

Sodium Arsenite Caused Mineralization Impairment in Rat Bone Marrow Mesenchymal Stem Cells Differentiating to Osteoblasts

Mohammad Hossein Abnosi ^{*1}, Zahra Jafari Yazdi¹

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ABSTRACT

Background: Sodium arsenite (SA) recently has been recommended to be used in malignancy therapy. Our studies showed, SA in short and long period of treatment caused reduction of rats Bone Marrow Mesenchymal Stem Cells (MSCs) viability and induced caspase dependent apoptosis. The aim of this study was to investigate the effect of SA on osteogenic differentiation of MSCs.

Methods: MSCs were extracted and expanded to third passage, then cultured in DMEM supplemented with osteogenic media in presence of 1 and 25nM of SA for 21 days. The viability and the level of mineralization were determined using MTT assay and alizarin red respectively. In addition morphology and nuclear diameter of the cells were studied with the help of fluorescent dye. Furthermore, calcium content and alkaline phosphatase activity also were estimated using commercial kit. Data was statistically analyzed and the $P < 0.05$ was taken as the level of significant.

Results: The viability and mineralization of the cells treated with SA reduced significantly ($P < 0.05$) after tenth day in compare with control. Also, chromatin condensation, reduction of nuclei diameter and cytoplasm shrinkage were observed in the cell treated with 1 and 25 nM concentrations. The calcium and alkaline phosphatase activity of the cells decreased significantly with 1 and 25 nM concentrations of SA when compared with control.

Conclusion: Adverse effect of SA was observed on osteogenic differentiation of MSCs at 1 and 25 nM due to disruption of mineralization. We strongly suggest more investigation to be run on this chemical with respect to the therapy of the malignant patients.

Keywords: Mesenchymal Stem Cell, Mineralization, Osteogenic Differentiation, Sodium Arsenite.

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INTRODUCTION

Arsenic, a naturally occurring element and by-product of copper, lead and other metals smelters, is the top environmentally hazardous substances, which were demonstrated to be a human carcinogen (1,2,3). Arsenic exist in several oxidative states but its pentavalent (arsenate, AS⁵⁺) and trivalent (arsenite, AS³⁺) forms are most prominent in the

environment which have toxicological significance (2). Base on the American FDA recommendation, arsenic trioxide (arsenite) has been used for the treatment of relapsed or refractory of acute promyelocytic leukemia in 2000 (4,5).

There are investigations which report the presence of $< 5 \mu\text{M}$ of sodium arsenite in the serum of malignant patients (6,7). Trivalent salt of arsenic (arsenite) is considered more toxic than pentavalent one

1. Department of Biology, Arak University of Medical Sciences, Arak, Iran.

*Corresponding Author E-mail: abnosi2002@yahoo.com

and the report indicated that the sodium arsenite causes genetic and epigenetic changes in mouse testicular leydig cells (8,9). Sodium arsenite also induces apoptosis in different type of cells such as rat midbrain neuroepithelial, CD4+ T cells, human neutrophils, Gclm mouse embryo fibroblasts and human bone marrow mesenchymal stem cell in micromolar concentration (10-13). Sodium arsenite readily react with thiol group of enzymes, receptors or coenzymes which may inhibit important biochemical events that could alter cellular redox status and eventually lead to cytotoxicity (14,15). Some other mechanisms including genotoxicity, alteration in DNA repair and methylation, oxidative stress, co-carcinogenesis, and tumor promotion also have been reported for arsenite toxicity (9,16,17).

Multipotent rat bone marrow mesenchymal stem cells (MSCs) representing <0.01-0.001% of the total nucleated bone marrow and posses two fundamental characteristics: the ability of extensive replication and the capacity of multilineage differentiation among bone, cartilage and adipose cell lineages (18-20). Yadev, et al. in their report showed that high concentration ($>5\mu\text{M}$) of sodium arsenite affect viability, DNA synthesis, morphology, cell cycle and apoptosis of human bone marrow mesenchymal stem cells (hMSCs) (13). Result of our previous studies showed that the sodium arsenite ($<5\mu\text{M}$) in 36hrs caused significant reduction of rat bone marrow mesenchymal stem cells (rMSCs) viability due to caspase dependent apoptosis in culture media (21). In addition, we have shown that much lower concentration of sodium arsenite (25nM) caused the significant reduction of BMCs viability after 21 days due to caspase dependent apoptosis, but there is no data available on the effect of sodium arsenite on differentiation property of MSCs (22).

Therefore, in this report, we investigated the effects of 1 and 25 nM of sodium arsenite on morphology, viability, calcium concentration, alkaline phosphatase activity and mineralization of rat bone marrow mesenchymal stem cells following its differentiation to osteoblasts.

MATERIALS AND METHODS

Marrow cell culture

In the present study, Wistar rats (6-8 weeks old) were purchased from Pastor Institute (Tehran, Iran) and kept in the animal house of Arak University under standard condition of light and food. The animals were sacrificed by excessive chloroform inhalation and then their tibia as well as femur were removed and cleaned from the adherent soft tissue. Then the two ends of the bones were cut off and bone marrow was flash out using 2 ml DMEM (Dulbecco's Modified Eagles Medium, Gibco, Germany) supplemented with 15% FBS (Fetal Bovine Serum, Gibco, Germany) and penicillin-streptomycin (Gibco, Germany).

Bone marrow content was centrifuged at 1200 rpm for 5 minutes and re-suspended in 5 ml DMEM containing 15% FBS and antibiotics then plated in 25-cm² flasks and incubated at 37 °C with atmosphere of 5% CO₂. Two days after culture initiation, the first medium replacement was performed and then medium was changed two times a week till the bottom of the flask was covered with the cells (till confluency). The cells were trepsinized (trypsin-EDTA, Gibco, Germany) and passed to another culture flask as the first passage and then the cultures were expanded through two additional subcultures for more purification of the mesenchymal stem cells which were used for further investigation.

Osteogenic induction

Mineralization was induced on confluent monolayers of cells by addition

of DMEM containing 15% (v/v) FBS, streptomycin-penicillin and osteogenic supplements [1mM sodium glycerophosphate, 50 µgmM L-ascorbate and 10^{-8} M dexamethasone (all the chemicals were purchased from Sigma-Aldrich company)]. Culture flasks were incubated for 21 days at 37°C with 5% CO₂ and their medium was changed every 3 days (23).

Exposure to sodium arsenite

To perform the assays, cells were cultured in separate culture dishes in presence of DMEM supplemented with osteogenic media for periods (days) according to the design of the test, which represented control and sodium arsenite treated (exposed to 1 and 25 nM of sodium arsenite) groups.

Cell viability assays

The viability test on control and treated cells was carried out in an ELISA plate using MTT (4, 5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), where after 4 hours of incubation the mitochondrial succinate dehydrogenase in the live cells convert yellow color tetrazolium into violet crystal of formazan. Then 100µL of DMSO was added to each well of the plate and formazan crystals were extracted in that following incubation for 30 min (1/2 hrs) in room temperature. The extracted solutions were transfer in another well and absorbance was measured on an automated microplate reader (SCO diagnostic, Germany) at 505 nm.

Analysis of morphological changes

Following sodium arsenite treatment in an osteogenic media for 21 days, the nuclear morphology of the cells was studied using Hoechst 33342 at room temperature after 5 minutes of incubation in the dark. The diameter of the cells was also measured in µm with the help of Motic Image software (Micro optical group company version 1.2). after 21 days Hoechst is a fluorescent dye which penetrate the cells through the intact

plasma membrane and stain the DNA , and where the changes in nuclear morphology such as chromatin condensation and fragmentation can be investigated (24).

The morphology of the cell cytoplasm was investigated using another fluorescent dye (acridine orange) which stains the nuclei green and the cytoplasm orange. The cells after staining were washed twice with PBS, examined and immediately photographed under an inverted fluorescence microscope (Olympus, IX70) equipped with camera using 40X magnification.

Detection and quantification of mineralization

Cells in 6-well plates were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) at room temperature for 15 minutes. The cells were then washed twice with excess of dH₂O and 1mL of 40mM alizarin red solution (ARS) (pH 4.1) was added per well. The plates were then incubated at room temperature for 20 minutes with gentle shaking. After which the excess of dye was poured off and the plates were washed four times with dH₂O. Stained cells were investigated under light microscopy using an inverted microscope. To quantify the level of absorbed alizarin red, 800µL of 10% acetic acid (v/v) was added to each well, and the plate was incubated at room temperature for 30 minutes with gentle shaking.

Then the loosely attached cells were scraped from the plate with a cell scraper and transferred to a 1.5-mL microcentrifuge tube. After vortexing for 30s, the slurry was overlaid with 500µL mineral oil (Sigma-Aldrich), heated at 85°C for 10 minutes, and then kept on ice for 5 minutes. The slurry was then centrifuged at 20,000g for 15minutes and 500µL of the supernatant was transferred to a new microcentrifuge tube and 200µL of 10% ammonium hydroxide (v/v) was added to neutralize the acid. An aliquots of

the supernatant (100 μ L) was read in triplicate at 405 nm in a microplate reader (SCO diagnostic, Germany) and quantified against standard graph (23).

In order to prepare Alizarin Red standards graph, working ARS (40 mM) was diluted 20 times with a mixture of 5:2 of 10% acetic acid and 10% ammonium to give a concentration of 2000 μ M. Then using serial dilution, standard solution of 2000 to 31.3 μ M was prepared and the absorption was taken at 405nm using a microplate reader. The concentration of the unknown samples was calculated using linear formula $Y=0.179X+0.094$ with $R^2=0.997$ where Y is the absorbance and X is the concentration (mM) of alizarin red.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity of control and treated cells in 6 well dishes was determined by p-nitrophenyl-phosphate (pNPP) hydrolysis method, using the ALP assay kit (Darman Kave, Iran). Cells were washed three times with PBS and homogenized in lysis buffer (0.25 M Tris-HCl, Triton X-100, PH:7.5) and the samples were centrifuged at 12000 rpm for 10 minutes at 4 $^{\circ}$ C (25).

The supernatant was kept in -20 $^{\circ}$ C for the analysis of ALP activity and protein content. The total protein content of each sample was determined according to Bradford, using bovine serum albumin (BSA) as standard. ALP activity was determined in protein lysate base on equal amount of protein using p-nitrophenylphosphate (pNPP) as substrate according to the kit instruction (Darman Kave, Iran). Absorbance at 410 nm was measured using spectrophotometer (T80+ PG instrument ltd, England) and then ALP activity was determined from a pNPP standard curve.

Calcium concentration

Cells in 6-well plates including control and treated ones were first washed twice with PBS and then their calcium

content was extracted in 50 μ l of 0.5 N HCl for 24 hours (26).

The amount of calcium was determined using commercial kit (Darman Kave, Iran) and the developed color was measured at 575 nm using spectrophotometer (T80+ PG instrument ltd, England).

Statistica analysis

Statistical evaluation of the data was performed using one and two-way analysis of variance (ANOVA) Tukey test, with the help of SPSS. Results were shown as mean \pm S.D and $P<0.05$ was accepted as the minimum level of significance.

RESULTS

Effect of sodium arsenite on cell viability

Cell viability assay (Table 1) showed that the 25 nM of sodium arsenite significantly decreased the viability ($p<0.001$) of bone marrow mesenchymal stem cell under osteogenic differentiation at 5th, 10th, 15th and 21th days as compared with control. Lower dose of sodium arsenite (1nM) showed no significant effect ($p>0.05$) on the viability of the cells at 5th and 10th days whereas at 15th and 21th days significant decrease of viability ($p<0.05$) was observed. Analysis of data using two-way ANOVA showed that the significant reduction ($P<0.001$) of viability depends on both dose and time of exposure (Table 2).

Sodium arsenite induced morphological changes of MSCs differentiated cells

Morphological study of the nuclei of differentiated mesenchymal stem cells treated with 1 and 25 nM of sodium arsenite after 21 days showed significant reduction ($p<0.05$) in nuclei diameter (Table 3) and chromatin condensation as well as nuclear breakage (Figure1-a2and3). It can be also noticed that sodium arsenite

at these concentrations caused remarkable changes in the morphology of cytoplasm (Figure1-c3) such as shrinkage and in

some cells complete disappearance of cytoplasm as compared to control cells.

Table 1. Effect of sodium arsenite on cell viability in osteogenic culture of BMCs base on MTT assay after 5, 10, 15 and 21 days of treatment.

Dose(nM)	Days			
	5	10	15	21
0	0.45 ^a ±0.26	0.53 ^a ±0.02	0.64 ^a ±0.32	0.67 ^a ±0.02
1	0.40 ^a ±0.02	0.48 ^a ±0.05	0.53 ^b ±0.02	0.57 ^b ±0.01
25	0.19 ^b ±0.01	0.26 ^b ±0.01	0.32 ^c ±0.03	0.38 ^c ±0.01

Values are means ± SD. Means with the different letter code differ significantly from each other (one-way ANOVA, Tukey test, $p < 0.05$).

Table 2. Viability of the Mesenchymal Stem Cells after 5, 10, 15 and 21 days of Treatment with 0, 1 and 25nM of sodium arsenite is Dose and Time Dependent

Dose (µM)	Days				P-Value
	5	10	15	21	
0	0.45±0.26	0.53±0.02	0.64±0.32	0.67±0.02	
1	0.40±0.02	0.48±0.05	0.53±0.02	0.57±0.01	0.001
25	0.19±0.01	0.26±0.01	0.32±0.03	0.38±0.01	
Dose and time					0.001

Values are means ± SD. (Two-way ANOVA, Tukey test, $P < 0.05$).

Table 3. Effect of 1 and 25nM of sodium arsenite on nucleus diameter (µM) of BMCs after 21 day of culture in osteogenic media

Dose (nM)	Nucleus diameter (µm)
0	11.0 ^a ± 0.4
1	9.1 ^b ±0.4
25	8.8 ^b ±0.2

Values are means ± SD. Means with the different letter code differ significantly from each other (one-way ANOVA, Tukey test, $p < 0.05$).

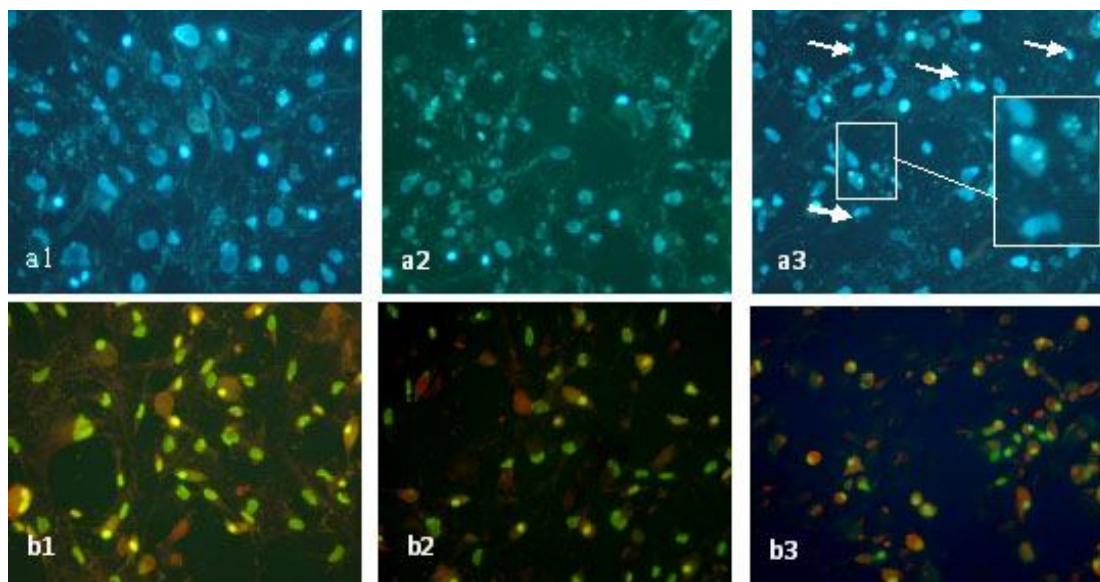


Figure 1. (a) Cells in osteogenic medium stained with Hoechst, (a1) control (a2) cells treated with 1nM of sodium arsenite for 21 days (a3) cells treated with 25 nM of sodium arsenite for 21 days. Nuclear condensation and DNA fragmentation (arrows and enlargement part) of cells was observed in cells treated with 25 nM of sodium arsenite. **(b)** Cytoplasm morphology of cells using Acridine orange staining, (b1) control (b2) cells treated with 0.5 μM (b3) cytoplasm shrinkage was observed in cells treated with 1 and 25 nM of sodium arsenite for 21 days (with 40X magnification).

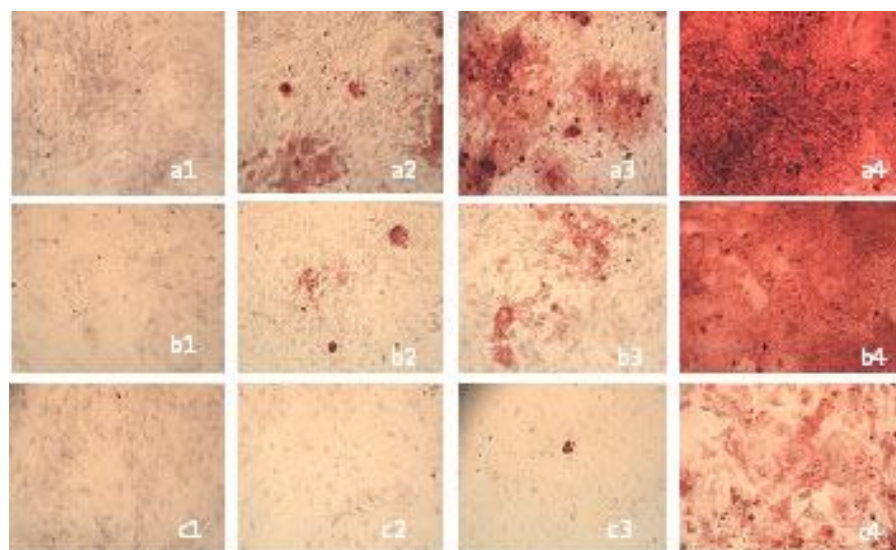


Figure2: Alizarin red staining for mineral deposition was performed for MSCs after 5, 10, 15 and 21 days of osteogenic induction. (a1-4) Control (b1-4) in presence of 1nM of sodium arsenite (c1-4) in presence of 25 nM of sodium arsenite (with 10x magnification).

Mineralization base on alizarin red staining

Data showed that the mineralization of cells under osteogenic differentiation is minimum at 10th day and reaches its maximum level at 21th day in control group (Figure 2 a1-a4). Treatment of the cells with 1 ($p<0.05$) and 25 ($p<0.001$) nM of sodium arsenite caused significant reduction in the mineralization from 10th day to 21th day based on quantitative (Table 4) as well as qualitative alizarin red estimation (Figure 2 c1-c4) as compared with control groups. Also, analysis of data using two-way ANOVA showed that the reduction of mineralization is time and dose depends (Table 5).

Calcium concentration and Alkaline phosphatase activity

Calcium concentration of the differentiated cells at 21th day was found to decrease significantly ($p<0.05$) in the group treated with 1 nM of sodium arsenite as compared to control. In addition a highly significant reduction ($p<0.001$) of calcium concentration was shown in the group treated with 25 nM when compared with control as well as 1nM group. Decrease of the activity of alkaline phosphatase enzyme was also in the same manner as calcium concentration, where 1 nM caused significant ($P<0.05$) and 25 nM caused highly significant ($P<0.001$) reduction when compared to control group (Table 6).

Table 4. Effect of sodium arsenite on mineralization of BMCs cultured in osteogenic medium based on quantitative Alizarin red(mM) staining.

Dose (nM)	Days			
	5	10	15	21
0	0.12 ^a ±0.04	0.40 ^a ±0.13	18.95 ^a ±0.27	24.33 ^a ±0.10
1	0.12 ^a ±0.03	0.29 ^b ±0.38	16.85 ^b ±0.23	19.95 ^b ±0.77
25	0.12 ^a ±0.08	0.23 ^b ±0.14	3.48 ^c ±0.30	6.35 ^c ±0.83

Values are means ± SD. Means with the different letter code differ significantly from each other (one-way ANOVA, Tukey test, $p<0.05$).

Table 5. Effect of sodium arsenite on mineralization of the Mesenchymal Stem Cells after 5, 10, 15 and 21 days of Treatment with 0, 1 and 25nM of sodium arsenite is Dose and Time Dependent

Dose (µM)	days				P-Value
	5	10	15	21	
0	0.12±0.04	0.40±0.13	18.95±0.27	24.33±0.10	0.001
1	0.12±0.03	0.29±0.38	16.85±0.23	19.95±0.77	
25	0.12±0.08	0.23±0.14	3.48±0.30	6.35±0.83	
Dose and time					0.001

Values are means ± SD. (Two-way ANOVA, Tukey test, $P<0.05$).

Table 6. Effect of sodium arsenite on Ca²⁺ concentration and Alkaline phosphatase activity of BMCs cultured in osteogenic medium.

Dose (nM)	mg/dl (Ca ²⁺ concentration)	ALP activity(U/L)
0	37.32 ^a ± 0.27	63.9 ^a ± 1.2
1	29.24 ^b ±0.20	45.91 ^b ±0.8
25	12.09 ^c ±0.48	20.60 ^c ±0.7

Values are means ± SD. Means with the same letter code do not differ significantly from each other (one-way ANOVA, Tukey test, $p>0.05$).

DISCUSSION

The present study was designed to investigate the effect of low dose (nM) of sodium arsenite on differentiation of MSCs to osteoblasts and attempt was to characterize the cellular and molecular nature of differentiated MSCs in response to this toxicant. Previous studies have shown that sodium arsenite enhance apoptosis in cell types such as rat midbrain neuroepithelial, CD4+ T cells, human neutrophils, Gclm mouse embryo fibroblasts and human bone marrow mesenchymal stem cell (10-13). In this study, the viability of differentiated MSCs in response to 1 and 25 nM of sodium arsenite have reduced significantly at 15th day onwards but no effect was observed at 5th day, though the 25 nM reduced the viability even at 5th day. As the low and high dose (1 and 25 nM) of sodium arsenite showed significant effect on viability, therefore we may say that there is only time limitation for sodium arsenite toxic effect. Since bone matrix is in direct contact with peripheral blood, therefore the presence of even low dose up to 1 nM might be of a great concern. In previous studies it was shown that the concentration of sodium arsenite was less than 5 μ M in the blood of malignant patients under therapy with this chemical (6,7). Since the MSCs are able to differentiate to osteoblasts, chondrocytes and adipocyte, therefore it is considered as the main source of bone regeneration and remodeling during its homeostasis (27-30). Thus it should be taken in consideration that, in presence of sodium arsenite the health of the bone might be in great danger.

We found also, 1 and 25 nM of sodium arsenite after 21 days of treatment caused chromatin condensation, nuclear diameter reduction and nuclear breakage as well as cytoplasm shrinkage which all together might be considered as sign of apoptosis and a reason for significant viability reduction (31). Many

investigators have shown that the sodium arsenite cause activation of caspases through internal and external pathway thus the viability reduction of MSCs under osteogenic differentiation might be due to apoptosis (8,32-34). Also, sodium arsenite induces oxygen free radicals production which might be another reason of nuclear breakage (17,35). In addition, differentiation of MSCs to osteoblasts is followed by changes in cytoskeleton content like actin, where it is well documented that the sodium arsenite can affect the cytoskeleton, which can be another reason for cytoplasm shrinkage (36-38).

Our finding showed that the level of mineralization in term of quantitative alizarin red, calcium concentration and alkaline phosphatase activity reduced significantly ($p < 0.05$) from the 10th day in the 1 and 25 nM group as compared to the control group. After a certain period of time, in vitro osteogenic mineralization starts, with respect to alkaline phosphatase activity resulting in the release of phosphate ion which bring about large influx of calcium ion into the cells (23,39). The influx of calcium is a necessary step in formation of hydroxyapatite crystal which is the prompt step of bone formation (40). At this point, with respect to viability and mineralization data it may be concluded that the effect of the sodium arsenite began as the osteogenic changes occurred in the cell somehow after the 5th day.

As mentioned earlier, researches has shown that sodium arsenite induces oxidative stress and the oxidative stress induced by oxygen free radicals inhibit osteogenic differentiation processes thus it might be another reason of why sodium arsenite caused impairment in osteogenic differentiation processes (17,35,41). Furthermore, osteogenic differentiation depend on Wnt signaling, where in this pathway in the presence of the β -catenin and ICF/LEF factor activation of alkaline phosphatase genes takes place (42, 43). Researches has shown that oxygen free

radical can inhibit expression of alkaline phosphatase gene by disrupting Wnt signaling which might be a reason for significant reduction of enzyme activity in this study (41). In addition, investigators showed that the free radicals can cause inhibition of calcium channel and disruption of calcium homeostasis which itself might be a reason for significant reduction of calcium influx due to sodium arsenite toxicity (44,45).

CONCLUSION

All together, it is to be mentioned that the MSCs are pluripotent stem cells that can differentiate to osteoblast and are also considered to be a major source of bone formation and remodeling, thus there health should be under a great consideration and attention (30,46). As sodium arsenite has been recommended to be used in therapy as well as it was reported that this chemical is presented in some food material such as rice and water therefore their consumption would increase the sodium arsenite concentration in human blood, thus it might have profound effect on bone homeostasis and remodeling (4,5,47,48). Therefore we strongly suggest more investigation to be carried out regarding the relationship between bone diseases such as osteoporosis and sodium arsenite toxicity in general population.

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