrual blood (David J Eve et al, 2008)

Mesenchymal Stem Cells-

Friedenstein et al. described a population of nonhematopoietic cells isolated from human bone marrow with the in vitro potential to adhere, proliferate, and differentiate into chondrocytes, osteoblasts, and adipocytes. Such cells were identified in small amounts in multiple tissues throughout the body (Friedenstein et al, 1966). Because of their differentiation properties and the ease of their isolation and in vitro expansion, these mesenchymal stem cells (MSCs) were considered an attractive candidate progenitor cell type for tissue engineering and regenerative medicine applications. (Yangzi Jiang et al)

MSCs have become widely studied over the past ~30 years for their interesting cell biology, broad-ranging clinical potential, and as a central building block in the rapidly growing field of tissue engineering. MSCs grow readily in the culture dish, have intrinsic differentiation potentials not found previously in other cells, and produce an abundance of useful growth factors and cytokines.

The isolation of MSCs from various tissue sources and their re-implantation at other sites raises questions about the natural in vivo MSCs and their ability to normally repair endogenous tissues, a process that clearly diminishes with age. Mesenchymal cell replacement in the large numbers needed to treat significant tissue injury requires engraftment, structural organization

and cellular differentiation—a complex process that has made much progress but remains unperfected. Friedenstein was first to culture bone-forming cells from guinea pig and Owen re-energized this inquiry by expanding such work to rats. The isolation and culture expansion of human bone marrow MSCs were reported in

1992 and their infusion into patients was begun as early as 1993 as reported in 1995. (Mark F. Pittenger et al, 2019)

Earlier, there was a debate among scientists about the stemness and nomenclature of MSCs. Some articles preferred mesenchymal "stromal" cells instead of stem cells. Some researchers attempted to change the name of MSCs to medicinal signaling cells because of their secretory role in the locations of diseases, injuries, and inflammations. However, later reports showed that prostaglandin E2 (PGE2) secreted by MSCs is responsible for maintaining the self-renewal ability and PGE2 is also involved in the immunomodulation of MSCs, creating a cascade of events, which proves the stemness of MSCs. Hence, the name given as Mesenchymal Stem Cells is justified.

There are various sources of isolation of MSCs such as adipose tissue, bone marrow, peripheral blood and neonatal tissues (umbilical cord, placenta, amniotic fluid, and amniotic membrane). The concentration of MSCs strongly depend on the source of isolation and it also decides the level of cytokines and certain expressed markers. However, the most basic identification markers of MSCs are CD73 (cluster of differentiation 73), CD105, CD90 but they should be CD34-, CD14-, CD45-, CD11b-, CD19- and lack HLAII (Human Leukocyte Antigen complex 2). Besides that, MSCs must express transcription factors such as octamer-binding transcription factor 4 (OCT-4) and homeobox protein NANOG. (Vineet Kumar Mishra et al, 2020)

Figure 1- Isolation and differentiation of MSCs
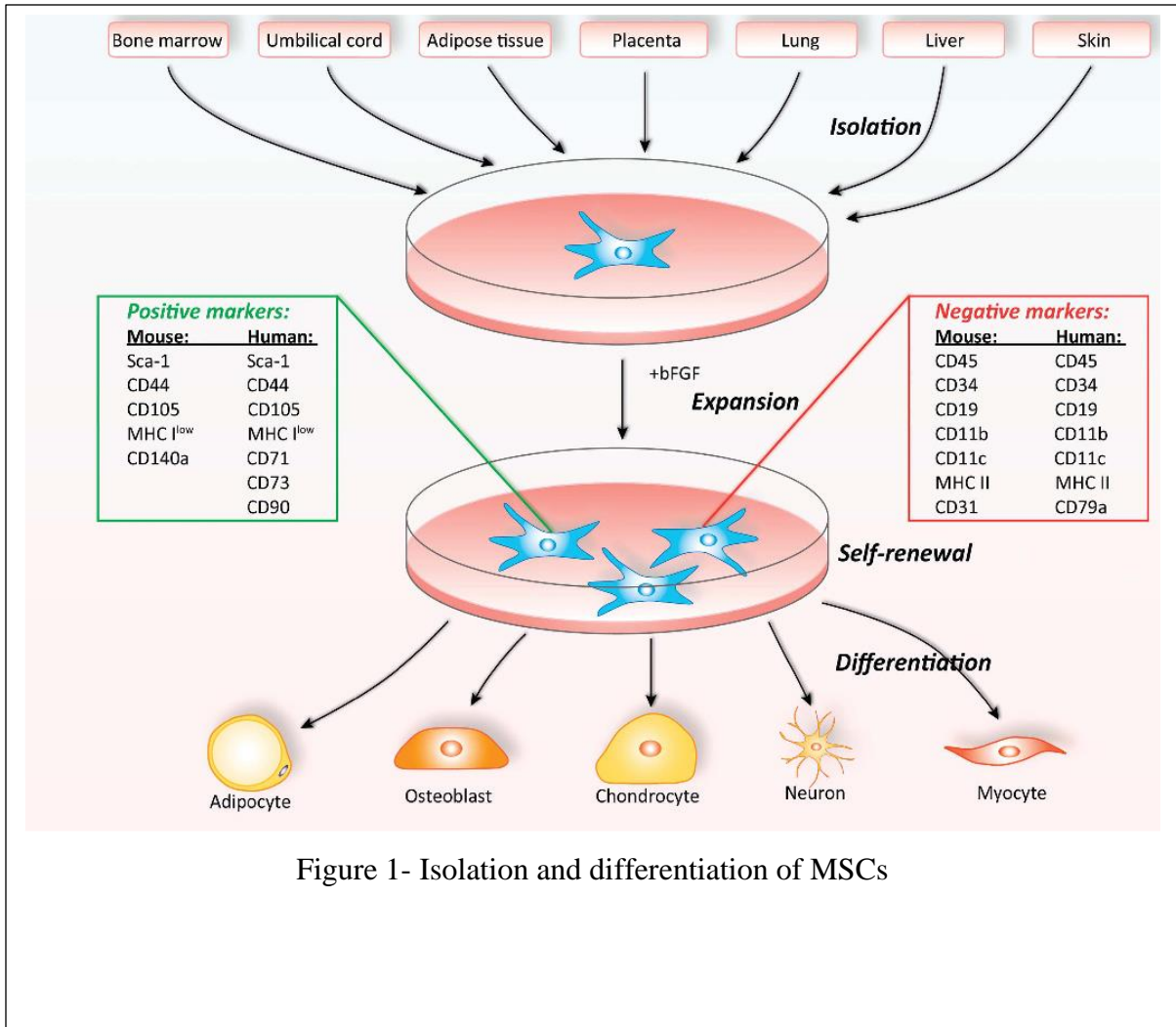
Signaling pathways in adipo-osteogenic differentiation of MSCs - The differentiation of MSCs is a two-step process, lineage commitment (from MSCs to lineage-specific progenitors) and maturation (from progenitors to specific cell types). Intensive studies in recent decades have demonstrated that a number of critical signaling pathways are involved in regulating the lineage commitment of MSCs, including transforming growth factor-beta (TGFβ)/bone morphogenic protein (BMP) signaling, wingless-type MMTV integration site (Wnt) signaling, Hedgehogs (Hh), Notch, and fibroblast growth factors (FGFs). As these pathways are well-established, we only briefly review their roles in MSC differentiation.

Cadmium also affects the differentiation lineage decision of MSCs. Various studies have also shown the data to justify this

Table 2 - Role of Cadmium in differentiation of MSCs

S. No.	Role of Cd	Range of conc. of Cd	Treatment time	Reference
1	Suppress osteogenesis	0,0.1,0.2 µM	1,3,7 days	Lu Wu et al., 2019
2	Induce Adipogenessis	0.5,2 µM	21 days	Latifa Kanani et al., 2019
3	Induce Adipogenessis	0, 10, 20, and 30 mol Cd/kg bw	6h	Takashige Kawakami et al., 2010
4	Induce Adipogenessis	0, 5, 10, and 20 mol Cd/kg bw	14 days	
5	Reduces adipocyte cell size	0, 5, 10, and 20 mol/kg bw	14 days	Takashige Kawakami et al., 2010
6	Inhibit lipid accumulation in differentiating 3T3-L1 preadipocytes.	0,0.3,1,3µM	6 days	Eun Jee Lee et al., 2011

Autophagy -

Under various physiological and pathological conditions, autophagy is the primary cellular mechanism for degrading and recycling intracellular proteins and organelles. (Sabrina Ceccariglia et al., 2020)

Autophagy, a self-eating cellular process, degrades cytosolic proteins and subcellular organelles in lysosomes to provide energy, recycles cytoplasmic components, and regenerates cellular building blocks; as a result, autophagy is essential for maintaining cellular and tissue homeostasis in all eukaryotic cells. Autophagy occurs as a self-eating cellular process in all eukaryotic cells to degrade cytosolic proteins and subcellular organelles in lysosomes, recycle cytoplasmic components, and regenerate cellular building blocks and energy, thereby maintaining cellular and tissue homeostasis. (Chenxia Hu et al, 2021)

Autophagy is characterised by the formation of a double-membrane vesicle that engulfs cytoplasm, malformed proteins, matured proteins, and other damaged organelles before fusing with lysosomes for degradation. The formation of double membrane vesicles is a complex process involving 16 autophagy-related proteins (Atg proteins). In addition, two ubiquitin-like conjugation systems are involved in the autophagy process. These systems generate modified

autophagy regulator complexes such as Atg8-PE, Atg5-Atg12-Atg16, and Atg7. This could influence the formation and size of the autophagosome.

The autophagy process then leads to the nucleation, expansion, uncoating, and completion of the autophagosome formation, which causes it to fuse with lysosomes. The molecular mechanism of autophagy involves several conserved Atg (autophagy-related) proteins, the majority of which were discovered in yeast. Two complexes are required to initiate autophagosome formation. A complex containing the class III PI3 K Vps34, Atg6/Beclin1, Atg14, and Vps15/p150.73. The other complex contains the serine/threonine kinase Atg1. Atg1's kinase activity is dependent on the function of two other autophagy proteins, Atg13 or Atg8, and Atg17.

Autophagosomes then fuse with lysosomes to form autolysosomes. The components encapsulated within autophagosomes are then degraded by lysosomal hydrolases, and the metabolites produced are the result of autophagy. They are recycled back into the cell as either energy sources or building blocks for the synthesis of new macromolecules. During the early stages of autophagosomal membrane formation, the endoplasmic reticulum is a key component of nascent autophagosomes.

Complex signalling pathways and the main autophagy proteins heavily regulate the autophagy machinery.

ATG genes were discovered through genetic screens for yeast autophagy-defective mutants, which was a significant accomplishment for the research at the time. More than 36 ATG genes have been discovered in yeast, and the vast majority of these have been conserved throughout evolution. The recruitment and hierarchy of ATG proteins in mammals have been studied. ATG proteins are one of the six functional groups of proteins that comprise the autophagy machinery and collaborate to form autophagosomes: Class III phosphatidylinositol 3-kinase (PtdIns3K), also known as vacuolar protein sorting 34 (Vps34), kinase complex; UNC-51-like kinase 1 (ULK1) kinase complex; PtdIns 3-phosphate (PtdIns3P)-binding FYVE or PX domain-containing proteins; the ATG5-12 ubiquitin- (Pasquier, Benoit)

Cellular sensors like mTORC1 (mammalian target of rapamycin complex 1) facilitate this coordination by promoting cell growth and directing metabolism into anabolic reactions. While inhibiting mTORC1 causes autophagy to activate, maintaining proper function halts the process. Indeed, the autophagy inhibitory compound MHY1485, an activator of autophagy,

has used this strategy pharmacologically. (Choi et al., 2012). The sequential activation of a series of protein complexes orchestrates the autophagic process. Several autophagic phases are proposed based on their order of participation. The "initiation phase" is characterised by the activation of the "ULK Initiation Complex," which is regulated by cellular sensors such as mTORC1 (D. Egan et al., 2011). Among other things, this complex contains key serine/threonine kinases like Unc-51-like kinase 1 (ULK1) or ULK2 and the scaffold protein FIP200. SBI-0206965 is an autophagy inhibitor that inhibits ULK1/2 kinase activity (D. F. Egan et al. 2015). The "initiation phase" of autophagy is followed by the "Nucleation phase," which also includes phagophore elongation.

This phase begins with the activation of the "VPS34 Nucleation Complex" at the sites of autophagosome formation. VPS34 (vacuolar protein sorting 34) is a class 3 phosphatidylinositol 3-kinase (PI3KC3) that forms a multiprotein complex with BECLIN-1. The generation of PI3P (phosphatidyl inositol 3-phosphate) by VPS34 and the recruitment of PI3P-binding proteins is a crucial step for the nucleation and elongation of the growing autophagosomes, which eventually seal to originate a mature vesicle (Jaber et al. 2012). 3-methyladenine has become a standard tool to test the involvement of autophagy in numerous biological paradigms through its blockage of VPS34 (Seglen and Gordon, 1982). Nonetheless, the high concentrations of 3-methyladenine required to block autophagy are facilitating its off-target effects (Wu et al. 2013). Furthermore, 3 methyladenine is not specific for VPS34, as class I PI3K (PI3KC1) is also inhibited (Wu et al. 2010). Furthermore, 3 methyladenine is not specific for VPS34, as class I PI3K (PI3KC1) is also inhibited (Wu et al. 2010). Cpd18 and SAR405, two inhibitors of VPS34 kinase activity with improved potency and selectivity, have been developed to overcome these limitations (Wu et al. 2013; Ronan et al. 2014; Pasquier, 2015). Spautin-1, on the other hand, inactivates the VPS34 complex by inhibiting two ubiquitin-specific peptidases (USPs), increasing BECLIN-1 ubiquitination and degradation (Liu et al., 2011). The autophagic cargo is loaded during the nucleation and elongation phases. It necessitates the involvement of specific ubiquitin-like conjugation enzymes, such as the E3-like complex. Some uncommon autophagy regulators that are not in general use are as follows –

Table 3- Non-traditional inducers and inhibitors of autophagy

S.No.	Compounds	Inducer/ Inhibitor	Model/Cell	Reference
1	Lithium Carbonate	Inducer	Hepatocyte cells	Roshan Dossymbekova et al. 2020
2	Copper	Inducer	Cardiomyocytes (myocardia pigs)	Quanwei Li et al. 2021
3	Fluorine	Inducer	Mice renal tissues	Qiang Guo et al. 2020
4	Arsenic	Inducer	Mice renal tissues	Qiang Guo et al. 2020
5	Statins	Inhibitor	NSC34 cells	Milad Ashrafizadeh et al. 2020
6	α-ketoglutarate	Inhibitor	Human osteosarcoma (U2OS), human neuroglioma H4	Elisa E Baracco et al. 2019
7	CyclosporinA	Inducer	Renal cells	Qinghua Wu et al. 2018
8	BisphenolA	Inhibitor	Neuro 2A cells	Jinglong li et al. 2022
9	BisphenolA	Inducer	Granulosa cell line (KGN cells) [Mouse & Human]	Miaoling Li et al. 2020
10	Doxorubicin	Inhibitor	Cardiomyocytes	Jian- An Pan et al. 2019
11	Cigarette smoke	Inducer	Granulosa cells (Murine)	Anne M. Gannon et al. 2013
12	Perfluoroctanoic acid(PFOA)	Inhibitor	Mice hepatocyte cells	Shengmin Yan et al. 2017
13	Prohibitin1	Inducer/Inhibitor	Intestinal epithilial cells/	Arwa S. Kathirai et al. 2012
14	Kynurenine	Inhibitor	Bone marrow mesenchymal stem cell	Dmitry Kondikov et al. 2020
15	SKP2 (S phase kinase associated protein 2)	Inhibitor	-	Nils C Gassen et al. 2019
16	Arsenic	Inducer	Leukemic cell lines	Sarmishtha Chatterjee et al. 2014
17	Cadmium	Inducer	MES-13 cells	Sarmishtha Chatterjee et al. 2014

18	Chromium	Inducer	Hematopoietic stem/ progenitor cell	Sarmishtha Chatterjee et al. 2014
19	Mercury	Inducer	Rat hepatocytes	Sarmishtha Chatterjee et al. 2014
20	Calcium	-	-	Yang Xi Hu et al. 2019
21	2,3,3',4,4',5- Pentachlorobiphenyl (PCB118)	Inducer	FRTL-5 cells(Fischer rat thyroid cell line-5)	Li Wang et al. 2020
22	Zinc	Inhibitor	MCF-7 breast cancer cells	Juan P Luizzi et al. 2014
23	PM 2.5	Inhibitor	Bronchial epithelial cells	Lu-Hong Kong et al. 2020
24	PM 2.5	Inducer	Human epidermal melanocytes	Pin Liyang et al. 2020
25	Canidin-3-glucoside (C3G)	Inducer	Caco 2 cells	Wen Chen et al. 2021
26	Hydrogen sulphide (H2 S)	Inducer	Hippocampal cells of rats	Hai-Yaio Liu et al. 2019
27	Chlorpyrifos	Inducer	SH - SY5Y	C Pellacani et al. 2018
28	Rotenone	Inducer	SH - SY5Y	C Pellacani et al. 2018
29	Fipronil	Inducer	SH - SY5Y	C Pellacani et al. 2018
30	Tri- ortho -cresyl phosphate (TCOP)	Inducer	SH - SY5Y	C Pellacani et al. 2018
31	2,3,7,8 - Tetrachlorodibenzo- p- dioxin	Inducer	SH - SY5Y	C Pellacani et al. 2018
32	Apatinib	Inducer	human osteosarcoma	Kuisheng Liu et al. 2017

Materials and Methods

Cell culture– Mesenchymal Stem cells of 9th passage, isolated from female mice, were cultured in α-MEM supplemented with 10% FBS and 1% antibiotic antimycotic solution in 37°C incubator with 5% CO_2 conditions.

Treatment - $CdCl_2.H_2O$ solution was made in MilliQ for 10mM stock concentration. Acute treatment for 24h was given to cells for various concentrations ranging from 0µM, 0.15625µM, 0.3125µM, 0.625µM, 1.25µM, 2.5µM, 5µM and 10µM.
Chloroquine treatment was given at the working concentration of 10µM.
Rapamycin treatment was given at the working concentration of 200nM.

MTT assay – MTT (3-(4,5- dimethylthiazol-2-yl)- 2,5- diphenyl tetrazolium bromide) reagent is a tetrazolium dye that binds with was added 100µl per well of the 96 well plate and incubated for 2 hrs. The formazon crystals thus formed were solubilized using SDS which was incubated for 24 hrs. The reading was taken at 570nm and 620 nm spectrometer.

Adipogenesis (differentiation) – Adipogenic medium was prepared by mixing Dexamethasone (1 µM), IBMX (0.5 µM) and Indomethacin (50 µM) 5µl per ml of complete α-MEM.

Nile Red staining – Nile red staining is a procedure done to stain the lipid droplets inside the cells. Cells were washed with PBS and fixed with 4% Formaldehyde at RT. Then stored with PBS at 4°C. Then the cells were incubated with DAPI (0.5µg/ml) - Nile red stain (200 ng/ml). And the cells were observed under fluorescence microscope.

Western blotting – SDS Polyacrylamide gel electrophoresis was run to quantify and analyze the desired proteins. 10% polyacrylamide gel was run to check for autophagy marker p62 and

15% gel was run to check for autophagy marker LC3 while 12% gel was run to check for adipogenic proteins i.e. CEBPα and PPARɣ
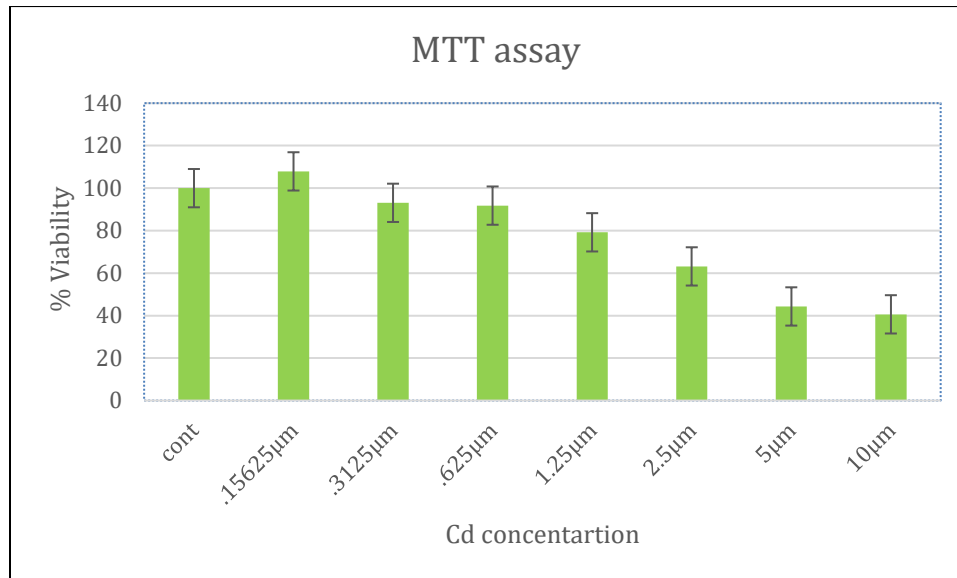
Results and discussion

1. MTT assay-

Figure 2 - Cell viability after acute (24hr) treatment

MTT assay was performed and the above data was achieved. The trend of cytotoxicity with increasing concentrations of Cadmium was observed. The highest experimental concentration i.e. 10µM lead to approximately 60% cell death (40% cell viability).

The working concentrations for further experiments were chosen to be 0.15625µM, 0.3125µM and 0.625µM so that there is almost 90% cell population viable to work with.

2. Adipogenesis levels-

Nile red	**DAPI**	**Nile red – DAPI merge**	
			Control
			0.1562μM
			0.3125μM
			0.625μM
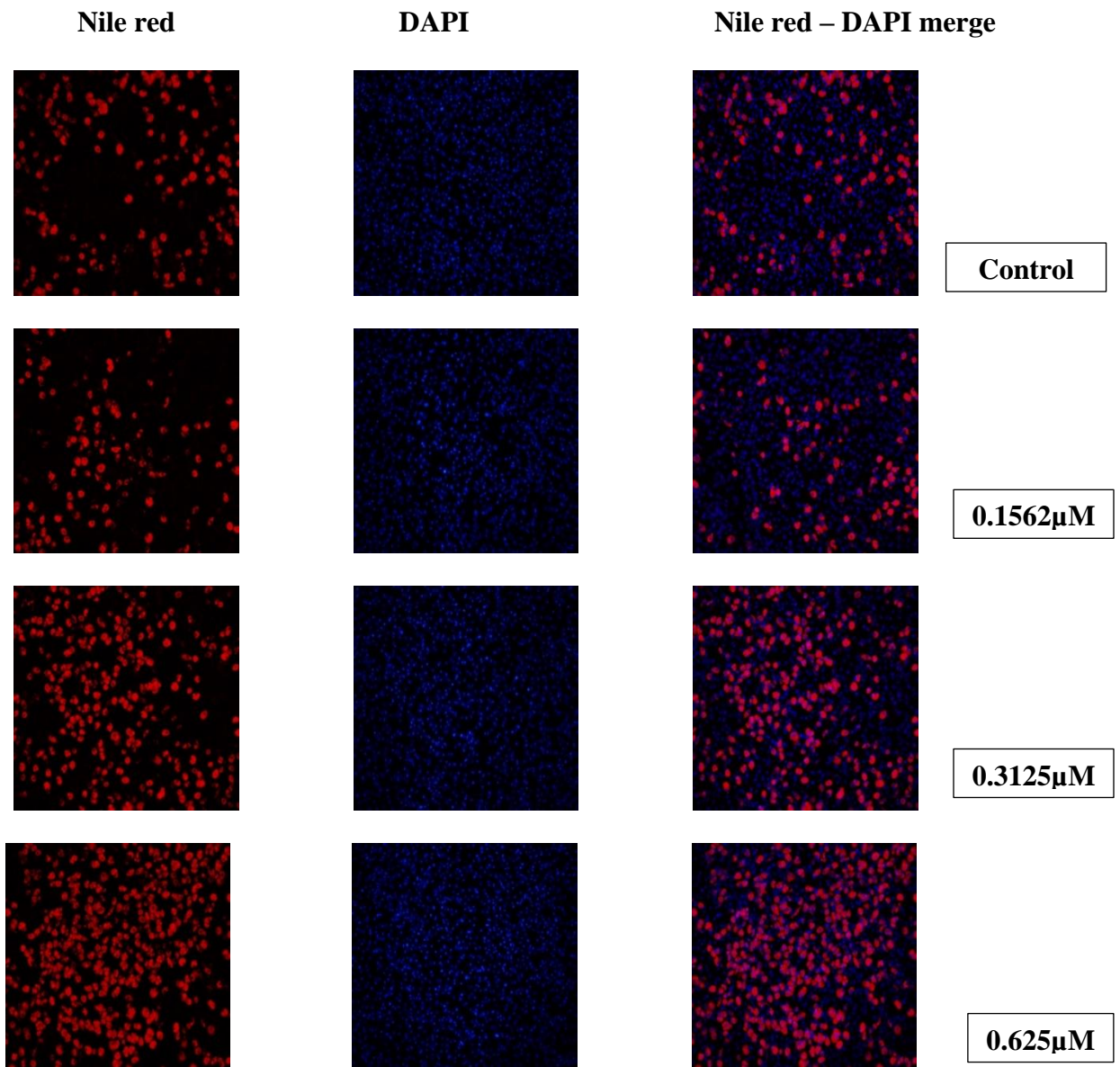
These are the immunofluoroscent images of MSCs treated with varying concentrations of Cadmium and stained with Nile Red dye and DAPI stain. These images confirmed an increase in adipogenesis after treatment with Cadmium in a dose dependent manner
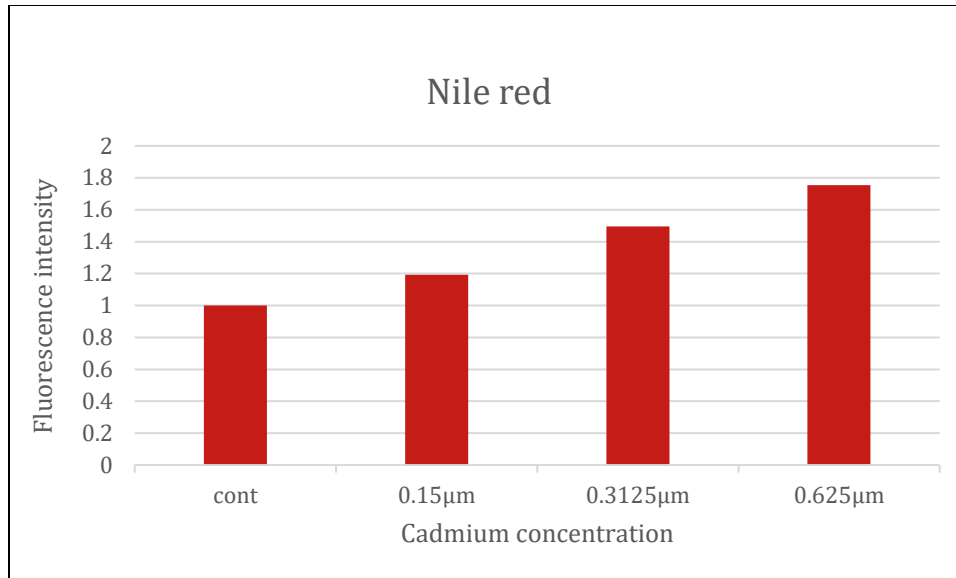
Figure 3- Adipogenesis measure

The results showed that treatment with Cadmium for 24 hours subsequently increased the process of adipogenesis. The 7 day process of adipogenesis was analyzed using Nile red staining method to stain the adipocytes and DAPI stain to stain the nuclei of the cells to mark the presence of cells. The differentiation of MSCs into adipocytes were increased in a dose dependent manner.

Thus, this concluded that Cadmium is shown to promote the process of differentiation into adipocytes.

3. Western blotting-

Western blotting technique was run to check for two hallmark markers of autophagy which are p62 and LC3 and of adipogenesis which are CEBPα and PPARϒ.

Quantification of Autophagy proteins
- **p62 expression levels-**
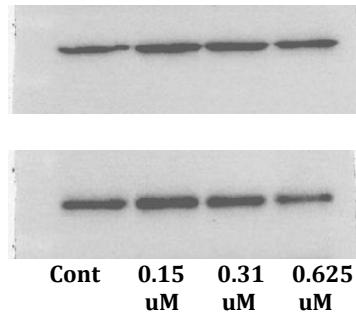
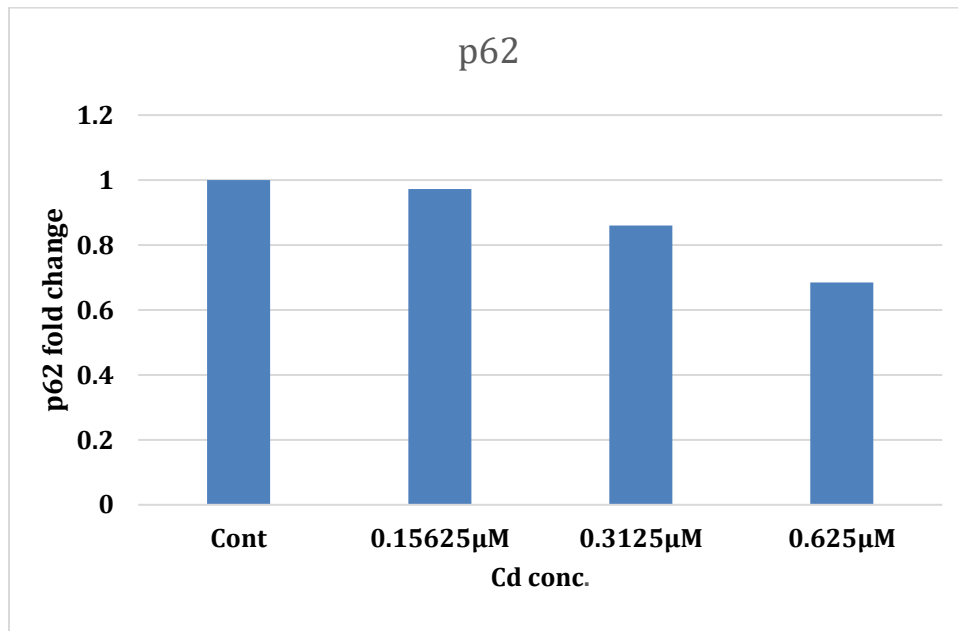Figure 4 - Blot for p62 (up) and its beta actin (down)after 24 hour cadmium treatment

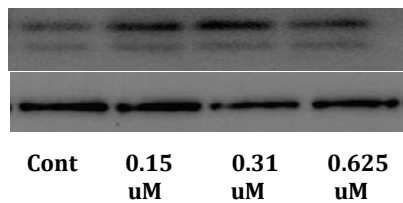Figure 5- Fold change of p62

- **LC3 expression levels –**

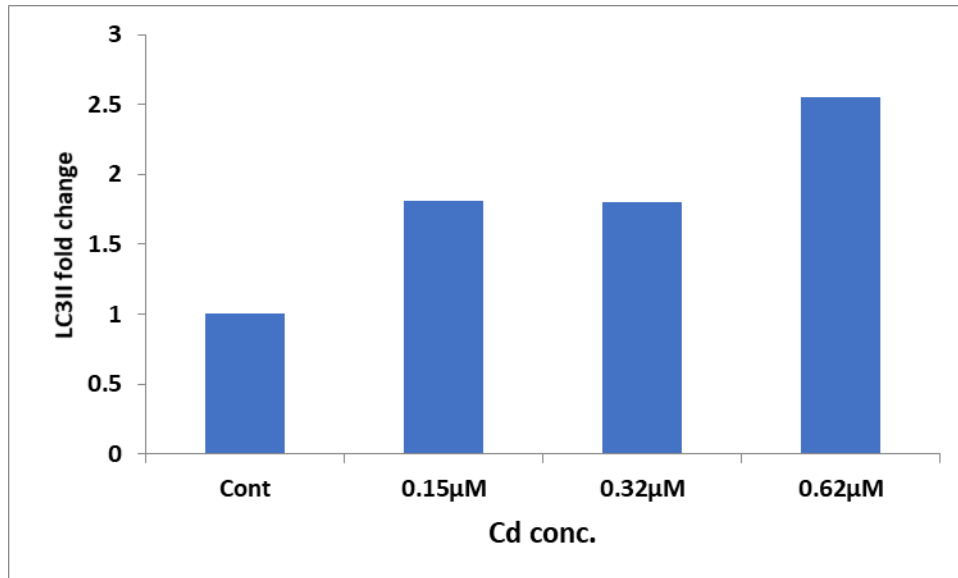Figure 6- Blot for LC3II(up) and its beta actin(down) after 24 hour cadmium treatment

Figure 7 - Fold change of LC3II

Decrease in the levels of p62 and increase in the levels of LC3II depict increase in autophagy after Cadmium treatment in a dose dependent manner. Both the markers were checked after 24 hour treatment with Cadmium.

These results showed increase in the level of autophagy after treatment with Cadmium. Cadmium proved to be an inducer of autophagy in Mesenchymal stem cells.

Flux analysis of autophagy proteins-
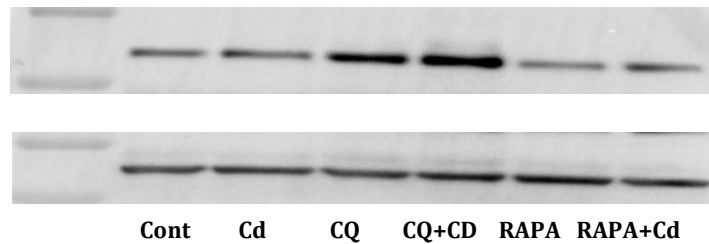
- **p62 expression levels**

Figure 8 - Blot for p62 (up) and its beta actin(down) with Autophagy promoters and inhibitors
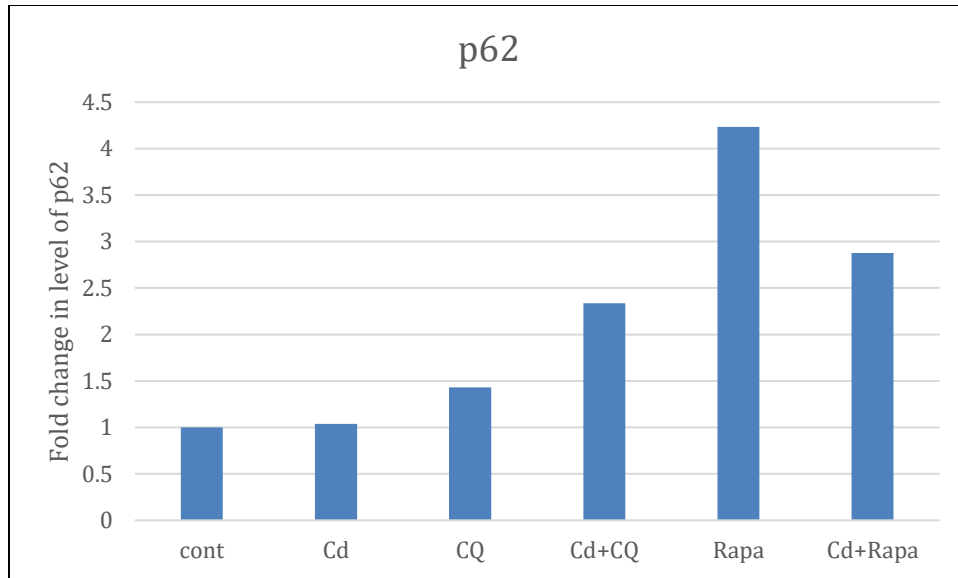
Figure 9 - Fold change of p62
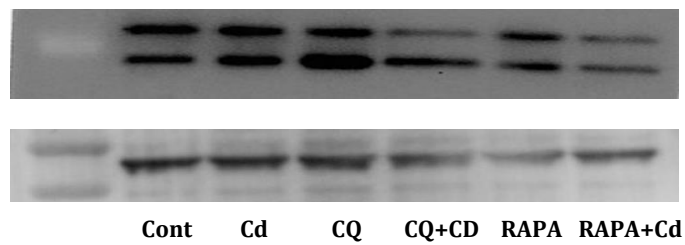
- **LC3II expression levels-**

Cont   Cd   CQ   CQ+CD RAPA RAPA+Cd

Figure 10- Blot for LC3II(up) and its bet actin(down) with autophagy promoters and inhibitors
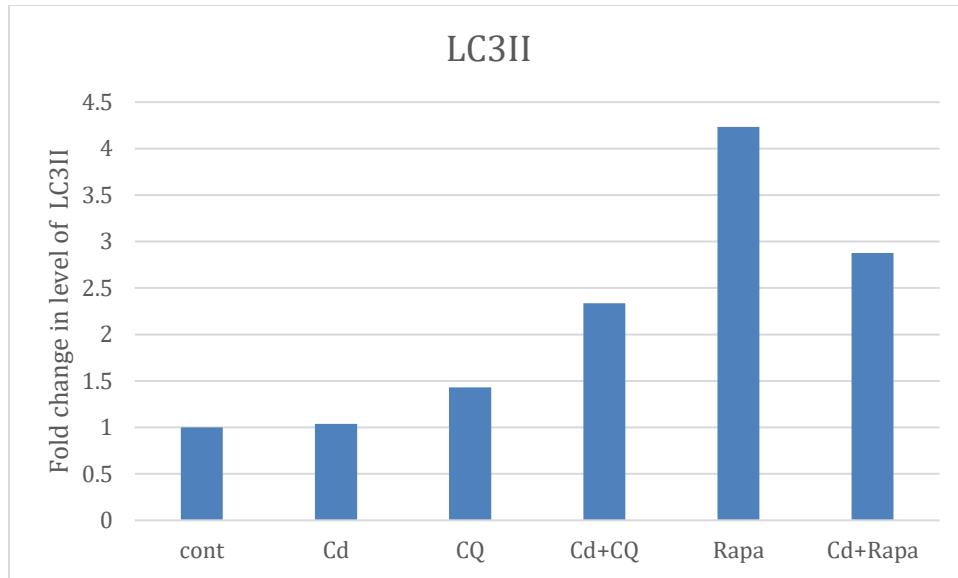
Figure 11 - Fold change of LC3II

Quantification of adipogenic proteins

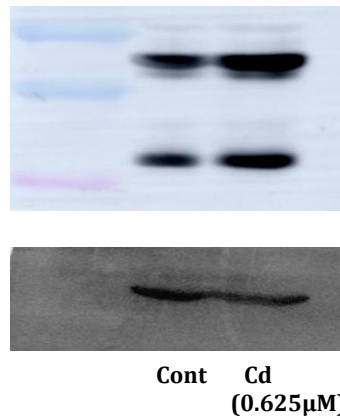- **CEBPα expression levels-**

Cont   **Cd**
(0.625μM)

Figure 12 - Blot for expression of CEBP alpha (up) and its beta actin (down) in control and Cd treated
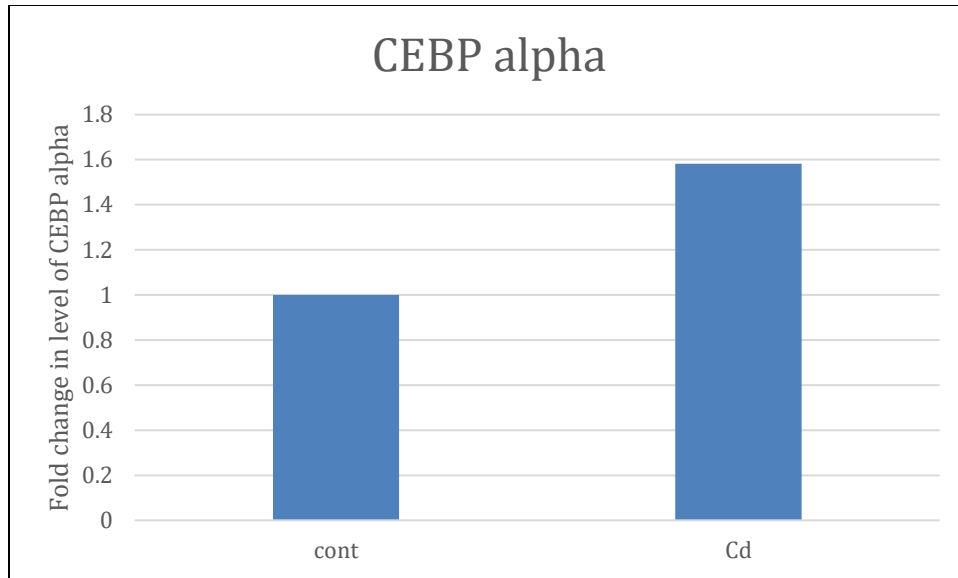
Figure 13 - Fold change of CEBP alpha

- **PPAR Ɣ expression levels –**
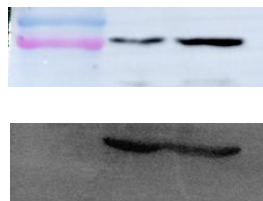
**Cont   Cd
(0.625μM)**

Figure 14 - Blot for expression of PPAR gamma (up) and its beta actin (down)

Figure 15 - Fold change of PPAR gamma

Both the adipogenic proteins have increased many folds after treatment with Cadmium.
These results showed that Cadmium induces adipogenesis. Hence, it affects the lineage differentiation decision of Mesenchymal stem cells.
(Due to shortage of cells experiments could not be repeated)

Conclusion

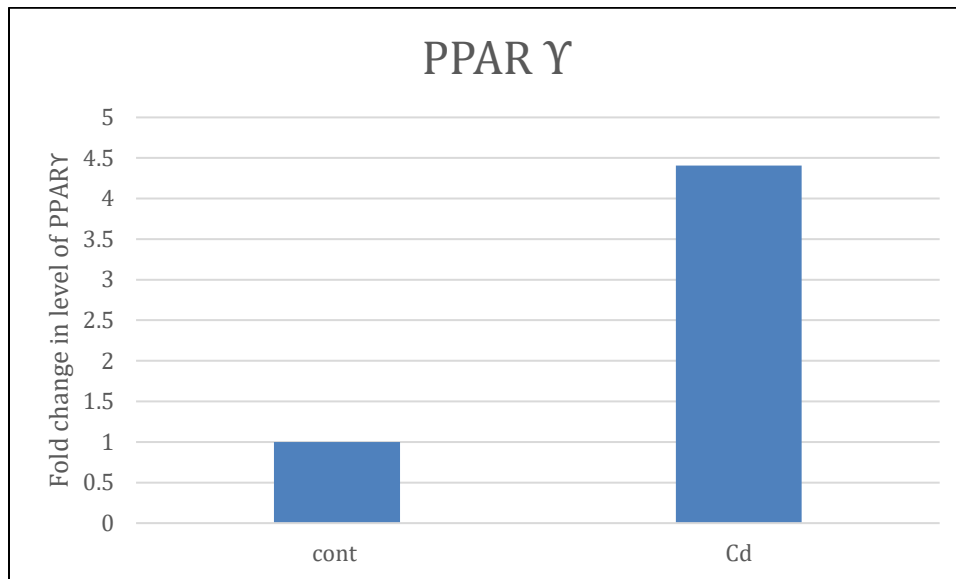The above data confirm the cytotoxic levels of acute Cadmium exposure that were lethal for the cells. However, some low dosages were safe enough to examine the effects of Cadmium treatment to the MSCs. After treatment with these dosages for 24 hours, the autophagy markers showed significant depiction of increase in autophagy levels in the cells. These increase were observed in dose dependent manner, implying Cadmium treatment does promote the process of autophagy.
The differentiating potential of the MSCs also increased with Cadmium treatment as observed in Nile Red imaging results. The protein markers of adipogenesis also showed increased levels after Cadmium treatment. Hence, this confirms that Cadmium induces autophagy and also increases the differentiating potential of the MSCs.

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