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# Myo-inositol at High Concentration Reduced Viability and Proliferation of Rat Bone Marrow Mesenchymal Stem Cells via Electrolyte Imbalance and Elevation of Aerobic Metabolism

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**Abstract**

Myo-inositol (MI) which is produced at low concentration is an essential substance for animal's natural growth. This study was performed to investigate the effects of MI on viability, proliferation and some biochemical factors of rat bone marrow mesenchymal stem cells (BMSCs). To investigate the cell viability using trypan blue assay, BMSCs after third passage were treated with different concentration of MI at 12, 24 and 36 hours. Then the samples were treated with 160 and 1280 µM of MI as selected concentrations for 36 hours to carry out further analysis including the proliferation ability via colony forming assay (CFA), publish doubling number (PDN) and the cells morphology. The level of $Na^+$ and $K^+$ was measured by flame photometer. In addition, the activity of ALP, LDH, AST, ALT as well as calcium concentration was evaluated using commercial kits. Data were evaluated using ANOVA, Tukey's test and $p<0.05$ was considered significant. Although MI in low concentration had no toxic effects at high concentration reduced the viability of the cells. MI reduced nuclei diameter, cytoplasm area, electrolytes as well as the activity of HDL and ALT. On the other hand, MI caused an increase in the activity of ALP and intracellular $Ca^{2+}$ level. High concentration of MI not only reduces cell viability via imbalance of electrolytes but also changed the cell morphology. In addition, we observed the elevation of aerobic metabolism via calcium dependent mechanism.

**Key words:** Mesenchymal stem cell; Myo-inositol; Viability; Morphology; Alkaline phosphatase

## Introduction

Myo-Inositol (MI) is the best known isomer of Inositol in nature. MI plays an important role in the basic structure of Eukaryotes secondary messenger. MI has also been found as an important compound in structural phosplipids, Phosphatidyl Inositol (PI) and its phosphate form, Phosphatidyl Inositol Phosphate (PIP) (Reynolds, 2009). In 1986 it was shown that myo-inositol monophosphate (IP) inhibits Parathyroid hormone-induced bone resorption probably by prevention of crystal dissolution (Reynolds *et al.*, 1986). Published study showed that inositol-1, 4, 5-triphosphate (I (1,4,5) P3) regulates penetration and transportation of $Ca^{2+}$ between intracellular supplies (Chen *et al.*, 2000). In a study via vast genomic association it was shown that PLCL1 (a gene which is coding a protein that attach to I(1,4,5)P3) inhibits $Ca^{2+}$ signaling mediated I(1,4,5)P3 (Liu *et al.*, 2008). In another study it was confirmed that low levels of IP6 is a risk factor of osteoporosis while high levels of IP6 showed protective effects versus osteoporosis (Lopez-Gonzalez *et al.*, 2008). The oral administration of $^{45}Ca$ in rats with or without MI and vitamin $D_2$ showed, MI increase the bone absorption of $^{45}Ca$ by 48% compared with control group during 24 hours (Angeloff *et al.*, 1977). In addition, investigation showed that rats with imperfect Sodium/Myo-Inositol co-transporter-1 significantly decreased MI level in the most tissues and the rats developed defective nervous system and died soon after birth as a

result of respiratory disorder. Adding MI into mother's drinking water before parturition could reduce the neonatal mortality (Dai *et al.*, 2011). MI is found in the cell culture media at low concentration (Soltani *et al.*, 2012) it is also one of the components of Dulbecco's Modified Eagles Medium (DMEM) (7.2 mg/l = 40 µM). There is no literature regarding the effect of high concentration of the MI on the stem cells which are cultured in the media *in vitro*.

Rat bone marrow mesenchymal stem cells (BMSCs) are progenitor cells with ability to proliferate and generate its own. They are also differentiated to other cell lines such as chondrocytes, hepatocytes and osteoblasts (Marion and Mao, 2006). These cells are in the bone marrow and other adult tissues which are differentiate to produce other cells that participate in tissue repair (Wu *et al.*, 2014). BMSCs are in direct contact with peripheral blood and therefore when MI are given to animal, it might come in close proximity with blood born chemicals which are entering the blood via intestinal absorption. Since investigation have shown that the MI influenced the mineralization and influence the $Ca^{2+}$ transportation, therefore one question has to be answered; what would be the effect of MI on the BMSCs, which are the cellular back up for osteoblast generation? In the present research we try to answer the effect of different concentration of MI on viability of BMSCs and then we evaluate the effect of certain dose of MI on proliferation and metabolic state of BMSCs.

## Materials and Methods

### Isolation and expansion of BMSCs

All the materials used in this study were purchased from Sigma, Aldrich Company (USA) unless it is mentioned elsewhere. In this experimental study, we used male Wistar rats (6-8 weeks old, weighing 140±20 gr). The rats were purchased from Pasteur Institute (Tehran, Iran) and maintained in the animal house of Arak University in the polyethylene cage under standard conditions at 27±3°C and convenient access to food and water. Rats were sacrificed by excessive chloroform (Merck, Germany) inhalation according to the animal laboratory ethical committee of Arak University. Under sterile conditions, their femora and tibia were then surgically removed and cleaned from surrounding connective tissue. Two ends of the bones were cut and bone marrow was flushed out using 2 ml of DMEM (Gibco, Germany) supplemented with 15% fetal bovine serum (FBS, Gibco, Germany) and penicillin/streptomycin (Gibco, Germany). Bone marrow content was centrifuged at 2500 rpm for 5 minutes, re-suspended in 5 ml culture media, then plated in culture flasks and incubated at 37˚C in an atmosphere of 5% $CO_2$. After 24 hours the supernatant containing non-adherent cells were removed and fresh culture media was added. The flasks were incubated for 14 days with the replacement of culture media every three days. When the bottom of the culture flask was covered with cells, the cells were trepsinized using trypsin-Ethylenediaminetetraacetic acid (Gibco, Germany) and washed with phosphate saline buffer (PBS). The fresh culture media was then added to the cells and divided in two similar flasks and kept in the incubator. To obtain a purity of 90-95%, two more passages were repeated and after the 3rd passage the BMSCs were used for further investigation.

### Exposure to myo-inositol

The cells were placed in an appropriate culture dish and allowed to attach for 24 hours. The cells were then treated with 20, 40, 80, 160, 320, 640, 1280 µM of MI (Merck, Germany) and incubated for 12, 24, 36 hours in the presence of control group (treated only with culture media). Each analysis was repeated three times in a bracket model.

### Cell viability assays

BMSCs were cultured at a density of 50000 cells per well in 24-well culture plates and culture media contained different concentrations of MI was added to the respective wells. After 12, 24, 36 hours, the cells were washed with PBS and using trypsin/EDTA the cells were detached from the culture flasks and subsequently collected by centrifugation at 2500 rpm for 5 minutes. Following centrifugation, the cells were re-suspended in the fresh culture media and 50 µl

of the cell suspension was mixed with equal volume of trypan blue and incubated for 2 minutes at 37˚C. Using a hemocytometer, the percentage of viable and dead cells was determined. Trypan blue is a dye which enters the cell via damaged cell membrane, thus the cells appears to be blue in color.

Based on the results of viability tests, two concentrations (160 and 1280 µM) of MI and 36 hours were chosen and following experimental groups were designed.

C-The control group, treated only with fresh culture media, $MI_{160}$, treated with 160 µM of myo-inositol, $MI_{1280}$, treated with 1280 µM of myo-inositol. Every analysis was repeated three times.

**Cell morphology assay**

To study the morphology of BMSCs, 10,000 cells were cultured in 24 wells plates and after 24 hours the cells were treated according to the above mentioned groups and then incubated for 36 hours. The plates were washed with PBS and then 10 µl of Hoechst solution (50 µg/ml) (Sigma-Aldrich, USA) was added to 100µl of the culture media for 15 minutes in dark at room temperature. To analyzed the cell cytoplasm the plates were incubated for 2 minutes with 10 µl of acridine orange (5 µg/ml) in a separate chamber. The cells were observed using an inverted fluorescence microscope (Olympus, IX70) equipped with a camera (DP72). The nuclei diameter (µm) and cytoplasm area ($\mu m^2$) of the cells were measured using Motic Image software (Micro Optical Group Company version 1.2).

**Quantification of cell proliferation ability**

**Colony forming assay**

To investigate the colony forming ability (CFA), cells at a density of 50,000 were cultured in 3 cm sterile plates and after 24 hours the cells were treated (according to the above mentioned groups) and incubated for 7 days with every 3 days of culture media replacement. After 7 days the cells were washed with PBS, and stained with crystal violet (0.5 g crystal violet in 100 ml methanol solution) at room temperature for 15 minutes. Using a light microscope equipped with a graticule the diameter (µm) and numbers of the colonies were determined.

**Population doubling number**

To investigate the population doubling number (PDN) 80000 cells were cultured in 3 cm sterile plates and after 24 hours the cells were treated (according to the mentioned groups) with contaminated culture media and incubated for 1, 3 and 6 days. Then the plates were washed with PBS and detached from bottom of the flasks using trypsin- EDTA. The cells were counted using a hemocytometer and the PDN was calculated using $PDN=\log N/N0\times 3.31$ where N0 was the initial number of the cultured cells and N was the number of harvested cells after 1,3 and 7 days.

**Cell content extraction**

Control and the treated BMSCs were incubated for 36 hours in culture flask. Then the cells were harvested using trypsin/EDTA and centrifuged at 2500 rpm for 5 minutes. The cells were washed twice with tris-HCl (20 mM tris-HCl, pH=7.2) and then kept at -20˚C overnight to break the cell membrane. Eventually the homogenate was thawed and centrifuged at 12000 g for 10 minutes. Using Lowry method, the total protein content of each sample was estimated. A standard graph was plotted using bovine serum albumin (BSA) and the linear formula,$Y=0.3169X+0.0001$, with $R2=0.999$ was used to calculate the concentration of the unknown protein samples. In the formula, Y represents the absorbance and X the concentration (µg) of the protein in each sample. The cell extract was used to estimate the biochemical factor as follow.

**ALT, AST and LDH activity determination**

To investigate the activity of alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) in protein lysate respective commercial kit (Pars Azmoon, Iran) was used. According to the company instruction and based on the same amount of protein the activity of the enzymes

was measured using the spectrophotometer (T80+, PG Instrument Ltd., England) at 340nm.

**Alkaline phosphatase activity determination**

Based on the same amount of protein and using p-nitrophenyl phosphate (pNPP) as substrate, the activity of the ALP was measured according to the commercial kit instructions (Pars azmoon, Iran), using spectrophotometer (T80+, PG Instrument, Ltd., England) adjusted at 410 nm.

**Intracellular calcium assay**

The intracellular calcium was measured using commercial kit (Pars Azmoon, Iran) based on colorimetric method. Calcium with Arsenazo in natural pH gives a blue color at which the color intensity is proportional to the concentration of calcium. Absorption was measured with the help of spectrophotometer (Model T80 + PG instrument manufacturing company UK) at a wavelength of 630 nm. The concentration was reported as mg/dl of extract.

**Sodium and potassium concentration determination**

The concentration of sodium and potassium in cell extracts was measured using a flame photometer (Model PFP7, England). $Na^+$ and $K^+$ emit light of different wavelengths, and emission can be measured using appropriate filters, which is correspondent to the respective concentrations. Using the same instrument a standard graph was plotted with different concentrations of NaCl and KCl and linear formula Y=0.0172X+0.0053 with R2=0.996 and Y=0.3169X+0.0001 with R2=0.999 were obtained for $Na^+$ and K+ respectively. Here, Y shows the absorbance and X stands for concentration of each electrolytes. The concentration was reported as µg/ml of the sample.

**Statistical analysis**

Statistical evaluation of the data was performed in SPSS (version 16, Sun Microsystems Inc., America) using one-way ANOVA and the Tukey's test. Results are shown as mean ± SD, and $P<0.05$ was considered significance.

**Results**

**Effect of MI on cell viability**

Results of trypan blue assay showed that MI after 12 hours had no significant effect ($p>0.05$) on cell viability, while after 24 hours a significant decrease was observed ($p<0.05$) from 640 µM onward compared to the control. However at 36 hours a significant decrease ($p<0.05$) was observed from 320 µM (Table 1).

**Population doubling number**

Treatment of the cells with 160 and 1280µM of MI caused no significant reduction ($p>0.05$) of PDN in 1 and 3 days as compared to the control group, whereas at day 6 a significant reduction ($p<0.05$) of PDN was observed. Results showed that MI in the both concentration was toxic and caused significant reduction ($p<0.05$) in PDN when compared to the control group at 6 days of treatment (Table 2).

**Colony forming assay**

In both of the treated groups, significant reduction ($p<0.05$) was observed in the number of colony compared to the control one. Whereas only treatment of the cells with higher concentration of MI (1280µM) caused significant reduction ($p<0.05$) of colony's diameter when compared to the control group. We found no significant differences ($p>0.05$) between the treated groups with respect to number and diameter of colonies (Table3).

**Morphology of the cells**

Results showed after 36 hours, the treatment of BMSCs with 160 and 1280 µM of MI caused chromatin condensation and cytoplasm shrinkage when compared to the control group (Figs 1and 2). In addition, statistical analysis of data (Table 4) confirmed the microscopic results and revealed a significant reduction ($p <0.05$) of nuclear diameter and cytoplasmic area of the cells.

**Table 1.** Mean percentage of BMSCs viability after 12, 24, and 36 hours of treatment with various concentrations of myo-inositol

| Time (Hrs) / Dose (µM) | 12 | 24 | 36 |
|---|---|---|---|
| 0 | 95.25$^{a}$±0.59 | 95.66$^{a}$±0.41 | 96.69$^{a}$±0.44 |
| 20 | 95.74$^{a}$±0.45 | 95.18$^{a}$±0.21 | 96.44$^{a}$±0.13 |
| 40 | 96.71$^{a}$±0.64 | 95.06$^{a}$±0.79 | 94.72$^{a}$±1.25 |
| 80 | 95.66$^{a}$±0.18 | 95.19$^{a}$±1.19 | 95.65$^{a}$±1.09 |
| 160 | 95.69$^{a}$±0.75 | 94.90$^{a}$±0.56 | 94.78$^{a}$±0.57 |
| 320 | 96.54$^{a}$±0.45 | 93.28$^{ab}$±0.57 | 91.24$^{b}$±0.66 |
| 640 | 96.64$^{a}$±0.91 | 92.16$^{b}$±1.28 | 92.14$^{b}$±0.65 |
| 1280 | 96.76$^{a}$±0.73 | 92.18$^{b}$±1.13 | 89.51$^{b}$±1.53 |

Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, P<0.05).

**Table 2.** Mean population doubling number of BMSCs after 1, 3 and 6 days of treatment with myo inositol.

| Time (Days) / Groups | 1 | 3 | 6 |
|---|---|---|---|
| C | 0.363$^{a}$±0.041 | 1.242$^{a}$±0.065 | 2.126$^{a}$±0.058 |
| $MI_{160}$ | 0.306$^{a}$±0.019 | 1.202$^{a}$±0.039 | 1.711$^{c}$±0.063 |
| $MI_{1280}$ | 0.328$^{a}$±0.090 | 1.211$^{a}$±0.092 | 1.569$^{bc}$±0.088 |

Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, P<0.05).

## Metabolic activity of the cells

Treatment of the cells with MI caused a highly significant reduction (p<0.001) in LDH activity and a significant reduction (p<0.05) in the activity of ALT but no changes were observed in AST activity when compared with the control group (Table 5). In the case of ALP activity, MI in both the treated groups showed a highly significant elevation (p<0.001) (Table 5).

## Intracellular calcium, sodium and potassium level

When compared to control group, a highly significant increase (p<0.001) was observed in the intracellular calcium due to treatment of the cells with MI. Intracellular sodium showed no changes with respect to treatment with MI in both the treated groups. But in case of potassium content, MI showed a significant reduction only in the group treated with 1280µM of MI (p<0.05) (Table 6).

**Table 3.** Number and colony diameter (mm) of BMSCs after 7 days of treatment with MI.

| Groups | Number of colony | Diameter of colony (mm) |
|---|---|---|
| C | 160.7$^{a}$ ±4.5 | 1.34$^{a}$ ±0.23 |
| $MI_{160}$ | 149.3$^{b}$ ±4.0 | 1.24$^{ab}$±0.24 |
| $MI_{1280}$ | 143.7$^{b}$ ±3.5 | 1.16$^{b}$±0.26 |

Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey test, P<0.05).

**Table 4.** Mean diameter (µm) and cytoplasmic area (µm$^2$) of BMSCs after 36 hours of treatment with myo-inositol.

| Groups | Nuclear diameter | Area of cytoplasm |
|---|---|---|
| Control | 22.09$^{a}$ ±1.37 | 3205.8$^{a}$ ±291.68 |
| $MI_{160}$ | 20.69$^{b}$ ±1.04 | 2847.7$^{c}$ ±355.72 |
| $MI_{1280}$ | 20.53$^{b}$ ±1.46 | 2805.8$^{c}$ ±327.80 |

Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, P<0.05).

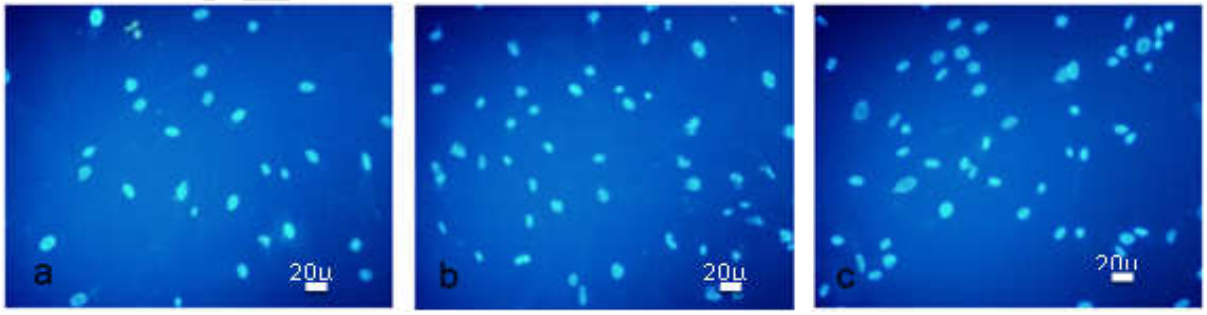


**Fig. 1.** Fluorescent micrograph of BMSCs stained with Hoechst, after 36 hours of treatment with MI. Differences in the size of the nuclei can be observed in both the treated groups (b and c) compared to control one (a). Magnification: 200X.

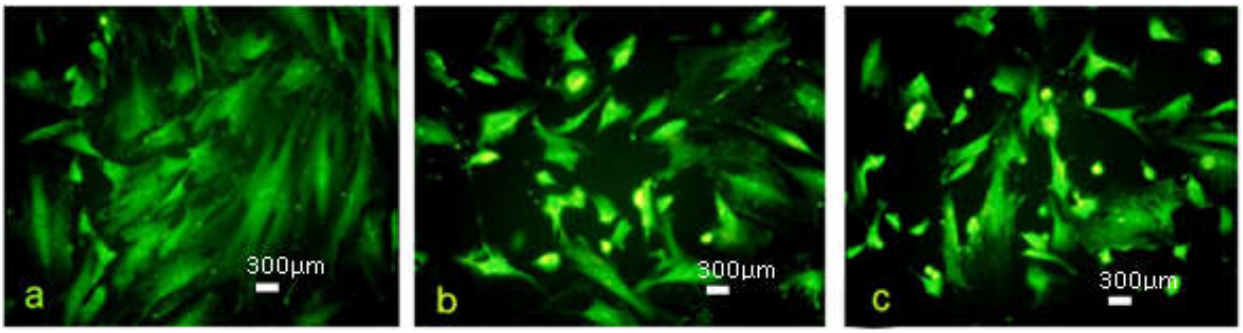


**Fig. 2.** Fluorescent micrograph of BMSCs stained with acridine orange, after 36 hours of treatment with MI. Differences in the shape of the cell cytoplasm can be observed in the groups treated with MI (b and c), where the cytoplasm are round instead of spindle in shape as compared with control group (a) .Magnification 200X.

**Table 5.** Mean activity (IU/L) of AST, ALT, LDH and ALP in BMSCs after 36 hours of treatment with myo-inositol.

| Groups | AST | ALT | LDH | ALP |
|---|---|---|---|---|
| Control | $21.94^{a}\pm0.09$ | $6.95^{a}\pm0.69$ | $2440^{a}\pm100.0$ | $84.67^{a}\pm3.97$ |
| $MI_{160}$ | $22.38^{a}\pm0.56$ | $4.37^{b}\pm0.87$ | $1670^{c}\pm91.6$ | $144.19^{c}\pm1.71$ |
| $MI_{1280}$ | $22.47^{a}\pm0.25$ | $3.37^{b}\pm0.48$ | $1580^{c}\pm40.0$ | $120.15^{d}\pm1.71$ |

Values are Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, P<0.05).

**Table 6.** Mean Total intracellular calcium (mg/dl), Na+ (µg/dl), and $K^{+}$ (µg/dl) levels in BMSCs after 36 hours of treatment with myo-inositol.

| Groups | Calcium | Sodium | Potassium |
|---|---|---|---|
| Control | $1.17^{a}\pm0.14$ | $2.35^{a}\pm0.29$ | $0.85\ ^{a}\pm0.05$ |
| $MI_{160}$ | $2.84^{c}\pm0.15$ | $2.25^{a}\pm0.34$ | $0.78\ ^{a}\pm0.01$ |
| $MI_{1280}$ | $2.43^{c}\pm0.21$ | $2.45^{a}\pm0.34$ | $0.66\ ^{b}\pm0.04$ |

Data are expressed as means ± SD. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, P<0.05)

## Discussion

In the present study, MI at low concentration had not effects on cell viability after 12, 24 and 36 hours while high concentration of MI could decrease cell viability after 24 and 36 hours. To our knowledge no study has reported the effect of high concentration of MI on the stem cells *in vitro* but MI is used as growth promoter in cell culture media (Soltani *et al.*, 2012) and also it is one of the components of DMEM (7/2 mg/l = 40 µM). Thus it is well proven that MI at low concentration has no toxic effects at all. The toxicity started at 640 µM in 24 hours and at 320µM in 36 hours, which are much more than the level presented in the culture media. Since trypan blue stain dose not penetrate the cells when the membrane function is restored (Tran *et al.*, 2011) therefore we might conclude that the cell membrane of the BMSCs has been disrupted by MI. Using the viability and proliferation (CFA and PDN) tests, we found that the plasma membrane of the cells was disrupted due to the treatment of the cells with high concentration at short time and even low concentration at long time. An important factor which ensures the appropriate function of the cell membrane is the balance of the electrolyte level (Kuroki *et al.*, 1981). The electrolyte analysis showed that the low concentration of MI did not change the level of sodium and potassium but high concentration of MI caused a significant reduction of potassium level but not sodium. MI enters the cell via Sodium/Myo-

inositol co-transporter 1 (SMIT1), which is an intra-membrane protein, responsible to enter one molecule of MI in return of two sodium ion out of the cell (Hager *et al.*, 1995). The sodium/potassium pump would work to balance the sodium/potassium level (Kuroki *et al.*, 1981), but when MI is presented in the culture media then the sodium/potassium pump might not be able to balance the level of electrolytes.

At a short time, 160µM of MI did not affect the PDN but at 6 and 7 days the proliferation of the BMSCs has been affected by 160 and 1280µM. Proliferation of the cells is a very complex mechanism in which many biochemical factors are involved. One of the most important factors is the energy state of the cell which ensures the harmony between other factors. When a cell leaves the G1 phase and enters the S phase of the cell cycle, needs lots of ATP to complete the synthesis of the necessary material for well going of the cell cycle. In addition to electrolyte imbalance, the presence of the MI caused significant reduction of LDH that ensures the cell metabolism have shifted to the aerobic condition (Gladden, 2008) which produce large amount of ATP. In addition, MI has caused the level of ALT to decrease significantly; which means that the entry of pyruvate to Crebs cycle is fast enough to be converted to alanine in enzymatic reaction (James *et al.*, 2011). Therefore the reduction of proliferation was not due to the shortage of energy but it might be due to changes in morphological states where chromatin condensation and cytoplasm shrinkage has been observed due to MI treatment. Chromatin condensation might be due to nuclease activation and protease activity (Zhang *et al.*, 1998), also cytoplasm shrinkage could be due to cytoskeleton misarrangement and protein synthesis reduction (Bortner and Cidlowski, 1998) which together with nuclear size reduction might be a sign of apoptosis (Elmore, 2007). We found no other research to mention the induction of apoptosis by MI, therefore we recommend more investigation to be run, but we speculate that the morphological changes might be a reason for significant reduction of proliferation. Myo-inositol at both concentration have caused elevation of calcium level. Calcium is necessary for activation of intracellular cascades which activate many cellular mechanisms such as elevation of metabolism and autophagy (Görlach *et al.*, 2015), another form of programed cell death. This might be another reason to direct us to presume the ability of MI to elevate the metabolic state of the cell and induce program cell death.

Along with calcium elevation there was a highly significant increase in ALP activity, which might itself be a reason of intracellular calcium elevation to compensate the phosphate level (Freethi *et al.*, 2016) as it is needed to phosphorylate the MI in the cell (Stephens and Stephens, 1990). Arriero et al showed that Inositol Hexakisphosphate (IP6) or phytic acid, increased the expression of ALP gene considered as an osteogenic marker in hUC-MSCs (Arriero *et al.*, 2012), which confirm our results.

In conclusion, our results showed that the presence of high concentration of MI is toxic and its toxicity mainly is due to electrolyte imbalance and morphological changes. We also conclude that the high concentration of MI would increase the aerobic carbohydrate metabolism via activation of calcium dependent intracellular mechanism.

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There are no conflicts of interest.

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