

Original Article

Exogenous Nitric Oxide Up-regulates the *Runx2* Via *Bmp7* Overexpression to Increase the Osteoblast Matrix Production *In Vitro*

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Article history:

Received: 3 Feb. 2022

Revised: 26 Feb. 2022

Accepted: 26 Feb. 2022

ePublished: 25 June 2022

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Abstract

Background: Nitric oxide (NO) is a signaling molecule that is required for the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). According to previous reports, high concentrations of sodium nitroprusside (SNP) inhibit the osteogenic differentiation of BMSCs, while its low concentration promotes this process.

Objectives: The present investigation focused on evaluating the underlying mechanism of the osteogenic differentiation of BMSCs treated with low concentrations of SNP as an NO generating agent.

Methods: The BMSCs after the 3rd passage was differentiated to osteoblasts when treated with 100 μ M for 1 hour every 48 hours until 5, 10, 15, and 20 days of incubation. Then, the matrix production was estimated by quantitative alizarin red assay and calcium determination. The expression of different genes involved in osteogenic differentiation was statistically determined using the reverse transcriptase polymerase chain reaction. Finally, alkaline phosphatase activity was measured by a commercial kit.

Results: The exogenous NO caused a significant ($P < 0.05$) increase in the matrix production of differentiated BMSCs from day 5 to 20. The results showed the elevation of alkaline phosphatase activity and the up-regulation of its gene. Eventually, an increase was observed in the expression of a cascade of other genes such as osteonectin, *Bmp7*, *Smad1*, *Runx2*, and *Raf1* in treated BMSCs.

Conclusion: Overall, short-time treatment with a low concentration of exogenous NO increases the matrix production via gene up-regulation and protein production, which might open a new window in treating the low-density bone complication.

Keywords: Mesenchymal stem cell, Cell differentiation, Nitric oxide, Gene expression, Calcium

Please cite this article as follows: Abnos MH, Sargolzaei J, Maleklou M. Exogenous nitric oxide up-regulates the *runx2* via *bmp7* overexpression to increase the osteoblast matrix production *in vitro*. Avicenna J Med Biochem. 2022; 10(1):58-64. doi:10.34172/ajmb.2022.08

Background

Three isoenzymes of nitric oxide (NO) synthase, namely, inducible NO synthase, epithelial NO synthase, and neural NO synthase (1) produce NO from arginine (2,3) and the first two are found in the osteoblasts (4). At the physiological level, NO causes the expression of the genes involved in the growth of the bone (5) and the maturation of osteoblasts (6) whereas the excess of NO causes cell cytotoxicity and induces apoptosis in a variety of cells such as mesenchymal stem cells (7), neurons (8), hepatocyte (9), and human sperm (10). The mesenchymal stem cell differentiates to osteoblast that plays a critical role in the prevention of osteoporosis and bone repair and remodeling (11). In the osteogenic differentiation of the mesenchymal stem cells, the expression of many genes (e.g., RUNX2, OSTRIX, SMADs, and BMPs) has to be regulated (12) that are directly or indirectly influenced by NO (13).

At low concentrations, NO is beneficial to the cell, but

its high concentration has been considered to cause cell toxicity, which is a matter of concern (14,15). Considering that NO has been prescribed to control complications such as hypertension, exogenous NO might be helpful in the treatment of osteoporosis or other bone-related complications. Many chemical agents such as sodium nitroprusside (SNP), S-nitroso-N-acetylcysteine, NO-aspirin, S-nitrosothiols, and the like are known to generate NO *in vivo* and *in vitro* (16). These chemicals have been used to investigate the effect of exogenous NO on the cellular mechanism such as the osteogenic differentiation of mesenchymal stem cells (17,18). Felka et al studied the inhibitory effect of exogenous NO released by SNP on the osteogenic differentiation of human mesenchymal stem cells and found that the high concentration of NO inhibited RUNX2 expression (19). In contrast, Abnosi and Pari revealed that the pre-treatment of the rat bone marrow mesenchymal stem cells (BMSCs) with 100 μ M of



SNP for 1 hour every 48 hours elevated matrix formation from day 10 of the differentiation process up to the 20th day of incubation (20).

Based on the previous report, the continuous exposure of BMSCs to low concentrations of NO might have beneficial effects. Therefore, the present study investigated the effect of continuously exposed MSCs to low concentrations of SNP as a NO liberating agent on the matrix formation, alkaline phosphatase (ALP) activity, and the expression of genes involved in the osteogenic differentiation of these cells *in vitro*.

Materials and Methods

BMSC Extraction

To conduct the experimental investigation, male Wistar rats (6-8 weeks) were purchased from Pasteur Institute of Iran (Tehran) and housed in a polyethylene cage at $27 \pm 3^\circ\text{C}$ with enough access to food and water. Based on the protocol approved by Arak University, the rats were anesthetized with the inhalation of chloroform in the airtight jar and then sacrificed. Next, the bones (tibias and femurs) were surgically removed, and the soft tissues were removed under sterile conditions. The ends of the bones were cut, and the bone marrow was extracted in 2 mL Dulbecco Modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 15% of fetal bovine serum (FBS, Gibco, Germany) and penicillin/streptomycin (Gibco, Germany). The extracted bone marrow was precipitated at 2500 rpm for 5 minutes, then suspended in a T25 culture flask containing 5 mL DMEM and incubated at 37°C under 5% CO_2 in an appropriated culture flask. The culture media were replaced twice a week until the bottom of the flask was covered with the cell. The cells were trypsinized (trypsin- Ethylenediaminetetraacetic acid; Gibco, Germany), and subcultured in fresh media after it was washed with phosphate saline buffer. The subculture was expanded for several passages to find the best generation and purity for further analysis using the flow cytometer (Germany, PARTEC; PAS).

Osteogenic Induction and Exposure to SNP

The stock solution of SNP was prepared in deionized water, and the pH was adjusted to 7.2. The BMSCs (5×10^4) after the 3rd passage were seeded in 6-well culture plates and allowed to cover the bottom of the plate up to 70%, and then cultured in osteogenic media containing DMEM, 15% (v/v) FBS, 1% (v/v) streptomycin- penicillin (10 mL/L), 1 mM sodium glycerophosphate, 50 $\mu\text{g}/\text{mL}$ L-ascorbate, and 10^{-8} M dexamethasone. The treatment was conducted with 100 μM of SNP (Merck, Germany) for 1 hour every 48 hours until 5, 10, 15, and 21 days of the incubation period in the presence of control groups. The analysis was repeated three times for each experiment.

Investigation of Mineralization

The plates were washed and fixed with formaldehyde (10% v/v, Sigma-Aldrich) for 10 minutes, then 1 mL of

the alizarin red solution (40 mM, pH=4.1) was added and kept at room temperature for 10 minutes. The excess dye was removed and washed with dH_2O , and then the stained cells were observed and photographed by a camera-equipped, inverted microscope. The cells were incubated at room temperature for 30 minutes, along with 800 μL of acetic acid (10%). Next, the cells were removed using a scraper and transferred to a 1.5 mL micro-centrifuge tube. The slurry was vortexed for 30 seconds, and 500 μL of the mineral oil (Sigma-Aldrich) was placed on it and kept in a water bath (85°C) for 10 minutes, followed by 5 minutes of incubation on ice. The extract was centrifuged for 15 minutes at 13000 rpm, and the supernatant was transferred to another micro-centrifuge tube and neutralized with 200 μL of ammonium hydroxide (10%). Subsequently, the absorption of 100 μL neutralized supernatant was measured at 450 nm in an ELISA reader, and then the concentration of alizarin red was calculated using the linear formula $Y = 0.168X + 0.112$ with $R^2 = 0.997$ where Y and X stand for the absorbance and concentration (μM) of alizarin red, respectively. The alizarin red standard graph was also prepared by diluting the alizarin red solution with the acetic acid solution (10%) and the ammonium solution (10%) in a ratio of 5:2. A series of diluted standard solutions from 31.3 to 2000 μM was prepared, then the absorption was measured at 450 nm using an ELISA reader.

Matrix Calcium Estimation

The plate was washed, and the cells were detached from the plate and transferred to a known weight micro-centrifuge tube using a scraper. Next, the weight of the samples was calculated by subtracting the weight of empty tubes and the weight of the tube containing the sample. The samples were treated with 50 μL of 0.5 N HCl for 24 hours and centrifuged for 10 minutes at 10000 rpm, and then the total calcium of the extract was measured using a commercial kit (Quantitative analysis of calcium: photometric method, Pars Azmoon Company, Iran) where theoretically calcium in neutral pH reacts with arsenazo, and the intensity of the produced blue color is proportional to its concentration after 5 minutes. Using different concentrations of CaCl_2 , the standard graph was plotted, and the unknown sample concentrations were determined by the linear formula $Y = 0.005X - 0.0009$ with $R^2 = 0.998$ (Y and X denote absorption and concentration of calcium, respectively). A spectrophotometer (T80 + PG Instrument Manufacturing Company, UK) was used to measure the absorption at 630 nm, and the calcium concentration was reported as mg/dL.

Cell Extract Preparation

After treatment, the cells were washed and scraped from the bottom of the flasks using Tris-HCl buffer (pH=7.4). The scraped cell was collected in a micro-tube, and then the content of the cells was released using freezing and thawing and centrifugation for 10 minutes at 12000 g. To estimate the total protein content of the samples, the Lowry method was employed, and the same method was applied

to plot a standard graph using bovine serum albumin as standard protein. To calculate the concentration of protein in the unknown samples, the linear formula $Y=0.0021X+0.0575$ with $R^2=0.9966$ was used, where Y and X represent absorbance and concentration (μg) of protein, respectively.

Estimation of ALP Activity

The ALP activity of the extracted samples was estimated based on the equal amount of protein using a commercial kit (Quantitative analysis of ALP: photometric method, Pars Azmoon Company, Iran). According to the instruction, the enzyme acts on p-nitrophenyl phosphate to release phosphate and p-nitrophenolate where the intensity of the developed color is proportional to the activity of ALP. The absorbance was measured at 410 nm using a spectrophotometer (T80+PG Instrument, Ltd., England).

Gene Expression Analysis

Following total RNA extraction, the gene expression was analyzed by a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using the Super RNA extraction kit (YT9080). The concentration of total RNA was determined using a spectrophotometer (T80+PG Instrument Manufacturing Company, UK), and the cDNA was synthesized according to the instruction given in the BioFACT commercial kit (BR631-096). Thermo Cycler (Eppendorf master cycler gradient) was employed to perform the PCR of ALP, glyceraldehyde dehydrogenase (GAPDH was taken as a housekeeping gene for internal control), SMAD1, osteonectin, Raf1, osteocalcin, BMP7, and Runt-related transcription factor 2 (RUNX2) genes with the following programmed using specific primers (Table 1):

First, 95°C for 5 minutes and 95°C for 1 minute as denaturation temperature, followed by the annealing temperature of the specific primer (Table 1) for 1 minute. The extension temperature was 72°C for 1 minute, and finally, 72°C for 7 minutes was applied as elongation temperature. The above cycle was repeated 40 times, and the agarose gel electrophoresis was used to run

the PCR product. The analysis was performed three times, and the intensity of the bands was determined by Gel Quant software (1.8.2). The data were analyzed by statistical software, and the results were presented as means \pm standard deviations (SD).

Analysis of Data

One-way analysis of variance (ANOVA) and Tukey's tests were applied to analyze data by SPSS software (version 20). Data were presented as the mean (\pm SD), and the $P<0.05$ was taken as the minimum level of significance.

Results

Estimation of Matrix Formation Based on Alizarin Red Assay

Alizarin red estimation showed that the matrix formation has started from day 10 and reached its maximum on day 20 in the control group, whereas it was observed to be started from day 5 in the treated group. In the treated group, statistical analysis revealed that the matrix formation was significantly increased ($P<0.05$) due to exogenous NO released by SNP when compared to the control group (Table 2). The microscopic analysis also confirmed the elevation of matrix formation during the incubation period from day 5 to day 20 (Figure 1) in the treated group.

Estimation of Calcium

The statistical analysis of the calcium concentration extracted from the matrix of the differentiated cells demonstrated that the exogenous NO released by SNP significantly ($P<0.05$) increased the deposited calcium

Table 2. The Mean Concentration of Alizarin Red (μM) Extracted From Control and Treated BMSCs After the Completion of the Incubation Period (5, 10, 15, and 20 Days)

	5 Days	10 Days	15 Days	20 Days
Control	0.94 ^a \pm 0.01	2.10 ^a \pm 0.18	3.10 ^a \pm 0.10	4.60 ^a \pm 0.02
100 (μM)	1.81 ^b \pm 0.05	3.90 ^b \pm 0.08	4.40 ^b \pm 0.05	5.17 ^b \pm 0.05

Note. BMSC: Bone marrow mesenchymal stem cell. Values are shown as means \pm standard deviations. In each column, the means with a different letter code denotes significant differences compared with the control group (Analysis of variance, Tukey's test, $P<0.05$).

Table 1. Primers Properties

Gene	Primers	Annealing Temperature	Product Length	Reference
<i>Alp</i>	F: CATGTTTCCTGGGAGATGGTA R: GTGTTGTACGTCTTGGAGAGA	58.4 59.4	144 bp	21
<i>Oc</i>	F: AACGGTGGTGCCATAGATGC R: AGGAGGCTCTCTGTCTCAC	60.5 62.5	294 bp	21
<i>Runx2</i>	F: CCGCACGACAACCGCACCAT R: CGCTCCGGCCCAAAATCTC	64.6 64.6	289 bp	21
<i>Raf1</i>	F: ATGTGCGGAATGGGATGAG R: TCGGTGTTCCAATCTAAGCG	54.4 58.4	139 bp	22
<i>Smad1</i>	F: CCGCCTGCTTACCTGCTCTGAA R: GAACGCTTCGCCACACGGTTGT	70.4 68.2	246 bp	21
<i>Bmp7</i>	F: AAGCCCAGATGGTACGG R: GCACCTCCAGGAAAAC	54.8 54.8	443 bp	21
<i>Gapdh</i>	F: TCGTCTCATAGACAAGATGG R: GTAGTTGAGGTCAATGAAGGG	56.4 59.4	136 bp	21

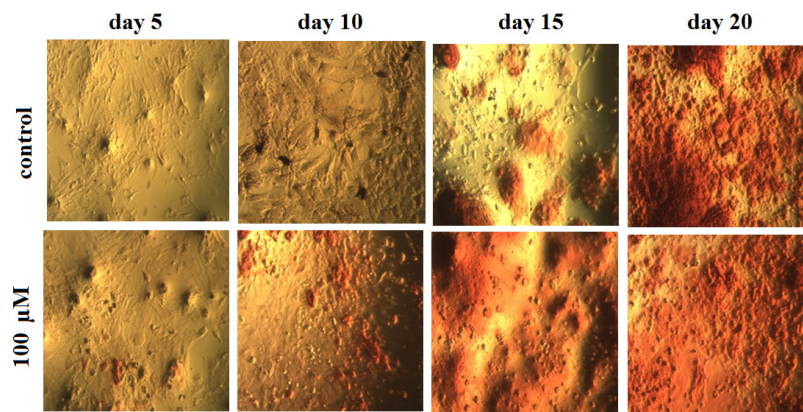


Figure 1. The Image of the Osteogenic Differentiated Cells Where the Differentiation Has Started From Day 5 in the Treated Group of the Cell When Compared to the Control One. Note. SNP: Sodium nitroprusside. The matrix deposition under the influence of SNP was observed to be significant (Magnification 20X).

Table 3. The Mean Concentration of Calcium (mg dl^{-1}) Extracted From the Matrix of Control and Treated BMSCs After the Completion of the Incubation Period (5, 10, 15, and 20 Days)

	5 Days	10 Days	15 Days	20 Days
Control	$2.80^a \pm 0.08$	$17.58^a \pm 0.21$	$26.08^a \pm 0.12$	$29.47^a \pm 0.21$
100 (μM)	$4.75^b \pm 0.17$	$18.09^b \pm 0.12$	$28.81^b \pm 0.26$	$31.04^b \pm 0.12$

Note. BMSC: Bone marrow mesenchymal stem cell. Values are indicated as means \pm standard deviations. In each column, the means with a different letter code implies differences which are significant compared to the control group (Analysis of variance, Tukey's test, $P < 0.05$).

(Table 3). It was also observed that the calcium deposition increased from day 5 as compared with the control group, and this elevation in calcium deposition continued until the end of incubation on day 20.

Estimation of Total Protein Concentration and ALP Activity

Based on the findings, the treatment of the cell with exogenous NO significantly increased ($P < 0.05$) the total protein from day 5 until day 20 when compared with the control group (Table 4). This increase was observed to be in comparison to each control group at its specific incubation time. It was noticed that the increase in the total protein content of the cell was highly significant ($P < 0.001$) from the 10th day of the treatment.

In addition, the results represented that there was a highly significant increase in the activity of ALP ($P < 0.001$) on day 5 when compared with the control group (Table 4). The continuous increase in enzyme activity was observed to rise sharply until day 20.

Expression of the Genes Involved in Osteogenic Differentiation

The statistical analysis of RT-PCR data indicated that the expression of ALP, osteocalcin (*Oc*), *Runx2*, *Raf1*, *Smad1*, and *Bmp7* was significantly ($P < 0.05$) up-regulated, while no significant ($P > 0.05$) changes were observed regarding the expression of *Gapdh* (Table 5). Figure 2 confirms the statistical results.

Discussion

Felka et al reported the osteogenic inhibitory effect of

Table 4. The Mean Concentration (μg) of Total Protein and Alkaline Phosphatase Activity (IU L^{-1}) Extracted From the Matrix of Control and Treated BMSCs After the Completion of the Incubation period (5, 10, 15, and 20 Days)

	Incubation Period (day)	Control	100 μM
Total protein concentration	5	$11.33^a \pm 0.14$	$14.66^b \pm 0.14$
	10	$21.42^a \pm 0.71$	$32.49^b \pm 0.35$
	15	$32.49^a \pm 0.35$	$41.30^b \pm 0.59$
	20	$35.95^a \pm 0.95$	$44.04^b \pm 1.70$
Alkaline phosphatase activity	5	$189.52^a \pm 3.43$	$374.16^b \pm 5.61$
	10	$346.82^a \pm 2.01$	$479.66^b \pm 4.72$
	15	$543.10^a \pm 4.95$	$806.85^b \pm 4.26$
	20	$726.91^a \pm 1.34$	$992.78^b \pm 3.46$

Note. BMSC: Bone marrow mesenchymal stem cell. Values are demonstrated as means \pm standard deviations. In each row, the means with a different letter code shows significant differences in comparison to the control group (Analysis of variance, Tukey's test, $P < 0.05$).

Table 5. The Mean Expression of the Genes Involved in the Osteogenic Differentiation of BMSCs after 20 Days of the Incubation period

	Control	100 μM
<i>Alp</i>	$0.74^a \pm 0.03$	$1.06^b \pm 0.08$
Osteocalcin (<i>Oc</i>)	$0.81^a \pm 0.05$	$1.10^b \pm 0.10$
<i>Runx2</i>	$0.86^a \pm 0.03$	$0.95^b \pm 0.03$
<i>Raf1</i>	$0.59^a \pm 0.01$	$0.69^b \pm 0.04$
<i>Smad1</i>	$0.74^a \pm 0.01$	$0.86^b \pm 0.02$
<i>Bmp7</i>	$0.63^a \pm 0.02$	$0.74^b \pm 0.01$
<i>Gapdh</i>	$0.98^a \pm 0.01$	$0.98^a \pm 0.00$

Note. BMSC: Bone marrow mesenchymal stem cell. Values are shown as means \pm standard deviations. In each row, the means with a different letter code represents significant differences compared to the control group (Analysis of variance, Tukey's test, $P < 0.05$).

exogenous NO at high concentrations (1 mM) in the early stage of osteogenesis, but in our previous study, we showed that the 100 μM of SNP as the NO-releasing agent induced the early matrix formation of BMSCs at day 5 in vitro (19,20). Some studies reported that the matrix formation starts from day 7 when the activity of ALP begins as an early gene (23,24). From the beginning of osteogenic induction, the *Bmp(s)* play an important role in starting the intracellular cascade, causing the up-regulation of the *Smad(s)* gene that induces the expression of *Runx2*, finally

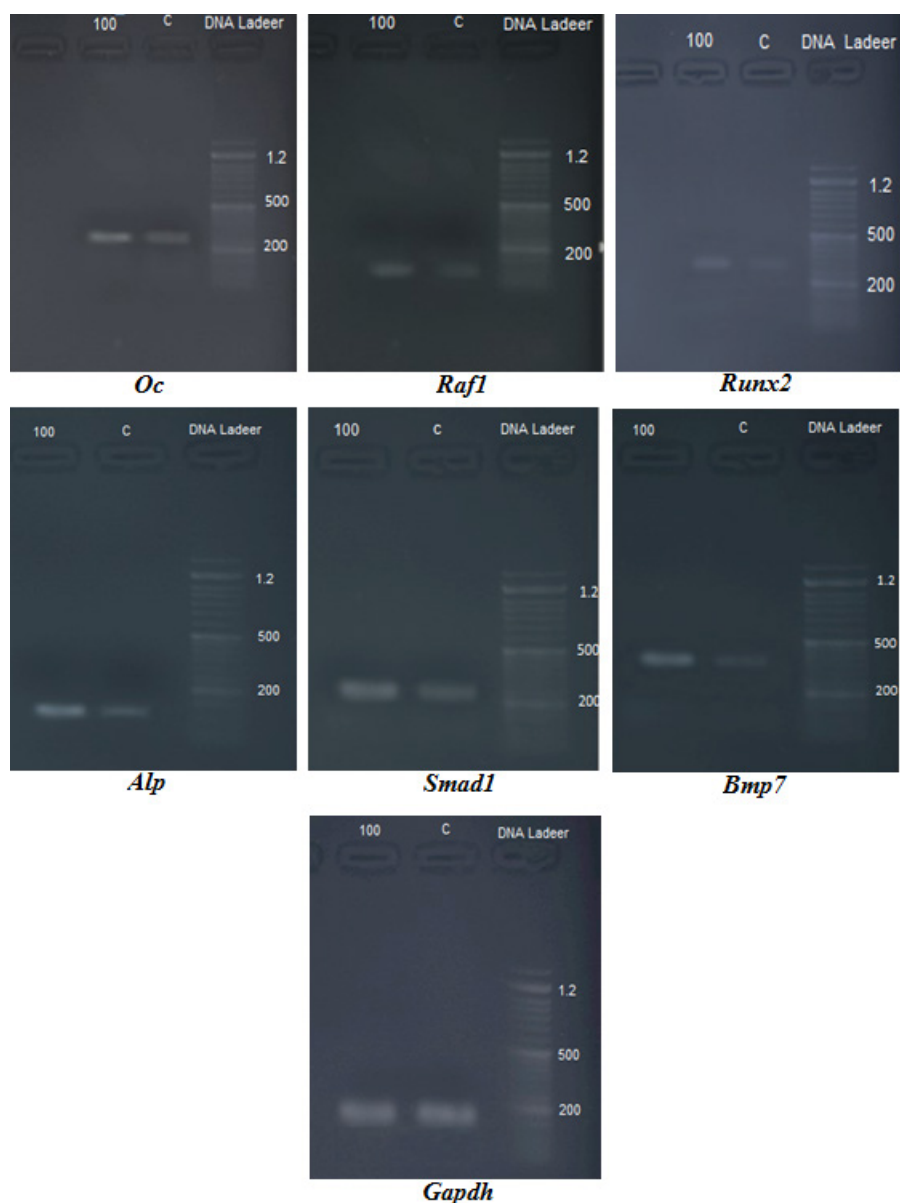


Figure 2. The Image From the Agarose Gel of the Genes Involved in the Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells.

leading to the up-regulation of osteonectin, osteocalcin, and ALP via *Osterix* gene expression (25). In the BMP family, the BMP7 is the main protein that activates the osteogenic cascade (26) by the activation of SMAD1 as a primary effector (27). The expression of *Alp* takes place in the early stage of the osteogenic differentiation (23), followed by the up-regulation of osteocalcin (*Oc*), osteonectin (*On*), and collagen I (*Coll 1*) which are necessary for matrix formation (21). To understand the mechanism of the NO effects on the osteogenic differentiation of BMSCs, the present study focused on determining the total protein and the activity of ALP, where it was revealed that the exogenous NO released by SNP induced and increased the total protein content of BMSCs, finally, elevated ALP activity. The elevation of the protein level was reported by other investigators with respect to collagen and other proteins required for bone matrix formation (28-30). In another study, the elevation of ALP activity was highlighted as the necessary equipment to deposit hydroxyapatite crystals (31).

In addition to the protein level, our findings revealed that the NO released by SNP induces the up-regulation of several genes involved in the osteogenic induction. The regulation of genes starts from *Bmp7* and continues with *Smad1* to elevate the expression of *Runx2*, finally up-regulating the expression of *Oc* and *Alp* genes (Figure 3). It was further found that NO released by SNP caused the activation of the MAPK gene related expression (data are not available), which may activate the expression of the *Runx2* gene (32,33) as well.

Based on the results of this study and other investigations (20, 34), it can be concluded that the exogenous NO might increase bone matrix formation by influencing the protein and gene expression of differentiated BMSCs and inducing early matrix formation in derived preosteoblasts. In our investigation, the effects of exogenous NO were started as early as the treatment began, from day 5 when the level of the total protein represented a significant increase. Proteins such as collagen I (35,36) are primarily required for the

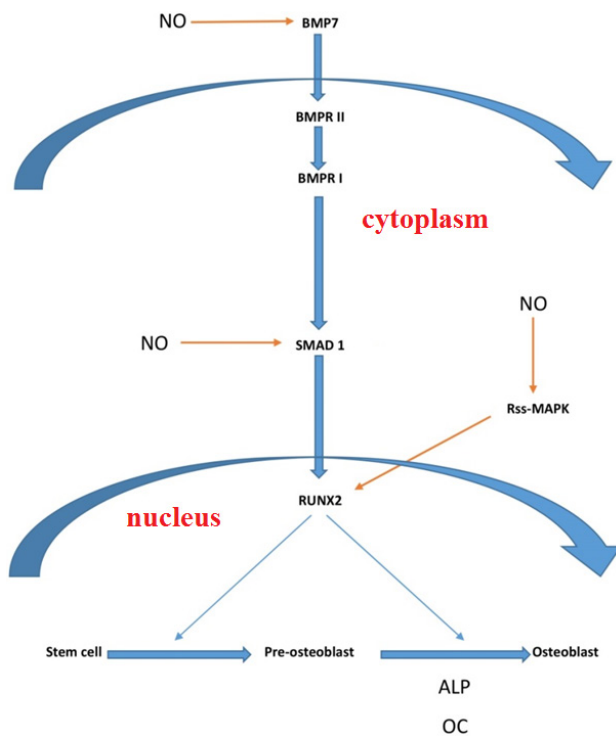


Figure 3. The Pathway of NO Release by SNP Affects the Expression of Genes involved in Osteogenic Induction of BMSCs *In Vitro*. Note. NO: Nitric oxide; SNP: Sodium nitroprusside; BMSC: Bone marrow mesenchymal stem cell.

deposition of hydroxyapatite in the matrix. Therefore, the elevation of the protein level, along with the up-regulation of osteogenic-related genes worked together to raise the production of the bone matrix where the alizarin red analysis and calcium measurement confirmed it.

Conclusion

In conclusion, it was found that the short-time exposure of BMSCs to low concentrations of exogenous NO would cause early osteogenic differentiation and mineralization through the elevation of protein production and the up-regulation of related genes. Accordingly, it is strongly recommended that more investigations be conducted to find out if the exogenous treatment with the NO-releasing agent could probably help compensate for low-density bone-related complications.

Acknowledgments

This project was performed in the Department of Biology, Arak University, as a M.Sc. research program; therefore, we wish to cordially acknowledge the authorities who granted this research work.

Authors' Contribution

Abnosi MH: Conceived of the study idea and were in charge of overall direction and planning, designed the experiments and wrote the manuscript. Sargolzaei J: carried out experiment and designed the primer. Maleklou M: carried out the experiments and analyzed the data.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

The study was approved by Arak Medical University ethics committee, Iran, and the approval number is IR.ARAKMU.REC.1397.110.

Funding/Support

The project was implemented with personal expenses and received no funding.

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