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*Basic Study*

**Zinc enhances the cell adhesion, migration, and self-renewal potential of human umbilical cord derived mesenchymal stem cells**

Zinc promote MSCs growth

**Abstract**

BACKGROUND

Zinc (Zn) is the second most abundant trace element after Fe, present in the human body. It is frequently reported in association with cell growth and proliferation, and its deficiency is considered to be a major disease contributing factor.

AIM

In the present study, we aimed to determine the effect of Zn on *in vitro* growth and proliferation of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs).

METHODS

hUC-MSCs were isolated from human umbilical cord tissue and characterized based on immunocytochemistry, immunophenotyping, and tri-lineage differentiation. The impact of Zn on cytotoxicity and proliferation was determined by MTT and Alamar blue assay. To determine the effect of Zn on population doubling time (PDT), hUC-MSCs were cultured in media with and without Zn for several passages. An *in vitro* scratch assay was performed to analyze the effect of Zn on the wound healing and migration capability of hUC-MSCs. A cell adhesion assay was used to test the surface adhesiveness of hUC-MSCs. Transcriptional analysis of genes involved in the cell cycle,

proliferation, migration, and self-renewal of hUC-MSCs was performed by quantitative real-time polymerase chain reaction (qPCR). The protein expression of Lin28, a pluripotency marker, was analyzed by immunocytochemistry.

## RESULTS

Zn at lower concentrations enhanced the rate of proliferation but at higher concentrations ( $>100\mu\text{M}$ ), showed concentration dependent cytotoxicity in hUC-MSCs. hUC-MSCs treated with Zn exhibited a significantly greater healing and migration rate compared to untreated cells. Zn also increased the cell adhesion rate, and colony forming efficiency (CFE). In addition, Zn upregulated the expression of genes involved in the cell cycle (CDC20, CDK1, CCNA2, CDCA2), proliferation (TGF $\beta$ 1, GDF5, HIF1 $\alpha$ ), (CXCR4, VCAM1, VEGF-A), and self-renewal (OCT4, SOX2, NANOG) of hUC-MSCs. Expression of Lin28 protein was significantly increased in cells treated with Zn.

## CONCLUSION

Our findings suggest that zinc enhances the proliferation rate of hUC-MSCs decreasing the population doubling time, and maintaining the CFE. Zn also enhances the cell adhesion, migration, and self-renewal of hUC-MSCs. These results highlight the essential role of Zn in cell growth and development.

**Key Words:** Human umbilical cord, Mesenchymal stem cells, Zinc, Cell proliferation, *In vitro* expansion.

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**Core Tip:** Zn efficiently increased the proliferation of MSCs with a significant increase in the rate of population doublings, and at higher concentrations, it was cytotoxic. MSCs of the test group maintained a higher frequency of mesenchymal progenitors. Upregulation of genes involved in cell cycle, proliferation, pluripotency, and migration, was also observed in test groups. Zn increased the migration capability of MSCs and cell adhesion ability of MSCs. Overall, the results showed that Zn at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion.

## **INTRODUCTION**

Preclinical and clinical research supports regenerative medicine as a promising therapeutic approach to address degenerative disorders, including cardiovascular diseases, dermal wounds, rheumatic diseases, traumas, immune system diseases, neuronal diseases, and cancers<sup>[1,2]</sup>. Cell-based therapy approach in regenerative medicine utilizes undifferentiated stem cells and possesses the potential to proliferate clonally and differentiate into other types of cells<sup>[3-5]</sup>. Among these stem cells, mesenchymal stem cells (MSCs) are the multipotent stem cells that act as immune modulators, lack HLA-DR, and down-regulate the antigen-presenting and co-stimulatory molecules<sup>[6]</sup>. These immune privilege properties make MSCs a potential candidate in regenerative medicine<sup>[7-9]</sup>. Currently, more than 1,000 registered clinical studies involve MSCs for the regeneration of tissues and organs<sup>[10]</sup>.

MSCs from perinatal sources, such as umbilical cord tissue, umbilical cord blood, and amniotic fluid, are highly proliferative with a sustained tendency of differentiation<sup>[11-13]</sup>. Though MSCs can be isolated from various body tissues, their low number has created a need for *in vitro* expansion. Clinically, 20 to 100 × 10<sup>6</sup> MSCs are required for a single therapeutic dose, which minimally takes 4 to 8 wk for *in vitro* expansion<sup>[13]</sup>. The expansion period and the quality of MSCs depend on the isolation and cell culture protocols. In addition, the biological properties of MSCs are influenced by the patient's

clinical history, including age and genetic makeup <sup>[14]</sup>. Non-physiological conditions, including cell density, oxygen level, passage number, culture medium ingredients, and senescence are also being reported to alter biological characteristics of MSC like stemness, morphological features, migration, and proliferative potential<sup>[13]</sup>.

Zinc (Zn) is a trace element that is widely reported for its role in growth and proliferation of cells, namely osteoblasts (MC3T3-E1 cells) <sup>[15]</sup>, endothelial cells <sup>[16]</sup>, smooth muscle cells <sup>[17]</sup>, CD8+T cells <sup>[18,19]</sup>. Its deficiency is directly proportional to stem cell proliferation <sup>[20]</sup>. Plasma contains more than 1% of the total Zn <sup>[21]</sup>. Biologically, Zn is involved in cell catalytic, structural, and regulatory functions <sup>[22]</sup>. It acts as a cofactor of more than 300 enzymes <sup>[23]</sup>.

Based on the role of Zn in cell growth and proliferation, we hypothesized that the addition of Zn in the growth medium would significantly increase the growth and proliferation rate of MSCs, which will be helpful in their *in vitro* expansion for therapeutic purposes. To the best of our knowledge, no study has addressed the effects of Zn on human hUC-MSCs. Thus, for the first time, we anticipate the potential role of Zn in the proliferation, migration, morphology, doubling time, and gene expression profile of hUC-MSCs.

## **MATERIALS AND METHODS**

### **2.1 Collection, Processing, and Culturing of Human Umbilical Cord Tissue Samples:**

Human umbilical cord tissue samples were collected during elective C-sections of healthy donors under formal consent from Zainab Panjwani Memorial Hospital, Karachi. The independent ethical committee of PCMD, ICCBS, University of Karachi, for human subjects has approved the protocol (IEC-009-UCB-2015). The samples were collected in phosphate buffer saline (PBS) containing 0.5% EDTA as an anticoagulant, and processed as reported previously<sup>[24,25]</sup>. The cord tissue was washed with PBS to remove blood clots and debris. After washing, the tissue was chopped into 3 mm<sup>3</sup> pieces or explants and transferred into T75 culture flask containing 10-12 mL Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS),

1% penicillin-streptomycin, and 1 mmol/L sodium pyruvate. The flask was incubated at 37°C in a humidified incubator set at 5% CO<sub>2</sub>. The culture medium was changed at the interval of three days. After 10-15 days, an outgrowth of cells was noted from the explants, that was allowed to grow and proliferate. hUC-MSCs upto passage 5 were used in the experiments.

## **2.2 Characterization of Mesenchymal Stem Cells (MSCs):**

MSCs were characterized based on tri-lineage differentiation, immunophenotyping, and immunocytochemistry.

### **Tri-lineage differentiation**

For differentiation towards adipogenic, chondrogenic, and osteogenic lineages, the cells at passage P3 were cultured in a 6-well plate and grown in DMEM until they achieved 60-70% confluency. Subsequently, DMEM was replaced with an adipogenic induction medium (200µM indomethacin, 1µM dexamethasone, and 10µM insulin), osteogenic induction medium (10µM beta glycerophosphate, 50µM ascorbic phosphate, and 0.1µM dexamethasone), and chondrogenic induction medium (20ng TGFβ1, 10ng insulin, 100µM ascorbic acid, and 1µM dexamethasone). Cells in induction media were incubated for three weeks. After incubation, cells were fixed with 4% Paraformaldehyde (PFA) and stained with Oil red O, Alcian blue, and Alizarin red S stain to detect oil droplets, glycosaminoglycan content, and calcium deposited in induced adipocytes, chondrocytes, and osteocytes, respectively. Images were captured under a bright field microscope with a CCD camera (Ti2, Nikon, Japan).

### **Immunophenotyping**

Immunophenotyping was performed for cell surface markers CD73, Vimentin, CD105, and CD45 (Cat # 562245, BD Bioscience). MSCs were incubated in blocking solution with primary antibodies at recommended dilutions for 2 h at 37°C, and finally with secondary antibody Alexa Flour 546 of 1:200 dilution for 1 h. Cells were analyzed on a flow cytometer (BD FACS Celesta, Becton Dickson, USA).

### **Immunocytochemistry**

MSCs were cultured in a chamber slide with a density of 5000 cells per well. Cells were fixed with 4% PFA for 10 min at RT and then permeabilized with 0.1% Triton X-100. Blocking was performed with 2% bovine serum albumin (BSA) for 20 min at RT. Primary antibodies for MSC antigens CD73 (550256, BD Pharmingen, USA), CD117 (32-9000, Zymed Laboratories, Inc., USA), CD29 (MAB-1981, Chemicon International, USA), CD105 (560839, BD Pharmingen, USA), Stro1 (14-6688-82, Molecular Probes, Invitrogen, USA), Vimentin (V6389, Sigma-Aldrich, Inc., USA), Lin28 (PA1-096, Molecular Probes, Invitrogen, USA), and CD45 (CBL415, BD Pharmingen, USA) were added at recommended dilutions and kept at 4 °C overnight. Following PBS washing, cells were incubated with Alexa fluor-546 sary antibody having 1:200 dilutions for 1 h at 37 °C. Nuclei were stained with DAPI for 15 minutes. Finally, cells were mounted and visualized under a fluorescent microscope (NiE, Nikon, Japan).

### **2.3 MTT Assay:**

MTT assay was performed, taking different concentrations of ZnCl<sub>2</sub> in complete DMEM to determine minimal toxic concentration. MSCs (2000 cells) per well were seeded in a 96-well plate with 200µL complete DMEM. The medium was aspirated, and cells were incubated with 5µM, 10µM, 20µM, 30µM, 50µM, 100µM, 250µM, and 500µM concentrations of ZnCl<sub>2</sub> for overnight. The next day, the medium was removed, and cells were incubated with 200µL of MTT dye (0.5 gm/mL stock) for 4 h and incubated at 37°C with 5% CO<sub>2</sub>. Then, the MTT solution was discarded, and 200µL of DMSO was added to dissolve formazan crystals formed by metabolically active cells. The plate was kept on an orbital shaker for 5 minutes at RT, and absorbance was measured at 570nm by spectrophotometer (SpectraMax, USA).

### **2.4 Alamar Blue Assay for hUC-MSc Proliferation Kinetics:**

To analyze MSC proliferation, 2000 cells per well were seeded in a 96-well plate and incubated with different concentrations of ZnCl<sub>2</sub> for 24 h, 48 h, and 72 h. After each time point, the medium was removed, and 0.02% Alamar blue solution was added to each well. Fluorescence intensity was measured using a fluorescence spectrophotometer with excitation at 560nm and emission at 600nm (Varioskan LUX,

Thermo Scientific). The relative fluorescence unit (RFU) was calculated by the given formula given below:

*Difference between treated and untreated (%) = (RFU of treated groups/RFU of untreated group) \*100*

#### **2.5 Proliferation Rate of hUC-MSCs:**

To analyze the proliferation rate of hUC-MSCs, 10,000 cells per well were seeded in a 24-well plate and incubated with 20 $\mu$ M ZnCl<sub>2</sub>. When cells attained 70-80% confluency, they were detached and counted by hemocytometer for the number of population doubling (NPD), and population doubling time (PDT) by the formulae given below:

$$\text{NPD} = 3.33 \times \log(N_t/N_i)$$

$$\text{PDT} = t \times \log(2) / [\log(N_t) - \log(N_i)]$$

Where, N<sub>i</sub> = no. of cells plated; N<sub>t</sub> = no. of cells harvested; t<sub>i</sub> = initial day (0 day) and t (harvesting day).

#### **2.6 Colony Forming Unit Assay (CFU-Assay):**

MSCs were seeded at 500 cells per cm<sup>2</sup> in a T25 culture flask. After two weeks of incubation, cells were fixed with 4% PFA, followed by incubation with 0.5% crystal violet prepared in 10% methanol. Cells were washed with distilled water, and colonies were visualized at 40X magnification under bright field microscope. A cluster of 100 cells was considered a colony. Results were analyzed by SPSS software.

#### **2.7 Transcriptional Profile of Genes Involved in Cell Cycle, Proliferation, Pluripotency, and Migration:**

Cells were harvested for RNA isolation, 1 mL Trizol reagent (15596026, Invitrogen) was added, followed by the addition of 500 $\mu$ L chloroform for phase separation and centrifugation at 12000rpm. The aqueous layer was collected and incubated with 1mL absolute ethanol for 2 h at -80 °C. The suspension was centrifuged, and the pellet was rinsed with 70% ethanol. Finally, the pellet was reconstituted in 15 $\mu$ L nuclease-free water, quantified by nanodrop spectrophotometer, and stored at -20°C. cDNA was synthesized using 1 $\mu$ g RNA by RevertAid first-strand cDNA synthesis kit (K1622, Thermo Scientific, USA). All the primers were designed by



<http://www.ncbi.nlm.nih.gov/tool/primerblast> and synthesized commercially. qPCR amplification was performed in a triplicate manner using qPCR master mix (A600A, Promega, Madison, WI, United States) for the genes mentioned in Table 1.

### **2.8 Scratch Assay:**

For cell migration, 4000 cells per well were seeded in a 24-well plate containing DMEM and incubated for 24 h. After monolayer formation, the scratch was developed using a 100 $\mu$ L pipette tip, and cells were incubated with 20 $\mu$ M ZnCl<sub>2</sub>. Migration of cells was observed at 2 h, 4 h, 6 h, 8 h, and 24 h of scratch development. Images were captured at different time points under a phase-contrast microscope (Eclipse Ts2, Nikon, Japan) and migration was measured in percentage with the help of the following formula:

$$\% \text{ of MSCs migration} = [(h_i - \Delta h) \div h_i] \times 100\%$$

Where,  $h_i$  = the area of scratch measured immediately after scratching, and  $\Delta h$  = is the area of scratch measured at 2, 4, 6, 8, and 24 h.

### **2.9 Cell Adhesion Assay:**

To analyze cell adherence in control and test groups, MSCs were harvested, seeded in a culture flask, and cell adhesion was determined by observing MSCs at different time points, i.e., at 0 minutes (immediately after seeding), after 15 minutes, 30 minutes, 1 h, and 2 h. Cell adhesion was evaluated by the given formula:

$$\% \text{Percentage} = \text{no. of cells adhered on the flask surface} / \text{no. of floating cells in media}.$$

### **2.10 Pluripotency Retention of hUC-MSCs:**


To analyze the pluripotency marker after 20  $\mu$ M ZnCl<sub>2</sub> treatment, MSCs were incubated with the primary antibody of Lin28 at 1:100 dilution and kept at 4 °C overnight. After PBS washing, cells were incubated with Alexa fluor-546 sary antibody of 1:200 dilutions for 1 h at 37 °C. Nuclei were stained with DAPI for 15 minutes and then visualized under a fluorescent microscope (NiE, Nikon, Japan).

## **RESULTS**

### **3.1 Human Umbilical Cord Processing and Culturing:**

MSCs were isolated from human umbilical cord tissue by the explant method (**Figure 1A**). Human umbilical cord tissue comprises one vein, two arteries, and Wharton's jelly (**Figure 1B**). MSCs started to outgrow the cord tissue after 10-15 days of cord processing. At this stage, cells adhered to the flask surface and appeared as trigonal shape (**Fig. 1C (a,b)**). After approximately 25 days, cells were observed to be interconnected with each other by their extensions, formed colonies, and exhibited elongated fibroblast-like morphology, which was termed P<sub>0</sub> (passage zero) MSCs (**Figure 1C (c,d)**).

### 3.2 Characterization of hUC-MSCs:

Cells isolated from the umbilical cord possessed adhesive properties and exhibited fibroblast-like morphology (**Figure 2A**). The isolated population of cells showed positive expression for MSCs specific markers, including CD73, CD117, CD29, CD105, Stro1, Vimentin, and Lin28, while negative for CD45, which is a hematopoietic marker (**Figure 2B**). Immunophenotypic analysis showed positive expression for CD73 (90%), vimentin (98%), and CD105 (80%), and less than 4% population was positive for CD45 (**Figure 2C**). The isolated cells successfully differentiated into adipogenic, osteogenic, and chondrogenic lineages as confirmed by Oil red O, Alizarine red S, and Alcine blue staining (**Fig. 2D**). 

### 3.3 Cytotoxicity Analysis and Proliferation Kinetics of hUC-MSCs:

ZnCl<sub>2</sub> concentrations ranging from 5 μM-100 μM did not show any cytotoxic effect on cells. ZnCl<sub>2</sub> was found to be significantly cytotoxic above 100 μM concentration, and its cytotoxicity was found to be higher in a concentration-dependent manner. The IC<sub>50</sub> of ZnCl<sub>2</sub> was found at 250 μM concentration (**Fig. 3A**). For proliferation analysis, MSCs were treated with 5μM-50μM ZnCl<sub>2</sub> for 24, 48, and 72 h. Fluorescence intensity was measured for each group at the respective time after incubating with Alamar blue for 4 h. Results of each temporal group were analyzed by one-way ANOVA between control and test groups. All test groups observed significantly increased proliferation after 48 and 72 h (**Fig. 4B**). MSCs in passage 5 were analyzed for NPD and PDT. After 10 days,

MSCs of the control and treated group (20  $\mu$ M ZnCl<sub>2</sub>), were counted, and significantly higher NPD was observed in 20  $\mu$ M ZnCl<sub>2</sub> treated group (**Figure 3C**), while PDT reduced significantly in the treated group in contrast to the control group (**Figure 3D**).

#### **3.4 CFU Assay and Transcriptional Analysis of Cell Cycle and Proliferation Genes:**

To analyze the presence of mesenchymal progenitors, CFU assay was performed. Colonies of hUC-MSCs were observed after 2 wk of cell seeding. The test group supplemented with 20  $\mu$ M ZnCl<sub>2</sub>, exhibited a significantly higher number of colonies than the control group (**Fig. 4A and B**). To analyze the transcriptional changes of 20  $\mu$ M ZnCl<sub>2</sub> treated MSCs to the untreated MSCs, fold change regulation ( $2^{-\Delta\Delta Ct}$ ) of cell cycle and proliferation genes was analyzed at 12, 24, 48, and 72 h. Expression of cell cycle genes, i.e., CDC20, CCNA2, and CDCA2, was comparatively increased in all temporal groups of 20 $\mu$ M ZnCl<sub>2</sub>, while CDK1 was upregulated after 24, 48, and 72 h. (**Figure 4C**). Among proliferation genes, expression of TGF $\beta$ 1 and GDF5 was significantly increased in all temporal groups of 20  $\mu$ M ZnCl<sub>2</sub>, whereas HIF1 $\alpha$  exhibited a significant upregulation after 24, 48, and 72 h (**Figure 4D**).

#### **3.5 Cell Migration Assay and Transcriptional Analysis of Genes Involved in Cell Migration:**

ZnCl<sub>2</sub> was also found to facilitate the migration of hUC-MSCs (**Fig. 5A**) and significantly increased ( $p \leq 0.01$ ) migration was observed in 20  $\mu$ M ZnCl<sub>2</sub> treated groups for 2-8 h, while scratch was completely healed after 24 h in both groups, i.e., control and ZnCl<sub>2</sub> treated groups. The percentage of area healed is plotted against time (**Fig. 5B**). Gene expression analysis of migration factors showed upregulation of CXCR-4 at all the time points, i.e., 24, 48, and 72 h, while V-CAM1, VEGF-A was significantly upregulated after 48 and 72 h. MMP-2 was found to be upregulated after 24 and 72 h (**Figure 5C**).

#### **3.6 Cell Adhesion Assay:**

To analyze the effect of ZnCl<sub>2</sub> on the adhesiveness of hUC-MSCs, *in vitro* cell adhesion assay, was performed. A significant increase in the cell adhesion rate of hUC-MSCs in the 20  $\mu$ M ZnCl<sub>2</sub> treated group was noted at 15 minutes, 30 minutes, 1 h, and 2 h,

compared to the control group (**Figure 6A**). Cell adhesion rate (%) is plotted against time (**Figure 6B**).

### **3.7 Retention of Pluripotency Markers:**

#### **Immunostaining**

Immunostaining of 20  $\mu\text{M}$   $\text{ZnCl}_2$  treated MSCs showed positive expression of pluripotency marker Lin28 at all the time points (**Figure 7A**). The quantified mean fluorescence intensity showed a significant increase in expression at 24 h (**Figure 7B**).

#### **Gene Expression Analysis**

Genes expression analysis showed that *OCT4* was significantly upregulated in all temporal groups (12, 24, 48, and 72 h), of 20 $\mu\text{M}$   $\text{ZnCl}_2$ . In contrast, the expression of *SOX2* and *NANOG* was significantly higher in temporal groups of 24 and 48 h and then fell to the level of control in 72 h (**Figure 7C**).

## **DISCUSSION**

Zinc is among the essential trace elements that is ubiquitously found in the human body. According to prior investigations, it is one of the trace elements that is majorly involved in cell growth and proliferation [16,17,26]. It stimulates cell division and proliferation by regulating enzymes such as deoxythymidine kinase and/or by regulating hormones including insulin-like growth factor-I (IGF-I) and nerve growth factors [23,27]. Zn acts as a signal molecule for both extracellular and intracellular pathways. It upregulates PI3 kinase pathway which activates AKT in fibroblast cells and transactivates EGF receptor by Src pathway in epithelial cells of the airway. It also regulates the ERK1/2 pathway with the induction of p21(CiP/WAF1), and cyclin D1 in colonocytes [21]. Studies reported the role of Zn on the proliferation of adipose derived MSCs [23,26]. A recent study reported the effect of Zn on human umbilical cord derived MSCs and showed its importance in MSC survival and differentiation properties. The study suggested that Zn could enhance antioxidative properties of MSCs by regulating Nrf2/Sirt3 signaling pathway[28]. In the present study, we analyzed the effect

of ZnCl<sub>2</sub> on human umbilical cord-derived MSCs (hUC-MSCs) in the context of its proliferation and growth, pluripotency, migration, and cell adhesion properties.

ZnCl<sub>2</sub> is reported to have a biphasic effect on the viability and proliferation of the cells and exhibited dose-dependent response, which varies for different cell lines [29]. Most of the studies found ZnCl<sub>2</sub> cytotoxic above 100 μM in different dividing cells, including coronary artery endothelial cells, vascular smooth muscle cells, and even MSCs [16,17,23]. Our findings showed cytotoxicity of ZnCl<sub>2</sub> above 100 μM, which is in line with the previous study [23]. The proliferation profile of MSCs reported by previous studies gave a bell shape biphasic curve whose peaks vary for ZnCl<sub>2</sub> concentrations for different cells. We also obtained a bell shape biphasic curve representing an increase in MSC proliferation with increase of ZnCl<sub>2</sub> concentration up to 30 μM, and then gradually decreasing upon further increase. However, up to 100 μM concentration of ZnCl<sub>2</sub> gave significantly increased proliferation of MSCs in test groups as compared to control. Increased population doublings (NPD) and reduced population doubling time (PDT) were observed in the treatment group in contrast to control MSCs.

MSCs are the progenitor cells that proliferate rapidly [30,31]. MSCs retain their multipotent ability, but the expanded cultured population of MSCs shows heterogeneous morphology of cells with variations in the frequency of early progenitor cells [30]. It is reported that human umbilical cord-derived MSCs exhibit the highest percentage of colony-forming units in contrast to the MSCs derived from bone marrow, adipose tissue, and dental pulp [32]. By CFU assay, we analyzed the impact of Zn on the colony-forming potential of MSCs derived from the human umbilical cord. Our results revealed that ZnCl<sub>2</sub> maintains a high pool of early progenitor cells in the MSC population, as many colonies were found in the treated group. ZnCl<sub>2</sub> treated group also exhibited denser colonies with spindle-shaped morphology which represented the rapidly self-replicating cells [30]. MSCs of the control group showed broad morphology with less dense colonies, which reflected slow self-replicating cells [30].

Gene expression analysis of treated MSCs exhibited a significant increase in cell cycle genes, including *CDK1*, *CDC20*, *CCNA2*, and *CACD2*. The level of *CDK1* remains

constant in entire cell division, but its activity depends on cyclins that are synthesized at different cell cycle stages [33]. CCNA2 or cyclin A2 is important for G1/S and G2/M transition, thus responsible for cell cycle progression [34]. CDK1 and CDC20 are the key mitotic regulators [35,36]. CDC20 is a cofactor of CPC/C (anaphase-promoting complex/Cyclosome), by activating CPC/C, CDC20 regulates the activity of CDK1 and promotes chromosome segregation by degrading securin, thus responsible for anaphase transition [36]. Whereas CDCA2 is essential for retaining chromatin structure in post-mitotic nuclei and regulates DNA damage responses for mitotic to interphase transition [37]. Upregulation of these genes required at different stages of the cell cycle showed progression of the cell cycle in the test group.

Few growth factors are pleiotropic and are found to be involved in multiple biological functions. For instance, TGF $\beta$  is one of them, and previous studies have reported the role of TGF $\beta$  in MSCs proliferation [38,39], and differentiation towards chondrogenic lineage; however, it inhibits terminal differentiation [39]. TGF $\beta$ 1 is found to promote hUC-MSCs proliferation without affecting the phenotype of MSCs [38]. Hence, our results are in the context of these findings as the ZnCl<sub>2</sub> treated group showed a significant increase in TGF $\beta$ 1 expression and stemness markers like *OCT4*, *SOX2*, and *NANOG*.

Few studies have reported the effect of Zinc on the expression of HIF1- $\alpha$ , but there is inconsistency, as some studies on cancer cells proved down-regulation of HIF1- $\alpha$  caused by Zinc [40,41], while in keratinocytes and prostate cells, Zinc is found to stabilize HIF1- $\alpha$  expression [41,42]. Our findings showed enhanced expression of HIF1- $\alpha$  in ZnCl<sub>2</sub> treatment group which is in agreement with later studies [41,42]. Overall, the significantly increased expression of *TGF $\beta$ 1*, *GDF5*, and *HIF1- $\alpha$*  suggested the increased proliferation and multipotency of MSCs in the test group.

Migration is an important factor that should be considered for better therapeutic effects of MSCs in regeneration and cell therapy. Studies reported that Zn enhances the migratory capability of cells [16,17,23,43]. For example, endothelial cells and smooth muscle exhibited enhanced migration and cell adhesion when treated with ZnCl<sub>2</sub>. These cells

showed upregulation of focal adhesion molecules, including integrin., vinculin, actin, ICAM1, and VEGFA [16,17]. We analyzed the effect of Zn on the migration ability of MSCs by *in vitro* scratch assay, which showed a significant increase in MSC migration after ZnCl<sub>2</sub> treatment. These results were further confirmed by gene expression analysis of migration factors, including *CXCR-4*, *V-CAM1*, *VEGF-A*, and *MMP2*. These are involved in chemotaxis, cell rolling, and cell invasion and play their role in different phases of cell migration [44]. *CXCR-4* and *VEGF-A* are the early migration markers, where *CXCR-4* is a chemokine receptor, and *VEGF* is a growth factor, and both are involved in chemotaxis and trafficking of cells. *V-CAM1* is a cell adhesion molecule, whereas *MMP2* is required for cell invasion [44]. Here we analyzed these genes to estimate the migration ability of MSCs after ZnCl<sub>2</sub> treatment, and we found that after 12 h, only *VEGF-A* showed high expression as compared to control, and after 24 h, all four migration markers exhibited significantly increased expression. After 48 h, *CXCR-4*, *V-CAM1*, and *VEGF-A* showed significantly increased expression, and after 72 h all of these genes showed significantly high expression, whereas *MMP2* showed the highest expression. Overall, up-regulation of these genes suggested that Zn also enhanced the expression of migration markers and promoted MSC migration, where fluctuations in their regulation may relate to their role at different stages of cell migration [44]. Studies reported that Zn promotes cell adhesion at lower concentrations in different cell types. Endothelial cells of coronary artery and smooth muscle cells exhibited increased cell adhesion ability when cultured in a ZnCl<sub>2</sub> supplemented medium [16,17]. We also found a considerably enhanced cell adhesion rate of MSCs when treated with 20μM ZnCl<sub>2</sub>.

*Lin28*, a pluripotency marker, was analyzed for its protein expression. It is an RNA binding protein that was first characterized in *C.elegans* as a heterochronic gene and was found to control developmental timing. In mammals, two homologs, including *Lin28a* and *Lin28b* are present in the cytoplasm as well as in the nucleus [45]. Structurally, *Lin28* comprises the N-terminal cold shock domain (CSD) and two zinc-binding motifs (CCHCx2) located at C-terminal. *Lin28* is highly expressed in ESCs, while upon differentiation, its expression is suppressed by miRNAs, namely *Let-7* and *Lin-4* [45]. It is

involved in post-translational modifications of specific subsets of mRNAs. In skeletal muscles, it binds to IGF-2 mRNA [46], while in mouse ESCs it is reported to bind with mRNAs of cell cycle-specific genes [47,48]. OCT4 mRNA is also reported as one of the targets of Lin28 for post-translational modifications [49]. Our results showed positive expression of Lin28 in all temporal groups, however, its expression exhibited fluctuations, i.e., after 24 h of treatment, it was significantly enhanced, and then at later time points, the expression decreased gradually and became approximately similar to control group. It might be because of its post-translational role. That is why it showed higher expression before, i.e., after 24 h than other pluripotency genes that exhibited their higher expression mainly after 48 and 72 h. Altogether, these factors make regulatory circuitry which is regulated through feed-forward loop and autoregulatory mechanism [49], and the fluctuations in their expression at time points may reflect this regulatory circuitry.

*OCT4*, *SOX2*, and *NANOG*, are the transcription factors that are conserved across mammalian species [50]. All three are highly expressed in ESCs and are responsible for the pluripotency and self-renewal of ESCs [51]. These transcription factors regulate gene expression in a highly cooperative fashion [52]. *OCT4* and *SOX2* form heterodimers and bind to specific promoters on DNA [53]. *NANOG* is a homeobox family gene that also forms heterodimer and regulates other pluripotency genes in feedback mechanism [53]. These are also found to have similar role in adult stem cells, including MSCs [11,50,54]. A study carried out by Hu *et al.*, 2016, saw a significant increase in the expression of *OCT4*, *SOX2*, and *NANOG* in mouse ESCs when treated with  $ZnCl_2$  for 48 h. The study further reported that as ESCs in humans and mice share the same key regulators, i.e., *OCT4*, *SOX2*, *NANOG* [55], Zn might exert similar effects regarding the acquaintance and maintenance of the pluripotency in human stem cells. Mnatsakanyan *et al.*, 2019 also showed significantly increased expression of *SOX2* in mouse ESC after long-term treatment, i.e., 30 days with  $ZnCl_2$  [56]. In our study, gene expression analysis of all three genes *OCT4*, *SOX2*, *NANOG* showed a significant increase in the test group after 24 and 48 h. After 12 and 72 h of treatment, *SOX2* and *NANOG* did not exhibit any



significant difference compared to the control, while *OCT4* represented a significant increase in all temporal groups.

Limitations of this study is that growth kinetics is analyzed by treating cells up to three days with zinc. Further temporal study is required to evaluate the long term effects of zinc. Also the signaling pathways that zinc utilizes to produce its effects are needed to be explored *via* protein quantification. An *in vivo* experimental evaluation is also needed to further support the *in vitro* findings.

## **CONCLUSION**

At lower concentrations, Zn efficiently increased the proliferation of MSCs with a significant increase in the rate of population doublings, and at higher concentrations, it was cytotoxic. MSCs of the test group maintained a higher frequency of mesenchymal progenitors. Upregulation of genes involved in cell cycle, proliferation, pluripotency, and migration, was also observed in test groups. Zn increased the migration capability of MSCs and cell adhesion ability of MSCs. Overall, the results showed that Zn at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion. Hence, Zinc could be a suitable constituent for cell culture medium that would help to enhance the quantity, quality, and rate of *in vitro* proliferation of MSCs for clinical and industrial-scale production. Though fewer studies are reported on Zinc effects on MSCs, further investigations are required to understand the mechanism that Zinc utilizes to induce its effects.

## **ARTICLE HIGHLIGHTS**

### ***Research background***

Zinc (Zn) is the second most abundant trace element after Fe, present in the human body. It is frequently reported in association with cell growth and proliferation, and its deficiency is considered to be a major disease contributing factor.

### ***Research motivation***

Trace elements are required to be supplemented in culturing media that will help to promote proliferation, cell division, and cellular growth, which will result in *in vitro* expansion of MSCs to achieve a desired number of cells for therapeutic dose.

### ***Research objectives***

In the present study, we aimed to determine the effect of Zn on *in vitro* growth and proliferation of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs).

### ***Research methods***

hUC-MSCs were isolated from human umbilical cord tissue and characterized based on immunocytochemistry, immunophenotyping, and tri-lineage differentiation. The impact of Zn on cytotoxicity and proliferation was determined by MTT and Alamar blue assay. To determine the effect of Zn on population doubling time (PDT), hUC-MSCs were cultured in media with and without Zn for several passages. An *in vitro* scratch assay was performed to analyze the effect of Zn on the wound healing and migration capability of hUC-MSCs. A cell adhesion assay was used to test the surface adhesiveness of hUC-MSCs. Transcriptional analysis of genes involved in the cell cycle, proliferation, migration, and self-renewal of hUC-MSCs was performed by quantitative real-time polymerase chain reaction (qPCR). The protein expression of Lin28, a pluripotency marker, was analyzed by immunocytochemistry.

### ***Research results***

Zn at lower concentrations enhanced the rate of proliferation but at higher concentrations ( $>100\mu\text{M}$ ), showed concentration dependent cytotoxicity in hUC-MSCs. hUC-MSCs treated with Zn exhibited a significantly greater healing and migration rate compared to untreated cells. Zn also increased the cell adhesion rate, and colony forming efficiency (CFE). In addition, Zn upregulated the expression of genes involved in the cell cycle (CDC20, CDK1, CCNA2, CDCA2), proliferation (TGF $\beta$ 1,

GDF5, HIF1 $\alpha$ ), (CXCR4, VCAM1, VEGF-A), and self-renewal (OCT4, SOX2, NANOG) of hUC-MSCs. Expression of Lin28 protein was significantly increased in cells treated with Zn.

#### ***Research conclusions***

Our findings suggest that zinc enhances the proliferation rate of hUC-MSCs decreasing the population doubling time, and maintaining the CFE. Zn also enhances the cell adhesion, migration, and self-renewal of hUC-MSCs. These results highlight the essential role of Zn in cell growth and development.

#### ***Research perspectives***

Zinc at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion. This will lead to produce large number of MSCs required for *in vivo* implantation in shorter period of time.

#### **ACKNOWLEDGEMENTS**

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SIMILARITY INDEX

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### PRIMARY SOURCES

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