

Effect of Lithium Chloride on Proliferation and Bone Differentiation of Rat Marrow-Derived Mesenchymal Stem Cells in Culture

*¹Mohamadreza Baghaban Eslaminejad, ²Mahmood Talkhabi, ²Bahman Zeynali

Abstract

Objective(s)

It is believed that the mesenchymal stem cell (MSC) differentiation and proliferation are the results of activation of wnt signaling pathway. On the other hand, lithium chloride is reported to be able to activate this pathway. The objective of this study was to investigate the effect of lithium on *in vitro* proliferation and bone differentiation of marrow-derived MSC.

Materials and Methods

In this experimental study, rat marrow cells were plated in a medium supplemented either with or without 2-10 mM lithium and expanded through three successive subcultures. To explore the impact of lithium on cell growth, doubling time (DT) of marrow cell population was determined for all the cultures. To determine the lithium effects on osteogenesis, the proliferation medium of passged-3 cells from all cultures were replaced by osteogenic media, with or without 2-12 mM lithium. Osteogenesis was then quantified by measurement of the amount of matrix mineralization and the expression of bone-specific genes.

Results

DT results indicated that the marrow cells in 4 mM lithium concentration were grown faster than the others ($P < 0.05$). Intensive matrix mineralization and abundance of bone specific gene expression were observed in the cultures with 10-12 mM lithium concentration. All these differences were statistically significant. According to the results, all lithium -treated cultures possessed more differentiation than the control. Moreover, lithium low concentration was associated with more proliferation and its high concentration with more differentiating effects.

Conclusion

Lithium chloride at 4 mM concentration promotes MSC proliferation and at 10-12 mM enhances MSC osteogenic differentiation.

Keywords: Lithium chloride, Mesenchymal stem cells, Osteogenesis, Rat

1- Stem Cell Department, Cell Sciences Research Center, Royan Institute, ACECR, Tehran, Iran.

* Corresponding author: Tel: +98-21-22413790; Fax: +98-21-22409314; email: eslami@royaninstitute.org

2- School of Biology, College of Science, University of Tehran, Tehran, Iran.

Introduction

Mesenchymal stem cells (MSCs) are defined as cells possessing the ability to undergo extensive replication and the capacity to generate multiple cell lineages (1). These cells were first discovered by Friedenstein *et al* who isolated the cells from marrow samples (2). MSCs have been described as plastic adherent cells growing in colonies and capable of differentiating into osteocytic, adipocytic and chondrocytic cell lineages in appropriate conditions (3, 4). So far, multiple nomenclatures are used to denote these marrow-resident cells. These include marrow stromal cells, marrow mesenchymal progenitor, and colony-forming unit-fibroblast. The most widely-accepted term would be the mesenchymal stem cells (MSCs) denoting that they are capable of producing mesenchymal cell lineages (5). Although, MSCs were first isolated from marrow tissue, but other tissues including fat, liver, amniotic fluid, umbilical cord blood have also been reported to contain MSCs (6-9). Nowadays, MSCs are considered to be a valuable cell source appropriate to be used in cell-gene-therapy and tissue engineering strategies.

While such strategy as tissue engineering needs an extensive quantity of cells, MSCs have been reported to be present in a low frequency in tissues. This requires that MSC to be multiplied in culture before they could be used in any cell-based application (10). In the other hand, Yoen *et al* have reported that the proliferation capacity of MSCs have been decreased following *in vitro* cultivation (11). To improve MSCs proliferation, scientists have evaluated several growth factor-based culture systems including that with fibroblastic growth factor (12).

One important characteristic of MSCs is their capability of bone differentiation, which has created great promise in orthopedic fields where the repair of extensive bone defects is regarded as a great challenge. Using these cells we have conducted experiments in which bone massive defects have largely been reconstructed in either small or large animals (14, 15). In different laboratories to induce bone differentiation, MSCs are usually plated

in a medium supplemented with dexamethasone, ascorbic 2-phosphate and beta-glycerol phosphate which results in the production of bone specific matrix and its subsequent mineralization (13).

To more improve the proliferation and differentiation protocols of MSCs, there are interests to examine molecular routes involved in MSCs proliferation and differentiation. Wnt signaling pathway as a conserved molecular mechanism in all multi cellular animals has been shown to be involved in cellular proliferation and differentiation. This signaling pathway is triggered by binding the secretive molecule of wnts on their cell membrane receptor followed by translocation of cytosolic β -catenin to the nucleus, where it mediates some of the downstream effects of wnt signaling. In the absence of Wnt signaling, the soluble pool of β -catenin is degraded by multiprotein complex that contains GSK3 β , axin, and the tumor suppressor protein adenomatous polyposis coli (APC) (16-18). Wnt signaling inhibits β -catenin degradation by deactivating GSK3 β . In a previous study, it has been shown that wnt proteins have enormous impact on stem cell proliferation, so that Willert *et al* suggested that these proteins can be used as growth factor in stem cell culture (19).

Lithium, an agent used to treat manic depressive illness, inhibits GSK3 β and has been shown to mimic the effects of Wnt signaling on cell proliferation and differentiation (20-22). Up to now, varying effects of lithium have been reported on cell proliferation in culture. While Yoshino *et al* reported that lithium enhanced *in vitro* proliferation of schwann cells (23), Smits *et al* indicated some opposite effects of lithium on P19 cell culture (24). Very limited information is available regarding the effects of lithium on MSC proliferation and differentiation. In this regard, the only report has been made by de Boer *et al* who examined the effect of 4 mM lithium on human MSCs culture and concluded that lithium could enhance MSCs proliferation and inhibit their bone differentiation (25). In the present study, we investigated the effects of lithium in rat MSCs

culture using a concentration range of 2-14 mM. In contrast to de Boer *et al* findings, our results indicated the positive effects of lithium on MSC osteogenic culture.

The objective of this study was to determine the effects of different concentrations of lithium on MSCs proliferation and bone differentiation in culture. For this purpose, rat marrow cells were plated in a medium containing varying concentrations of lithium and expanded by several subcultures during which doubling time (DT) as an index of cell growth rate was studied. For quantification of the amount of bone differentiation, the expression level of the bone specific genes as well as the amount of osteogenic culture mineralization were measured and statistically compared.

Materials and Methods

Marrow cell culture

In the present study, 7 wistar strain rats, 6-8 weeks old, purchased from Pastor Institute (Tehran, Iran) were used. The use of animals was approved by Ethic Committee of Royan Institute. The animals were sacrificed by excessive chloroform inhalation, the tibia and femur were removed, cleaned off from adherent soft tissue and placed into DMEM (Dulbeco's Modified Eagles Medium, Gibco, Germany) supplemented by 15% FBS (Fetal Bovine Serum, Gibco, Germany) and 100 IU penicillin (Gibco, Germany) and 100 IU streptomycin (Gibco, Germany) in a 15 ml tube. The tubes were transferred into laminar hood to perform the rest of the procedure: the two ends of the bones were clipped off, a 18 gauge needle inserted into one end and 2 ml DMEM medium supplemented with 15% FBS and antibiotics was injected into the bone canals to wash out the marrow from the other end into a 15 ml tube. Bone marrow cells were centrifuged at 1200 rpm for 5 min, then resuspended in 5 ml DMEM containing 15% FBS and antibiotics, counted with hemocytometer, and finally plated at 10^5 cells/cm² in 25-cm² flasks. Several flasks with 2, 4, 7 and 10 mM lithium and a culture without lithium supplementation were defined. All cultures were incubated at 37 °C with

atmosphere of 5% CO₂. Two days after culture initiation, the first medium replacement was performed and then medium change was done two times a week till confluency. Confluent cultures were passaged at 10^5 cells/cm² in media supplemented by above-mentioned lithium concentration. The cultures were expanded through two additional subcultures.

Doubling Time (DT)

Doubling time for a cell population in culture is defined as the time during which the number of cells is doubled. This is an important index of cell growth rate in culture. For the present study, DT was calculated for cultures during the passages 1-3, applying the equation $DT = \text{Culture duration} / \text{Population doubling number (PDN)}$. To determine PDN, the cells from each passage were plated at 10^5 cells in 25-cm² flasks and allowed to grow for 5 days. At this point the cultures terminated by trypsinization, the cells lifted and counted with hemocytometer. Using these data, PDN was calculated for each passage by the equation $PDN = (\log N/N_0 \times 3.31)$ where N is the number of cells at the end of culture period and N₀ is the number of cells at culture initiation. Total population doubling number (tPDN) then determined using $tPDN = PDN_{p1} + PDN_{p2} + PDN_{p3}$. In this study the culture period for determining DT was 15 days (5 days per each three passages).

Osteogenic culture

Confluent passaged-3 cells in 6-well culture plates, provided with osteogenic medium containing the lithium with 2-14 mM concentration. The cultures with osteogenic medium, without lithium were taken as control. Osteogenic medium, DMEM contained 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM β-glycerol phosphate (Sigma, USA). The cultures incubated at 37 °C in an atmosphere of 5% CO₂ for a period of 21 days. At the end of this period the cultures quantified in terms of the amount of culture mineralization as well as the expression level of alkaline phosphatase (ALP) and osteocalcin genes by alizarin red

staining and the semi-quantitative RT-PCR analysis respectively.

Quantification of bone differentiation

Measurement of matrix mineralization

Alizarin red is a dye that specifically stains the mineralized matrix of bone; more mineralization is associated with more intensive staining. Using this staining method, an osteogenic quantification method has been developed commercially to measure and compare the amount of mineralization in different osteogenic cultures. In this study, osteogenic cultures were stained with alizarin red quantification kit (Chemicon, USA) according to the manufacturer instruction. Briefly, the cultures fixed in 10% formaldehyde for 15 min and followed by staining with alizarin red for 10 min. The cultures then washed with distilled water and their red matrix precipitate was solubilized in 10% acetic acid (Sigma, Germany). Optical density of the solutions, which was the representative of the amount of mineralization, finally recorded at 405 nm with a micro plate reader. The cultures were then statistically compared in terms of the OD values.

Semi-quantitative RT-PCR

Total RNA was collected from the cells, using RNX-Plus™ solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription, the RNA samples were digested with DNase I (Fermentas) to remove contaminating genomic DNA. Standard reverse-transcription reaction performed with 5 µg total RNA, using Oligo (dT)₁₈ as a primer and RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacture's instructions. Subsequent PCR was as follows: 2.5 µl cDNA, 1× PCR buffer (AMS), 200 µM dNTPs, 0.5 µM of each primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). The primers indicated in Table 1 were utilized to detect differentiation. Each PCR performed in triplicate and under linear conditions. Products electrophoresed on 1.7% agarose gels. The gels stained with ethidium bromide (0.5 mg/ml) and photographed on a UV transilluminator (Uvidoc, UK). Gel images

analyzed using the UVI band map program (Uvitec, Cambridge, UK). For the semi-quantitative determination of mRNA levels of the candidate genes, transcript levels were normalized to the corresponding GAPDH. Data analyzed by SPSS 13.0 for windows.

Statistical analysis

ANOVA test was used to determine the statistic significance of the differences. All measurement tests were performed for the cells from 7 rats. All values stated as means ± standard deviations. A *P*-value of <0.05 was considered to be statistically significant.

Evaluation of adipogenic differentiation

Since MSCs are cells with several differentiation capacities, the studied cultures were further examined in terms of their capability in producing adipocytic cells in addition to their previously examined bone differentiating capacity. For this purpose, the medium of the confluent passaged-3 cultures were changed with adipogenic medium containing 100 nM dexamethazone (Sigma, USA) and 50 mg/ml indomethacin (Sigma, USA). The cultures incubated for 21 days at 37 °C, 5% CO₂ with medium change of three times weekly. Adipose differentiation examined by oil red staining. To do this, the culture fixed with 4% formalin at room temperature, washed with 70% ethanol and stained by oil red (Sigma, USA) solution in 99% isopropanol for 15 min. At the end, the stained solution was removed and the cultures washed with 70% ethanol, before they were observed by the light microscopy.

Results

Cell culture

Marrow cells in the primary cultures were mainly fibroblastic in appearance and kept this morphology during the passages (Figure 1, upper row).

Doubling time

According to the results, the growth rate of the marrow cells in cultures with 4 mM lithium appeared to be significantly increased compared with that in the control group

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($P < 0.001$). The cell population in this culture was doubled in average of 29.30 ± 0.302 hr (Figure 1, lower row). In this regard, the culture with 10 mM lithium had the slowest rate of growth with DT value of about 86.24 ± 2.94 hours which was even higher than that in the control group, with DT of 49.30 ± 1.05 hr ($P < 0.05$). Such DT values mean that the marrow cells in cultures reached confluence in varying time. Our observation was in agreement with this conclusion, since we observed that the subcultures with 2, 4, 7 and 10 mM lithium became confluent in about 9, 7, 8 and 15 days, respectively.

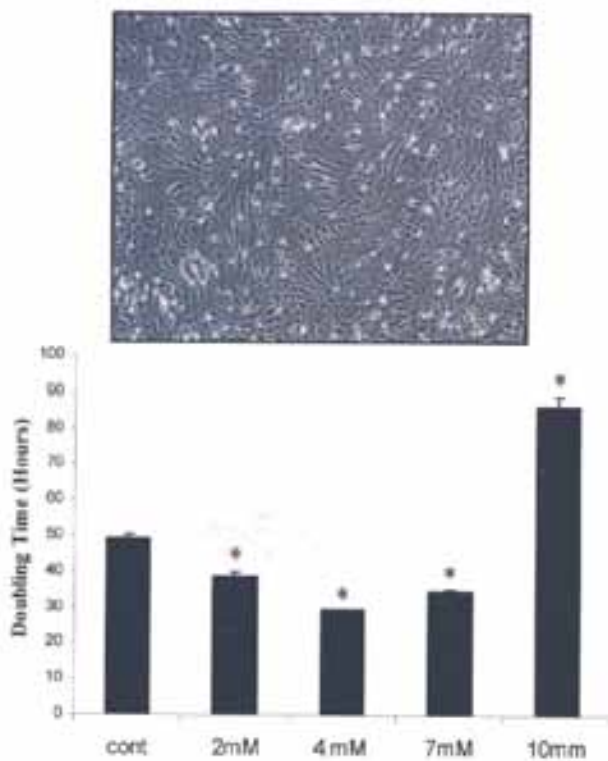


Figure 1. Upper: The cells during the cultivation period had approximately fibroblastic-like morphology. Lower: The graph indicating the doubling time (DT) of the marrow cell population in different culture groups. The cell population in culture with 5 mM Lithium doubled in faster rate than that in other cultures. * indicates the significant difference of the lithium-treated cultures compared with the control ($P < 0.05$).

Amount of culture mineralization

The osteogenesis ended up with matrix mineralization. Examination of the matrix of osteogenic culture indicated that different concentrations were associated with different amounts of mineralization. The highest

mineralization was observed at the culture with 12 mM Lithium concentration ($P < 0.05$) (Figure 2). After that point the mineralization rather was decreased by increasing the lithium concentration.

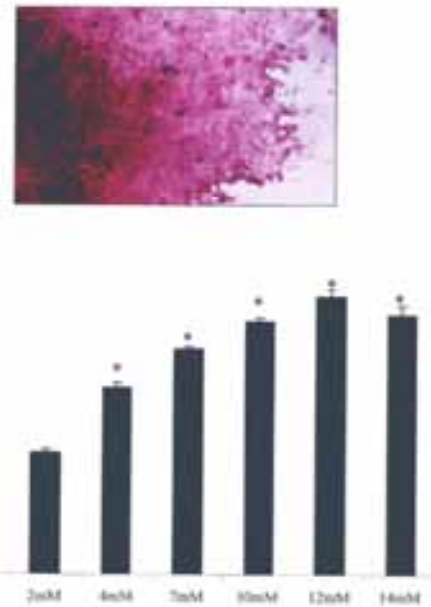


Figure 2. Measurement of the mineralization in the cultures with and without lithium. Upper: Alizarin red staining of the mineralized matrix in osteogenic cultures. The image was prepared after red matrix precipitate was solubilized in acetic acid. Lower: Graph indicating the OD of different cultures. The value of OD represents the amount of the mineralization being occurred at culture. In this term, the culture with 12 mM lithium exhibit heavily mineralization than the others. * indicates the significant difference of the lithium-treated cultures compared with control ($P < 0.05$).

Semi-quantitative RT-PCR

According to the results, osteocalcin and ALP genes appeared to be differentially expressed at the cultures. The highest level of osteocalcin expression observed at the culture with 12 mM lithium concentration (Figure 3). Up to 12 mM, the expression was concentration-dependent (progressively increased) but after that point, the expression was decreased. All differences with the control group were statistically significant ($P < 0.05$). The pattern of ALP expression was somehow similar to osteocalcin expression (Figure 4). In contrast to the osteocalcin expression, the peak expression level of the ALP was at 10 mM concentration.

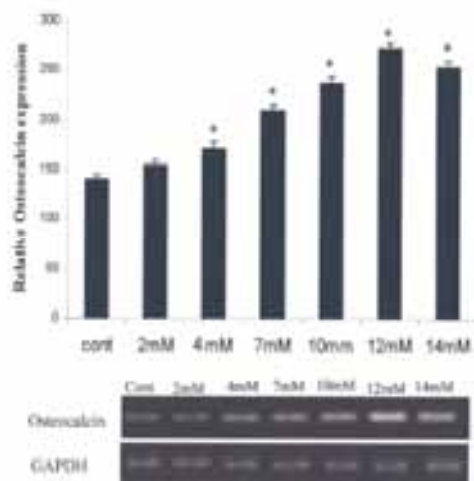


Figure 3. Osteocalcin expression in osteogenic cultures. Upper: the graph showed the relative expression of osteocalcin in different cultures. The cultures with 12 mM lithium appeared to be produced more osteocalcin mRNA than the others. Lower: semi-quantitative RT-PCR for osteocalcin expression. * indicates the significant difference of the lithium-treated cultures compared with the control ($P<0.05$).

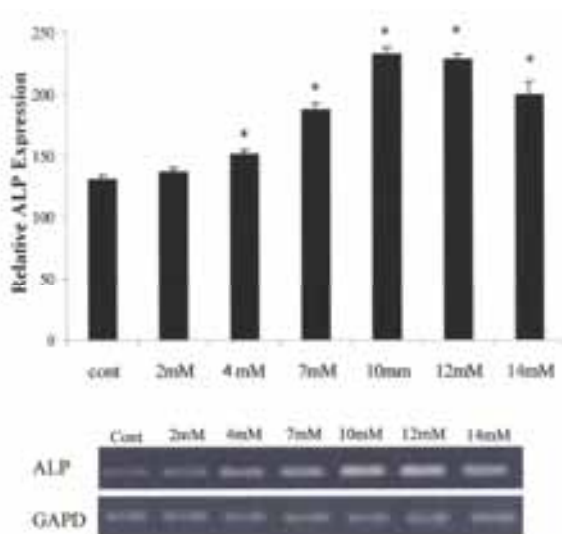


Figure 4. Alkaline phosphatase (ALP) expression in osteogenic cultures. Upper: the graph showed the relative expression of ALP in different cultures. The cultures with 10 mM lithium appeared to be produced more ALP mRNA than the others. Lower: semi-quantitative RT-PCR for ALP expression. * indicates the significant difference of the lithium-treated cultures compared with the control ($P<0.05$).

Adipogenesis

Upon oil red staining method, lipid droplets that were produced within the cytoplasm of differentiated cells, positively stained red (Figure 5).

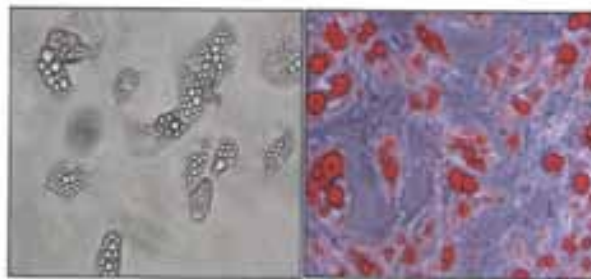


Figure 5. Adipose differentiation of the studied cells. The cultures were able to differentiate into adipose cells as evidenced in the appearance of lipid droplets in their cytoplasm (upper, left) which were positively stained with oil red (upper, right).

Discussion

In this study, effects of addition of lithium chloride on proliferation and osteogenic characteristics of MSCs were studied. The results indicated that lithium can enhance the rate of MSC *in vitro* proliferation as well as their bone differentiation. Such findings would be of great interest, since with increased MSC *in vitro* proliferation, sufficient cells could be provided for tissue engineering strategy: With enhanced MSC bone differentiation, cell-based therapy for bone defects would benefit from terminally differentiated cells which desired by clinicians who believes that in cell-therapy the use of undifferentiated cells may be accompanied with unwanted non-bone differentiation of MSCs in defect spot (26, 27).

The present report is not the first on lithium osteogenic effects. De Boer *et al* in 2004 have reported the inhibition of human MSCs bone differentiation in an osteogenic medium supplemented with 4 mM lithium (25). Our results are in contrast with the findings by de Boer *et al* in that, at our culture set up, 4 mM lithium concentration appeared to have stimulatory effects on MSC *in vitro* bone differentiation. This discrepancy would be the results from the difference in osteogenic culture period of the two studies. While in this study passaged-3 rat MSCs were plated in osteogenic medium for 21 days period, de Boer *et al* cultured human MSCs for only 4-5 days at osteogenic conditions. Therefore, the cells in the present study have been exposed to lithium for a comparatively longer period than those in de Boer *et al* experiment. This issue i.e. the effect of the exposure duration to

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lithium on MSCs osteogenesis, however, needs further investigation.

In the present study, the effects of lithium in bone differentiation of MSCs were investigated through the measurement of the level of expression of the alkaline phosphatase (ALP) and osteocalcin genes. ALP is a bone-specific enzyme, the presence of which is necessary for bone calcification (28). Osteocalcin is a glycoprotein that is specifically found in bone matrix binding hydroxyapatite, cell and collagen together (29). In this study, during *in vitro* bone differentiation, wnt signaling pathway was not directly dissected rather its terminal phases i.e. target gene expression were investigated. Since, previous investigations have provided some evidences that lithium exerts its effects through wnt pathway (20-22); therefore, the effects described here for lithium could be mediated by its GSK3 β -inhibitory effect. This, however, is remained to be further investigated.

In addition to its effect on *in vitro* bone differentiation of rat MSCs, in this study, the effect of lithium was further investigated in terms of cell expansion rate. The results indicated that the presence of lithium in rat MSC culture is associated with decreased doubling time, hence, with increased expansion rate. Similar findings have previously been reported by de Boer *et al.* (25). The mitogenic effect of lithium has also been reported at non-stem cell cultures including schwann cells (23). Former experiments have shown that lithium can also, improve renal epithelial cell viability in culture and maintains murine and human embryonic stem cell pluripotency. Lithium has reported to be able to protect renal epithelial cell culture against induced apoptosis (30, 31).

Cellular proliferation and differentiation are thought to be contrasting phenomena; proliferating cells possess limited differentiation potential and differentiating cells are rarely undergone proliferation (32). Our findings were consistent with this notion in that, among the lithium concentration studied, 4 mM concentration appeared with high mitotic effects, and 10-12 mM with highest osteogenic impact. In other words,

lithium in low concentration increased cell proliferation and at high concentration enhanced bone differentiation.

In this study, the optimum concentrations for lithium to emerge the highest mitogenic and osteogenic effects were 4 and 10-12 mM respectively. Above and below these concentrations, the cellular responses appeared to be decreased. There would be two possible explanations for such effects. One possibility is that the concentrations other than 4 and 10-12 mM might be toxic for MSCs culture, but this could not be the case because, for example, while the proliferation rate was significantly slowed down in the expansion cultures with 10 mM lithium, the presence of this concentration at osteogenic culture generates significant osteogenic effects. Lithium with cytotoxic effect could not exhibit significant osteogenic property. The other possibility is that the concentrations different from 4 and 10-12 mM may activate some signaling pathway with specific negative feedback inhibition on MSC proliferation or bone differentiation respectively. The involving of such a mechanism has previously been indicated by Garcia-Maya *et al* who investigated the effect of different concentrations of fibroblast growth factor (FGF) on fibroblastic cell culture (33).

In this study, one objective was to find the best lithium concentration that produces the highest differentiation effect. To do so, we initially examined the same range of lithium concentration as for proliferation experiments i.e. 1-10 mM, but this failed to determine the concentration with the highest effect because the 10 mM appeared to be the one with maximum osteogenic effects. Therefore, two additional concentrations including 12 and 14 mM were considered.

In spite of the considerable attempts that have been made to define the antigenic profile of MSCs from animals, no definitive single marker has so far been introduced. In almost all studies, the isolation of the cells is mostly performed on the base of their plastic adherence properties. After isolation, it is required to indicate their mesenchymal-stem cell nature. In the lack of specific marker it

was proposed that the golden standard to identify MSCs is their ability in differentiation into two or more cell lineages (34-37) which were considered in the present investigation.

Conclusion

Taken together it seems that lithium as an activator of wnt signaling pathway is able to increase rat marrow-derived MSC *in vitro*

proliferation in low concentration (4 mM) and enhances their *in vitro* bone differentiation in high concentration(10-12 mM).

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