

The effects of zinc oxide nanoparticles on differentiation of human mesenchymal stem cells to osteoblast

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Abstract

Objective(s): The mesenchymal stem cells (MSCs) have been introduced as appropriate cells for tissue engineering and medical applications. Some studies have shown that topography of materials especially physical surface characteristics and particles size could enhance adhesion and proliferation of osteoblasts. In the present research, we studied the distinction effect of 30 and 60 µg/ml of zinc oxide (ZnO) on differentiation of human mesenchymal stem cells to osteoblast.

Materials and Methods: After the third passage, human bone marrow mesenchymal stem cells were exposed to 30 and 60 µg/ml of ZnO nanoparticles having a size of 30 nm. The control group has received no ZnO nanoparticles. On day 15 of incubation for monitoring the cellular differentiation, alizarin red staining and RT-PCR assays were performed to evaluate the level of osteopontin, osteocalcin and alkaline phosphatase genes expression.

Results: In the group receiving 30 µg/ml of ZnO nanoparticles, the expression of osteogenic markers such as alkaline phosphatase, osteocalcin and osteopontin genes were significantly higher than both control and the group receiving 60 µg/ml ZnO nanoparticle. These data also confirmed by alizarin red staining.

Conclusion: It seems the process of differentiation of MSCs affected by ZnO nanoparticles is dependent on dose as well as on the size of ZnO.

Keywords: Differentiation, Mesenchymal stem cell, Osteoblast, Zinc oxide

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Introduction

Nanotechnology and biomedical treatments using stem cells are among the latest conduits of biotechnological research (1).

The application of nanotechnology to stem-cell biology would be able to address the challenges of disease therapeutics (2). Nanostructures of ZnO are equally as important as carbon nanotubes and silicon nanowires for nanotechnology and have great potential applications in nano-electronics, opto-electronics, sensors, field emission, light-emitting diodes, photocatalysis, nano-generators, and nanopiezotronics (3). Adult or somatic stem cells are undifferentiated cells with renewal property.

Adult stem cells are derived from bone marrow, umbilical cord, adipose tissue and other sources (4). Bone marrow mesenchymal stem cells (MSCs) could be differentiating to osteoblasts, neuron, chondrocyte and other cell type. Osteoblast cells express osteocalcin, osteopontin and alkaline phosphatase genes (5). Some studies have proven that material topography especially physical surface properties and particle size can be increased viscosity and osteoblast proliferation (6).

For example, increased adhesion and proliferation of MSCs in culture media containing titanium nanoparticles with defined size has been observed (7). MSCs have been introduced to appropriate cells for medical applications. Some studies have shown that topography of materials especially physical surface characteristics and particles size could enhance adhesion and proliferation of osteoblasts. In the present research, we investigated the distinction effect of 30 and 60 $\mu\text{g/ml}$ of ZnO in differentiation of human mesenchymal stem cells to osteoblast.

Materials and Methods

Properties of ZnO nanoparticles

ZnO nanoparticles used in this study are dry and granulated powder in white color. Using X-ray diffraction, the approximate

size of these nanoparticles are 30 nm and their purity are designated 99%.

These particles have elongated morphology, 5.6 g/ml of net density and 35-50 m^2/g of specific surface area. All analyses have been carried out according to the standard testing procedures of University of Alicante and Lurederra Technology Centre which guarantee the accuracy of the results. These nanoparticles were produced in TECNAN Spanish Company which was purchased from Neutrino Company (Table 1 and Figure 1).

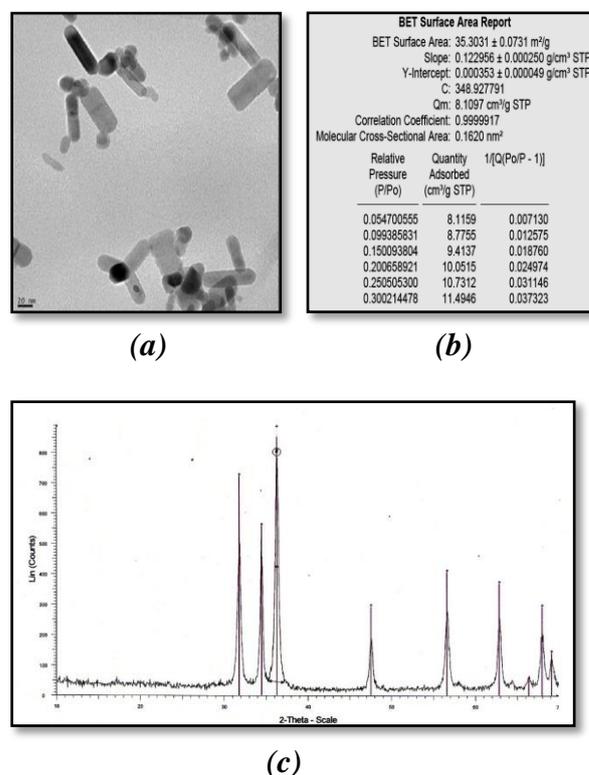


Figure 1. Properties of ZnO nanoparticles; (a) TEM image of ZnO nanoparticles. (b) results of the Specific Surface Area (SSA). (c) X-Ray diffractogram of nano zinc oxide (XRD).

Cell culture and exposure to zinc oxide nanoparticles

Bone marrow mesenchymal stem cells in the second passage were purchased from Royan Institute and were incubated in medium containing DMEM and 10% FBS (Gibco) and 1% penicillin-streptomycin at 5% CO₂ and 37°C. The medium changed every two days. The cells were subcultured into 24-well plates with a density of 5000 cells/well.

Table 1. The purity of zinc nano-Oxide (ICP-MS).

Components	Contents (%)
Al	0,0010010%
Fe	0,0030410%
Cu	0,0088810%
Si	0,0018520%
Ag	0,0000000%
Hg	0,0000000%
Sb	0,0000117%
Pb	0,0005711%
As	0,0012180%

Cells were incubated for 24 h in culture medium containing bone factors, ascorbic acid, beta-glycerophosphate and dexamethasone. Then, osteogenic medium together with ZnO nanoparticles were added to each well.

ZnO nanoparticles with 30 nm in size were suspended in osteogenic medium diluted to appropriate concentrations (30 and 60 µg/ml, Figure 2).

Quantitative analysis of gene expression for osteocalcin, alkaline phosphatase and osteopontin with RT-PCR

After 15 days of treatment with ZnO nanoparticles, total RNA of cells was extracted with 400 µL TRIzol (Invitrogen). The concentration of extracted RNA was determined by spectrophotometry.

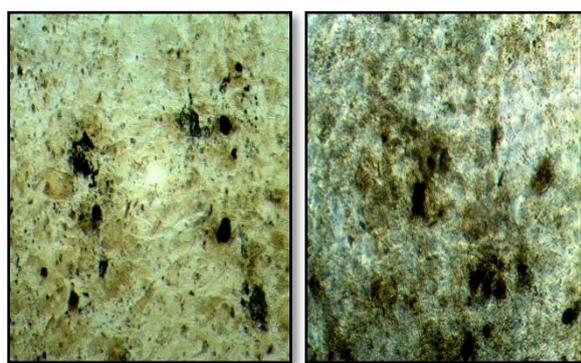


Figure 2. SEM mages of MSCs exposed to ZnO nanoparticles; (a) Sample with concentration 30 µg/ml of ZnO. (b) Sample with concentration 60 µg/ml of ZnO.

Then RNAs were reverse transcribed to complementary DNA (cDNA) using the Reverse Transcriptase 1st-Strand cDNA

Synthesis Kit (Takara Biotechnologies). The primer sequences specific for osteopontin, osteocalcin and alkaline phosphatase used for RT-PCR are listed in Table 2. The cDNA was subjected to RT-PCR with SYBR Green PCR master mix (Applied Biosystems) using the primers targeting the respective genes under the following conditions: 40 cycles at 95°C for 15s and then 60°C for 34s.

Mineralization measurements (Alizarin Red Test)

Cells were fixed with paraformaldehyde (1%, v/v) for ten min.

Then, cells were stained with solution of alizarin red (2%) for 45 min at pH 4.1.

At the end, cells were washed with sodium chloride solution (0.9%, w/v) twice.

Table 2. Sequences of primers and the products.

Gene name	Sequences of primers
Osteocalcin	F5' TGAGAGCCCTCACACTCCTC 3' R5' ACCTTTGCTGGACTCTGCAC 3'
Osteopontin	F5' CGCAGACCTGACATCCAGT 3' R5' GGCTGTCCCAATCAGAAGG 3'
Alkaline phosphatase	F5' TCACACTCCTCGCCCTATTGG 3' R5' GATGTGGTCAGCCAACTCGTCA 3'
GAPDH	F5' AGCCACATCGCTCAGACAC 3' R5' GCCCAATACGACCAAATCC 3'

Statistical survey

For data analysis software Image Tool was used (www.sourceforge.net).

Results

Alizarin red staining

The results of Alizarin red staining showed that the ossification process where observed reddish purple mass in some areas of culture indicated a positive trend of osteogenesis in human bone marrow mesenchymal stem cells.

The masses were observed in all groups, including controls, 30 and 60 µg/ml of nanoparticles (Figure 3) which were shown in figure 4.

Figure 4 shows that while the rate of osteogenesis in 30 µg/ml group increased significantly compared with other two

groups, the osteogenesis in the 60 µg/ml group was lower compared with other groups.

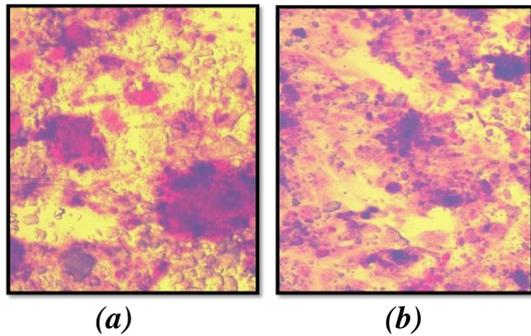


Figure 3. Light microscopic image of mineralization test; (a) cells exposed to 30 µg/ml of ZnO nanoparticles. (b) cells exposed to 60 µg/ml of ZnO nanoparticles.

RT-PCR reactions

Figures 5, 7 and 9 indicated the bands corresponding to the expression of osteocalcin, osteopontin and alkaline phosphatase in all three groups; control, 30 and 60 µ/ml of ZnO nanoparticles.

As it is evident in all three figures, expression of osteocalcin, osteopontin and alkaline phosphatase in control group was observed as a clear band. However, the width of the band containing 30 µg/ml of ZnO nanoparticles showed significant increase while it was significantly reduced in groups containing 60 µg/ml of ZnO nanoparticles. The results of the RT-PCR reactions are quantitative; it only showed the expression of osteocalcin, osteopontin and alkaline phosphatase in control group, and treatment groups contained 30 and 60 µg/ml of nanoparticles. In order to quantitatively evaluate the results and to infer the expression levels of genes in each of the categories with Image Tool software, the number of pixels in each of the bands was obtained and the charts were drawn by Excel Software. Figures 6, 8 and 10 show the density of the bands obtained from different groups related to each gene.

Survey of the results showed that there was significant difference between groups.

The results showed significant increase in expression of all three genes, osteocalcin, osteopontin and alkaline phosphatase in group containing 30 µg/ml of ZnO nanoparticles in comparison with both the control group without nanoparticles and the group containing 60 µg/ml of nanoparticles.

The results revealed that the expression of three genes in samples containing 60 µg/ml of ZnO nanoparticles were significantly reduced compared to other groups.

Discussion

Specific populations of stem cells within the bone marrow have the potential to differentiate into different types of cells (8).

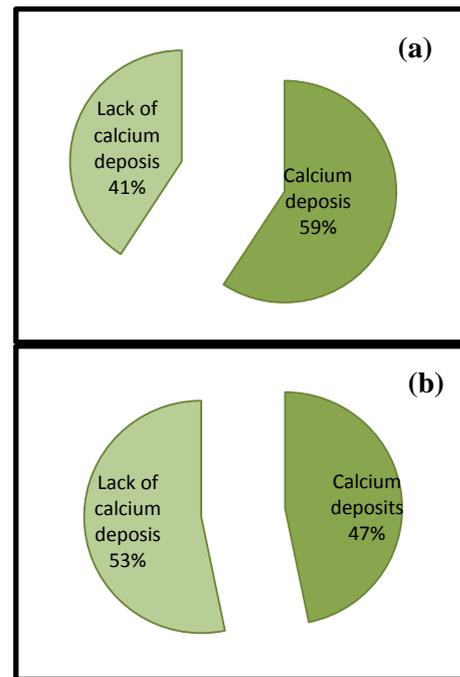


Figure 4. Represents the amount of calcium deposits in osteogenic medium; (a) samples containing 30 µg/ml of ZnO nanoparticles. (b) samples containing 60 µg/ml of ZnO nanoparticles.

MSCs could be differentiated into osteoblast in specific medium culture (9, 10).

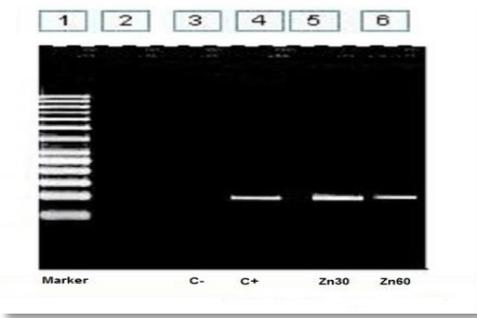


Figure 5. The expression gene of osteocalcin in 3 groups control (4), 60 µg/ml (5) and 30 µg/ml.

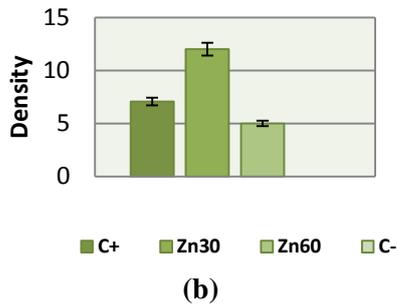
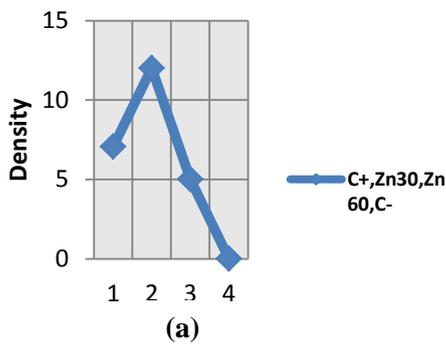


Figure 6. Expression levels of osteocalcin; (a) line chart. (b) column chart.

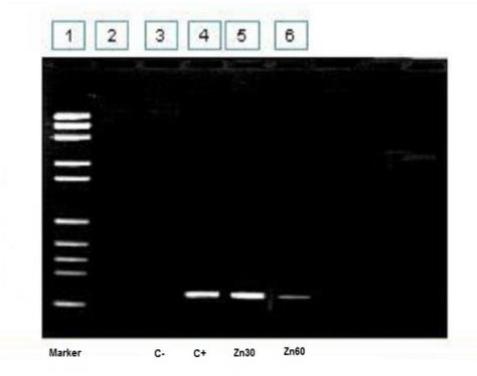


Figure 7. The expression gene of osteopontin in 3 groups control (4), 60 µg/ml (5) and 30 µg/ml (6).

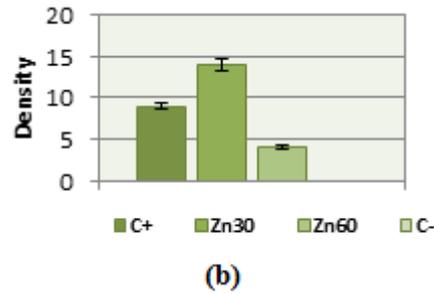
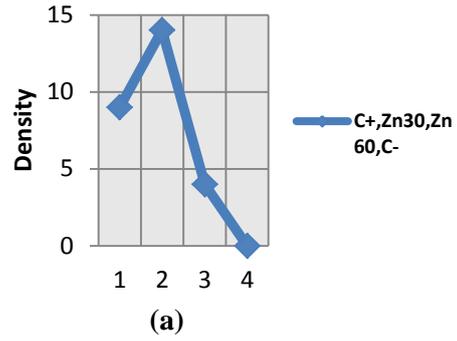


Figure 8. Expression levels of osteopontin; (a) line chart. (b) column chart.



Figure 9. The expression gene of alkaline phosphatase in 3 groups control (4), 60 µg/ml (5) and 30 µg/ml (6).

Topographic parameters such as geometry, size, distance and surface chemistry are important for direction of stem cell behavior. These parameters affect the adhesion, growth, proliferation and differentiation of stem cells (11).

Our results showed the MSCs cultured in osteogenic medium in both control and experimental group were differentiated into osteoblast lineage at day 15.

The data was demonstrated by alizarin red staining and RT-PCR assay of genes coding for osteopontin, osteocalcin and alkaline phosphatase.

Mesenchymal stem cell differentiation by zinc oxide nanoparticles

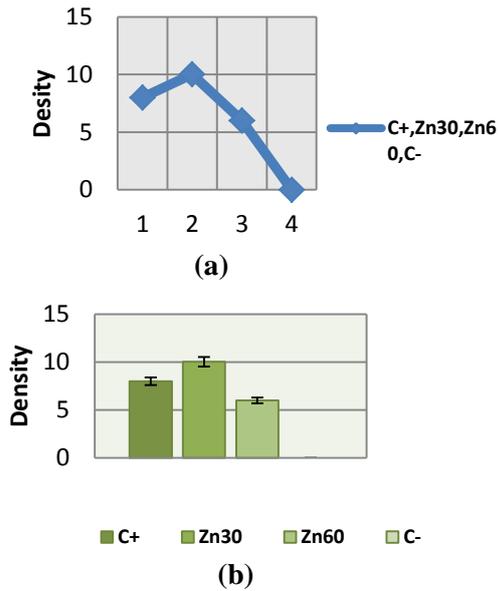


Figure 10. Expression levels of alkaline phosphatase; (a) line chart. (b) column chart.

Based on the findings of the present study, the osteocalcin, osteopontin and alkaline phosphatase expression in 30 $\mu\text{g/ml}$ ZnO group were significantly ($p < 0.05$) higher than those in 60 $\mu\text{g/ml}$ ZnO group after 15 day of incubation.

These differences imply that the higher doses of 30 $\mu\text{g/ml}$ ZnO increases ossification processes. Jones and his colleagues showed that ZnO particles with size 8 nm are more toxic than larger particles with size of 50-70 nm (12).

Hanley and colleagues have also found that there is an inverse relationship between nanoparticle size and cytotoxicity of nanoparticles in mammalian cells probably because of induction of reactive oxygen species (13). While Deng and his colleagues have shown that the toxic effects of ZnO nanoparticles on neural stem cells were dose-dependent (6).

It seems that process of ossification in MSCs affected by ZnO nanoparticles are dependent on dose in addition to size of ZnO. Indeed, when the size of nanoparticle is reduced, the ratio of surface atoms to interior atoms increases. In fixed size, lower doses of the nanoparticle showed less toxicity. Based on previous studies, the size of tested nanoparticle is one of the determinants of the toxicity of nano-

particles. Our data indicated that the level of toxicity in group treated with 30 $\mu\text{g/ml}$ of ZnO nanoparticles was less than that of treated with 60 $\mu\text{g/ml}$.

Significant differences between the group treated with 30 $\mu\text{g/ml}$ and the control group suggested that the dose of ZnO nanoparticles has a threshold on the differentiation of MSCs to osteoblast lineage.

Conclusion

It seems that the process of differentiation in MSCs affected by ZnO nanoparticles is dependent on dose in addition to size of ZnO. Also the dose used of ZnO nanoparticles has a threshold on the differentiation of MSCs to osteoblast lineage.

Acknowledgements

This study was supported by Iran Nanotechnology Initiative Council.

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