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IMPACT OF ZINC CHLORIDE ON CELLULAR VIABILITY OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent cells with an inborne ability of renewing themselves and differentiate into various cell types. Over the past five decades, MSCs have been extensively studied and have remained to be the focus of intense research. Despite promising potential, the therapeutic use of bone marrow (BM) MSCs has been limited due to difference in number of cells required for a therapeutic infusion ($1–100 \times 10^6$ cells/kg of patient) and the number of cells that can be obtained from bone marrow samples. Several efforts have been carried out for the improvement of expansion techniques to increase the proliferative capability of MSCs. Zinc is one of those substances which is known for its cytoprotective and antioxidant properties. A scanty amount of studies have focused on the role of zinc in stem cells, none of them was on bone marrow derived stem cells. We designed an experiment to analyze the cytotoxicity of $ZnCl_2$ on BM MSCs at late passages. We used various concentrations (10µM, 30µM, 50µM, 100µM & 300µM) of $ZnCl_2$ for 48 hours and performed MTT assay. Cells treated with lower concentrations (10µM, 30µM, & 50µM) showed no cytotoxic effect; instead cell viability was found to be increased. With higher

concentrations (100µM & 300µM) of $ZnCl_2$, we observed significant cytotoxicity and a drastic decrease in cell viability. We advocate further studies to investigate the effects of zinc on cellular morphology, proliferation, and migration potential to counteract the limitation of BM-MSCs in a clinical setting.

Keywords: Bone Marrow, Mesenchymal Stem Cells, Zinc Chloride, Cytotoxicity, Late Passages

INTRODUCTION

Mesenchymal stem cells (MSCs) are undifferentiated cells that exists in adult bone marrow and have the ability to self-renew and differentiate into different types of cells and tissue. MSCs plays important roles in cellular homeostasis **[1]**. The experiments of renowned scientist, Sir Alexander Friedenstein (1924–1998), who first isolated MSCs from bone marrow, postulated the theory that commenced the development of various approaches for cellular therapy **[2]**. Since their discovery in 1968, various other sources were also identified, which includes foetal origin tissues (umbilical cord blood, umbilical cord and placenta), and a wide variety of adult tissues including blood vessels, synovial membrane, endometrium, dermis, dental pulp, periosteum, adipose tissue and many more **[3-7]**. In addition, almost all types of tissues and organs home mesenchymal stem cells (MSCs) or MSC-type cells **[8]**.

MSCs are shaping up to be the foremost promising source for supply of stem cells in treatment of various immune disorders (arthritis, multiple sclerosis), trauma or ischemic injuries (kidney failure, myocardial ischemia, spinal injury), congenital defects (myelomeningocele, congenital heart disease, congenital diaphragmatic hernia) and degenerative diseases (muscle degeneration, intervertebral disc degeneration, sensori neural hearing loss) **[9]**. As of october 2019, about 528 clinical trials using MSCs are present on "ClinicalTrials.gov" database, presented by the National Institutes of Health (USA), and these includes investigations on the above mentioned diseases **[10]**. Persistent progresses in the understanding and application of adult mesenchymal stem cells (MSC) have revolutionized regenerative medicine **[11]**.

Since their discovery, bone marrow MSCs have been thoroughly analyzed from a clinical perspective. The therapeutic use of MSCs is hampered by several obstacles including low yield of BM MSCs acquired from bone marrow aspirate **[12, 13]**, large number of cells being required for transplantation **[14, 15]**, limited in-vitro life span, senescence in late passages **[16]**, and inhospitable in-vivo environment **[17]**. Such major limitations have hindered BM

MSCs from being practiced into a clinical setting. Several factors have been investigated to overcome these limitations and still many are under consideration, few of them are, appropriate culture, supplementation, preconditioning with different compounds and hypoxic preconditioning **[18]**. However, zinc has been widely studied in several cell lines where it showed a cytoprotective role **[19-23]**.

Zinc is an essential & the second most abundant metal in body **[24]**. Its total body content is approximately 1.4 to 2.3 g. The plasma concentration of Zinc is only 0.1% while 95% of it is intracellular **[25]**. Of the 95% intracellular Zinc, 10 to 20% is part of the cell wall, 30 to 40% is found inside the nucleus and about 50% is in the cytosol & vesicles **[26]**. Forty four percent of Zinc is used in DNA transcription. Zinc is a constituent of about 10% of all the proteins found in the human body **[27]**. Zinc is necessary for normal functioning of transcription factors, several enzymes and other proteins **[28]**. In view of these properties of zinc, we designed an *in-vitro* study to investigate the effects of $ZnCl_2$ on BM-MSC in late passages. Moreover, the cytotoxic analysis after treatment with different concentrations of $ZnCl_2$ was carried out to optimize the effective concentration of this compound.

MATERIALS & METHODS

Study Setting:

The in-vitro experimental study was conducted on commercially purchased PromoCell® Tech, adult BM-MSCs at Dr. Panjwani Center for Molecular Medicine (PCMD) and Drug Research, International Center for Chemical and Biological Sciences (ICCBS).

Human Bone Marrow Mesenchymal Stem Cells:

Commercially available human BM-MSCs were purchased from (PromoCell GmbH, Heidelberg, Germany). Cryovial was then thawed in warm water at 37°C after sterilization with 70% ethanol. Cells were shifted to falcon tube containing 9ml of complete medium (high glucose Dulbecco's Modified Eagle's Medium, DMEM) supplemented with 10% fetal bovine serum, 1mM L-glutamine, 1mM sodium pyruvate, and 1% penicillin streptomycin. After centrifugation for 8 minutes at 1000 rpm, the supernatant in the falcon tube was removed. Cell pellet was resuspended in fresh medium and then shifted to T75 flask. The flask was then incubated at 37 °C with 5% CO_2 for attachment, growth and proliferation.

Sub-Culturing of Bone Marrow MSCs:

On reaching 70-80% confluency, medium was removed, and carefully cells were rinsed twice with 1X PBS for complete removal of medium. 1mL of 1X trypsin-EDTA (0.25%) added and flasks were then

incubated 3-4 minutes at 37°C in CO_2 incubator. Following incubation, under phase contrast microscope (TE 2000S, Eclipse, Nikon, Japan) flasks were observed to assess cellular detachment. If 50% cells start floating, 3mL complete medium was added to cease the proteolytic activity of trypsin. Then, all the cells of T 75 flask was shifted to falcon tube and centrifuged at 120×g (1000 rpm) for 8 minutes (Centrifuge 5804R, Eppendorf, Germany). Supernatant in the falcon tube was discarded & 2mL fresh medium was added to resuspend the pellet. Two new T75 flasks were then filled with 9 mL of complete medium and same quantities of resuspended cells were transferred into them. Flasks were incubated under optimal conditions in CO_2 incubator. Now, the culture was labelled as P_4 or Passage four. Flasks were observed under microscope after every 2-3 days for cells attachment & confluency. The medium was changed after every 3-4 days. Once the flask achieves 70-80% confluency, culture was again sub-cultured. This procedure was repeated to reach P-5 for our experimental procedures.

Cytotoxic Analysis:

To evaluate cellular cytotoxicity in response to different concentrations of $ZnCl_2$, we performed (MTT) colorimetric assay. In a 96 well-plate, BM MSCs were seeded with different concentrations (10µM, 30µM, 50µM, 100µM & 300µM) of $ZnCl_2$ in triplicates. The 96 well-plate was incubated for 48 hours at 37˚C in 5% CO_2 in a humidified incubator. After incubation, the growth media was discarded from wells. MTT dye was added and incubated again for 4 hours under same conditions. The dye was carefully removed and replaced with equal amount of Dimethyl Sulfoxide (DMSO) to solubilize the crystals. For 5-7 minutes, the plate was placed on a shaker. Finally, the absorbance was measured at 570 nm spectrophotometrically (Spectra Max, Molecular Devices, USA). MTT is light sensitive hence the procedure was executed in dark.The experiment was carried out in triplicates.

Statistical analysis of data:

Results were presented as mean ± SD. Statistical software IBMSPSS program (version 25.0 USA) was used for analysis. For data processing & entry both, Microsoft Excel (360) & IBM SPSS (version 25.0 USA) program was used. To obtain the difference between groups we used one-way ANOVA followed by Tukey's post hoc test. P value of <0.05 designate to be statistically significant. (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

RESULTS

This study was carried out to evaluate the cytotoxic concentrations of zinc chloride in stem cell culture at late passages. Initially

we expanded commercially purchased bone marrow derived MSCs that displayed capability to adhere to plastic surface of tissue culture flask and proliferated under optimal conditions. **Figure 1** shows MSCs at different stages of P4, P5, P6 and P7. At passage 4-5 (P4, and P5) spindle shaped cellular morphology was observed with no significant morphological change **(Figure 1A-1B)**. Whereas, at passages 6(P6) cells turn out to be large and wide **(Figure 1C)**, while at passage 7 (P7) cells were widened and subsequently flattened **(Figure 1D)**. These wide and flat cells at P7 were treated with different concentrations of $ZnCl_2$ for 24 hours in a 96 well-plate and analyzed by MTT assay. Cells treated with concentrations of 10µM, 30µM & 50µM displayed no cytotoxic effect; instead cell viability was found to be increased **(Figure 2)**. However, $ZnCl_2$ at 100µM & 300µM concentrations indicated significant cytotoxicity and drastic decrease in cell viability was observed **(Figure 1)**.

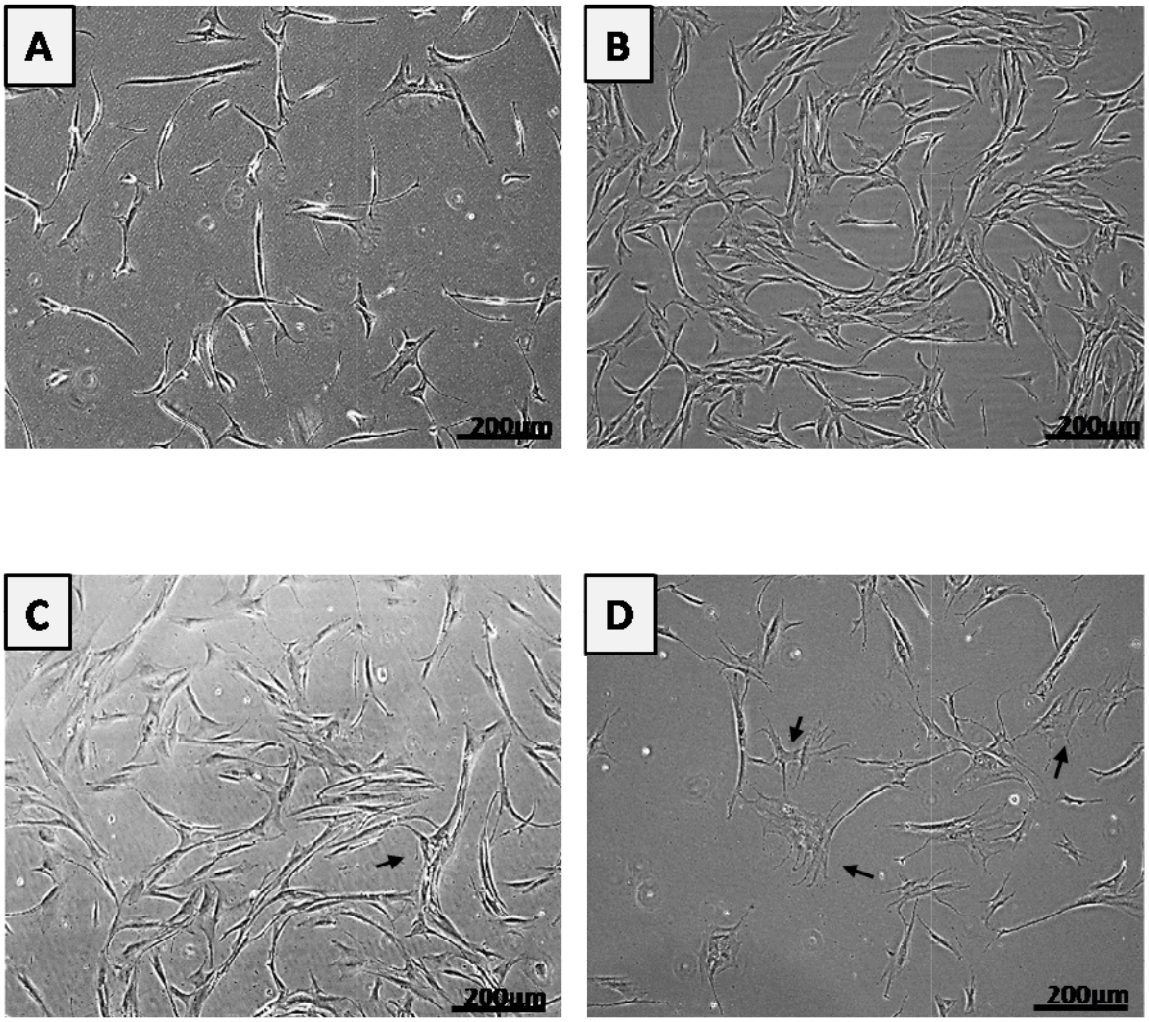

Figure 1: Morphological Features of Bone Marrow MSCs at different Passages
(A) Cells at passage 4, (B) Cells at passage 5, (C) Cells at passage 6, and (D) Cells at passage 7. No significant morphological change were observed in figure A & B. In picture C cells are large and widen, while in figure D several cells have shown widened morphology. These images were taken via phase contrast at 4X magnification

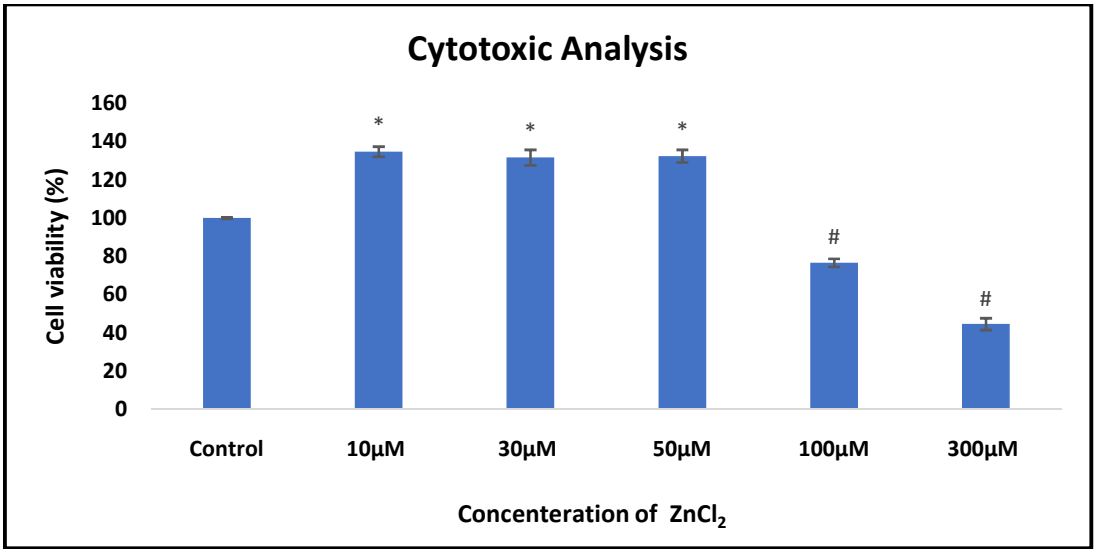

Figure 2: Cytotoxicity Analysis of $ZnCl_2$ by MTT Assay:
At low doses (10 µM, 300 µM & 50 µM) culture displayed significant increase in MSC metabolic activity. However, at high doses (100 µM and 300 µM) MTT assay exhibited significant decrease in viability of BM MSCs. All the values are denoted as Mean- ±- SEM; level of confidence $p \leq 0.05$. (*= $p < 0.05$ upregulated and #= $p < 0.05$ downregulated)

DISCUSSION

This study was focused on investigating the cytotoxic effect of different concentrations of Zinc in BM MSCs. Using MSCs, this study established that *in-vitro,* zinc environment can affect cellular viability. Excess amount of zinc chloride produces reduction in viability. We observed cytotoxicity at 100 µM & 300 µM concentrations. However, lower concentrations of zinc chloride may increase cell viability. Here, we found that concentrations of zinc chloride 10µM, 30µM & 50µM are well tolerated by bone marrow derived stem cells. Moon *et al*., in his experiment demonstrated cytotoxicity of adipose derived mesenchymal stem cells at concentration of 300µM which is similar to one of our finding. He also demonstrated that 100µM dose of zinc increases cellular viability. This contradicts with our findings where we observed significant decrease in cell viability at 100µM concentration. He also demonstrated increase in cellular viability at lower doses **[29]**. This might be due to the difference of stem cell source. In morphological analysis we found spindle shaped, fibroblast like morphology at early passage. We sub-cultured MSCs to passage 7 and we found change in morphology, where cells became large, wide, flat and loses it connections with other cells. This agrees with previous reports that exclaim, BM MSCs undergoes replicative senescence after a few passages in cultures, and show morphological abnormalities proceeding to proliferation arrest. This may restrict its clinical use **[16, 30]**. Tsai *et al*., also revealed that increase in number of passage is inversely proportional to the proliferation of MSCs cultures **[31]**. For regenerative medicine, stem cell use is vital

& has a potential to be used in large number of pathalogical conditions. Use of stem cells is not just restricted to life threatening conditions. Instead, it has been proposed that it may be used in multiple disorders to improve quality of life. However, for clinical use a large number of cells is required **[14]**. In view of above findings, it is beneficial for cultures in late passages to be used with low concentrations of zinc for increased cellular viability.

CONCLUSION

We have concluded that Zinc Chloride in low concentration (10µM, 30µM & 50µM) has no cytotoxic effects, whereas cytotoxicity was found at higher concentration (100µM & 300µM). Moreover, treatment with $ZnCl_2$ at low concentration may be used *in-vitro* to increase cell viability at late, and obtain desirable number of cells. Further studies are required to check the potential beneficial effects of zinc on morphology, proliferation, and migration rate to counteract the limitation of BM-MSCs in clinical setting.

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