

Nano-TiO₂ particles inhibit the biological behavior and mineralization of bone marrow mesenchymal stem cells transfected with CXCR4

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Abstract. – OBJECTIVE: Bone marrow mesenchymal stem cells (BMSC) are widely used as experimental cells with potential differentiation function. Nanomaterials are currently a research hotspot. We assessed nano-TiO₂ particles' effect on the biological behavior and mineralization of CXCR4 transfected BMSCs.

PATIENTS AND METHODS: After transfection of BMSC with CXCR4, cells were divided into blank group (no transfection), control group (transfection with CXCR4) and observe group (transfection with CXCR4 containing nanoparticles). Then, cell proliferation and ALP staining were measured along with analysis of Runx2 and BGP level by Western blot or RT-PCR and mineralization detection.

RESULTS: With increased culture time, the observed fractionation on day 14 showed significantly reduced activity; 3 mn nano-TiO₂ particles significantly inhibited cell proliferation and bone formation after CXCR4 transfection with an inhibitory effect on the osteogenic ability of CXCR4-transfected BMSC cells in a time-dependent manner. The longer the culture time, the more significantly inhibitory effect; 3 mn nano-TiO₂ particles can inhibit the mineralization of BMSCs after transfection of CXCR4 to a certain extent.

CONCLUSIONS: TiO₂ nanoparticles have an inhibitory effect on the biological behavior and mineralization of BMSC cells transfected with CXCR4. The longer the culture time, the greater the inhibitory effect on osteogenic differentiation of BMSC cells transfected with CXCR4.

Key Words:

Nano-TiO₂ particles, CXCR4 transfection, Mesenchymal stem cells, Mineralization.

Introduction

Mesenchymal stem cells (MSCs) have a wide range of sources, mainly in bone marrow, skeletal muscle, umbilical cord blood and other tissues. They are widely used for treating several diseases. Under certain experimental conditions, they can effectively differentiate into osteoblasts, immune cells, muscle cells and fat cells etc¹. It can be extracted from animal embryos, baby umbilical cord blood, etc., and is a very mature adult stem cell for experimental studies. In the body's inflammatory response, CXC receptor 4 (CXCR4) can specifically bind inflammatory chemokines and produce a series of corresponding reactions; and cause a large number of inflammatory cells to aggregate and activate. When BMSCs with positive expression of CXCR4 on the cell membrane surface are attracted, they can migrate to the damaged site, differentiate into various cells, and participate in bone defect repair². However, in clinical applications, if considering the treatment of diseases, it is necessary to face the combined use of drugs and special materials to enhance the effect, but there are few related studies. Studies have confirmed that nano-TiO₂ particles are a good biocompatible material. In the field of medicine, TiO₂ nanoparticles can cause tumor cell damage and can enhance the effect of particles on penetrating cell membranes, and cause membrane rupture. After TiO₂ nanoparticles enter the body, a series of biochemical reactions

can occur, thereby affecting the normal function of the body. After it acts on the body, it can cause cytotoxicity, which can lead to disorders of cell survival, proliferation and function to varying degrees, and trigger a variety of adverse reactions. The main reason may be that the substance disturbs the concentration balance of ions, proteins and other essential substances inside and outside the cell³. This article explores the inhibitory effects of nano-TiO₂ particles on biological behavior and mineralization in the application of CXCR4-transfected BMSCs, and provides a theoretical basis for the safety evaluation of nano-biomaterials in the human body⁴.

Materials and Methods

Cell Transfection

Cell transfection was performed as described previously^{5,6}. 0.4 mL CXCR4 was used to transfect cells using Lipofectamine 2000.

Cell Group

BMSCs were divided into blank group (no transfection), control group (BMSC cells transfected with CXCR4) and observation group (transfected with CXCR4 containing nanoparticles). TiO₂ nanoparticles with a uniform tube diameter of 3 nm was added to the observation group and the blank group to co-culture with BMSC cells. During the experiment, three mediums with exactly the same conditions were selected, and appropriate doses of penicillin (100 U/mL), 10% fetal bovine serum, and streptomycin (100 µg/mL) were sequentially added. Then, dexamethasone, sodium glycerophosphate, ascorbic acid and other ingredients to each medium was added to induce BMSCs differentiation.

Cellular ALP Staining

On the 7th and 14th day after culture, some BMSC cells were washed twice with PBS solution, fixed in 4% paraformaldehyde solution for 30 min followed by washing and addition of 200 µL dye solution for 20 minutes under dark. Then, NBT/BCIP for light-proof reaction (Merck Millipore, Burlington, MA, USA) was performed followed by observation under an optical microscope.

Cell Proliferation Detection

After culture for 24 hours, 10% MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added

to cells for 4 hours followed by addition of 500 µl DMSO and subsequent measurement of absorbance (A) at 490 nm.

Western Blot

On the 14th day, BMSCs were collected to extract cell protein using RIPA lysis buffer which was then quantified by BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 50 µg protein was separated on SDS-PAGE for Western blot analysis by blotting the PVDF membrane with LPL antibody (Cell Signaling Technology, Waltham, MA, USA) and HRP-labelled mouse anti-rabbit secondary antibody (Cell Signaling Technology, Waltham, MA, USA).

RT-PCR

Total RNA was isolated and synthesized into cDNA followed by qPCR analysis of the expression of Runx2, BGP, and CXCR4 mRNA. GAPDH was used as internal control. The primer sequences were: Runx2-F-CCTGAAGTCTGCACCAAGTC and Runx2-R-GAGGTGGCAGTGTCATCATC; BGP-F-CAGACAAGTCCCACACAGCAGC and BGP-R-TGTTCACTACCTTATTGCCCTCC; CXCR4-F-TCAGTGGCTGACCTCCTCTT and CXCR4-R-CTTGGCCTTTGACTGTTGGT; GAPDH-F-TTCCTTCTGGGTATGGAAT and GAPDH-R-GAGCAATGATCTTGATCTTC.

Mineralization Test Detection

Culture medium was replaced for 2 days/time. The entire culture time was 21 days and the sample was subjected to the mineralization test. After removal of the culture medium, sample was fixed in 2.5% glutaraldehyde for 30 min and then stained with the 40 mM Alizarin Red solution for 20 min followed by washing and left for 30 min. After heating the cells for 10 min at 85°C, the cells were centrifuged to remove the supernatant followed by addition of a solution of 10% NH₄OH to the supernatant. Then, the optical density value was monitored at 405 nm using a microplate reader.

Statistical Analysis

SPSS 26.0 software was utilized for analyzing data (IBM Corp., Armonk, NY, USA). Counting data was expressed as % and assessed by Chi-square test. Measurement data were shown as mean ± SD and assessed by *t*-test. *p* < 0.05 indicates a significance.

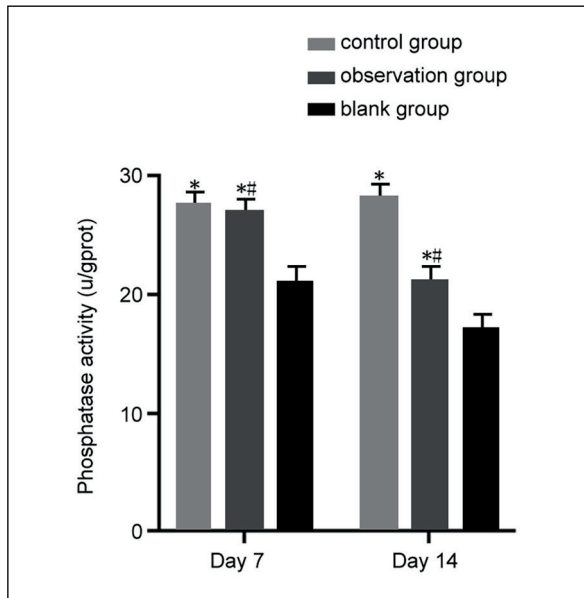


Figure 1. Phosphatase activity. *Compared with blank group at the same time point, $p < 0.05$; #Compared with control group at the same time point, $p < 0.05$.

Results

As the Cell Culture Time Is Prolonged, the Observed Fractionation 14d Shows Significantly Reduced Activity

After 7d and 14d, the ALP activity was significantly different ($p < 0.05$). Further analysis showed that ALP activity of observation group was significantly lower than that of the control

group ($p < 0.05$); after 7d and 14d culture, ALP activity of observation group was significantly lower than that on 7th day. Compared with control group, ALP activity was significantly reduced, indicating the inhibitory effect of 3 nm nano-TiO₂ particles on ALP activity (Figure 1).

3 nm Nano-TiO₂ Particles Inhibit Cell Proliferation After Transfection of CXCR4

There was no significant difference in cell viability between observation group and control group and blank group at 1 day after inoculation (Figure 2). In order to further detect the number and activity of cell proliferation, we performed the MTT experiment. On day 1, the difference between three groups was not significant ($p > 0.05$); on the 2 and 3 days, cell proliferation of observation group was significantly inhibited with reduced cell activity ($p < 0.05$), indicating that the observation group cells present significant cytotoxicity (Figure 2).

3 nm Nano-TiO₂ Particles Inhibit Bone Formation in BMSC Cells Transfected With CXCR4

After 7d and 14d of culture, the differences in the expression of Runx2 and BGP of BMSC cells in each group were significant. The Runx2 and BGP level in observation group were significantly lower than those in control group ($p < 0.05$); after 7d and 14d of culture, compared with blank group, Runx2 and BGP expression was significantly reduced in observation and control group ($p < 0.05$).

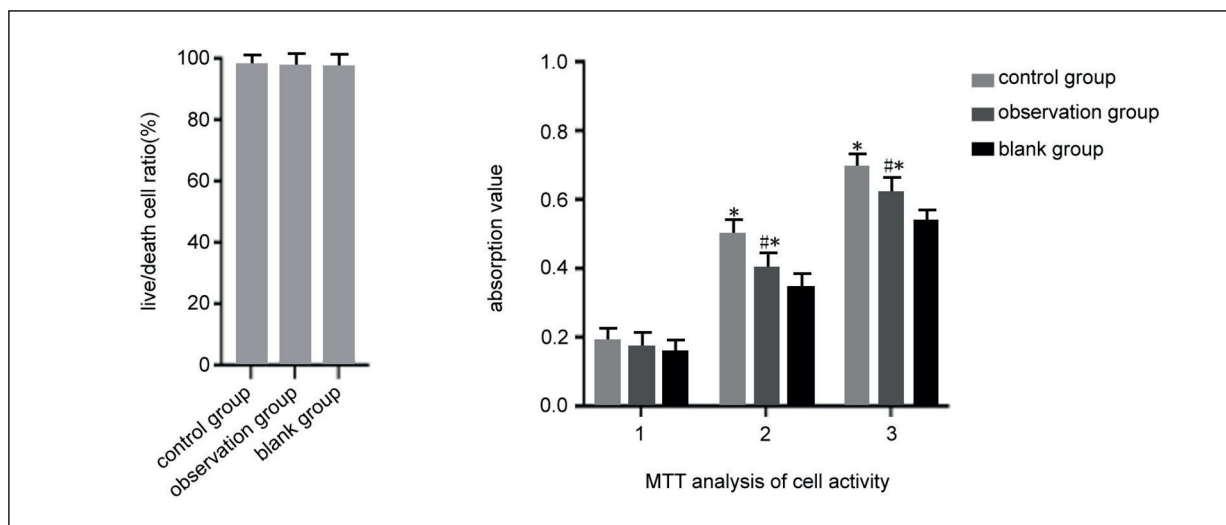


Figure 2. Live and dead cell experiment (fluorescence microscope $\times 200$); MTT method for determination of cytotoxicity. *Compared with blank group, $p < 0.05$; #Compared with control group, $p < 0.05$.

We found that the LPL of observation group was significantly higher than control group; while blank group showed significantly lower LPL than other groups ($p < 0.05$) (Figure 3).

3 mn Nano-TiO₂ Particles Have an Inhibitory Effect on the Osteogenic Ability of CXCR4 BMSCs Cells

After 7d and 14d of culture, the Runx2 mRNA and BGP mRNA of observation group were lower than those of control group ($p < 0.05$); after 7d and 14d of culture, compared with blank group, Runx2 mRNA and BGP mRNA of observation and control group was significantly decreased ($p < 0.05$) (Figure 4).

3 mn Nano-TiO₂ Particles Can Inhibit the Mineralization of BMSCs Transfected with CXCR4

After 21 days of cultivation, the mineralization test results showed that control group's mineralization ability and its outstanding ability were significantly better than the other groups, with observation group showing significantly higher mineralization than blank group ($p < 0.05$) (Figure 5).

Discussion

The high expression of CXCR4 can improve the migration ability of MSCs to a certain extent,

but has no significant effect on differentiation ability. Its gene transfection has no significant effect on MSCs proliferation ability^{7,8}, and it can participate in the growth of BMSCs to a certain extent. Studies have found that in the BSMC transport system of CXCR4 encapsulated by TiO₂ nanoparticles, TiO₂ nanoparticles can not only bind BSMCs, but also play a protective effect to ensure that the CXCR4 receptor can smoothly reach the target site and bind to the target regulatory gene. However, the size of nanoparticles has a certain impact on the biological processes⁹, but whether they have good biological safety is worth exploring. At present, we co-cultured nano-TiO₂ particles with a diameter of 3 nm with CXCR4-transfected BMSCs. Nano-TiO₂ particles are wrapped on the surface of BMSC cells, which can reduce the enzymatic hydrolysis and corrosion of intracellular enzymes and acids¹⁰. During the induction of osteogenic differentiation of BMSCs, the degree of osteogenic differentiation has a certain relationship with nano-TiO₂ particles.

After TiO₂ is added, it will cover the cell surface, but if it enters the cell, it will cause corresponding cytotoxicity, and the cell will be further swallowed due to the entry of nano-TiO₂ particles, changing the acidity of cell, and then the cell viability and proliferation are inhibited. The results of the study showed that 3 mn nano-TiO₂ particles had inhibitory effects on BMSCs while the observation group showed certain cytotoxicity. 3 mn nano-TiO₂ particles inhibited

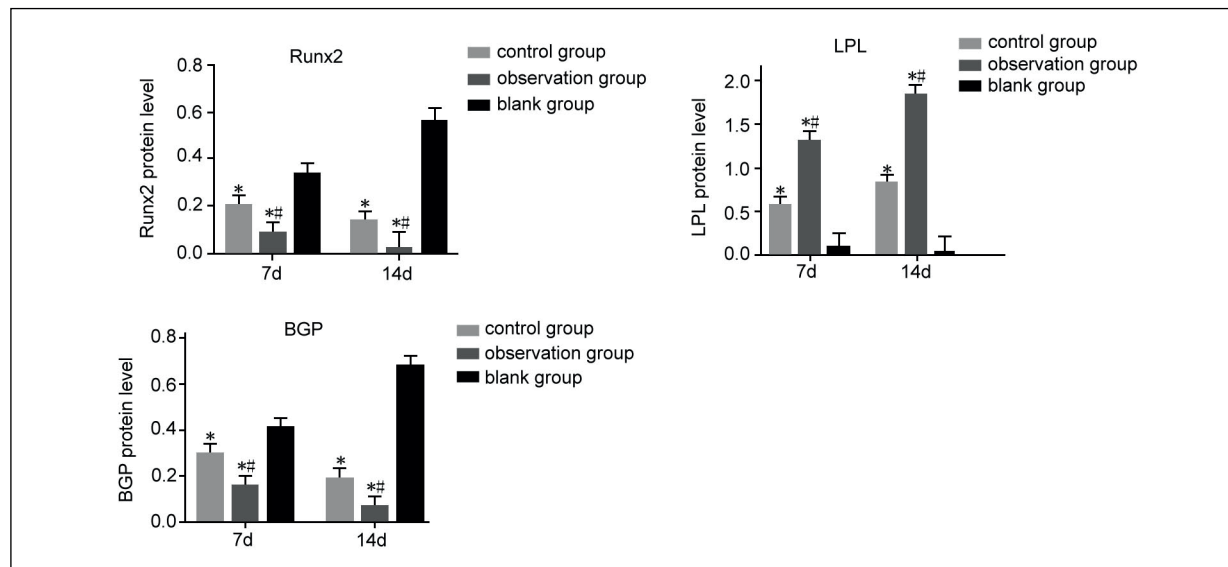


Figure 3. LPL, Runx2, BGP protein expression. *Compared with blank group, $p < 0.05$; #Compared with control group, $p < 0.05$.

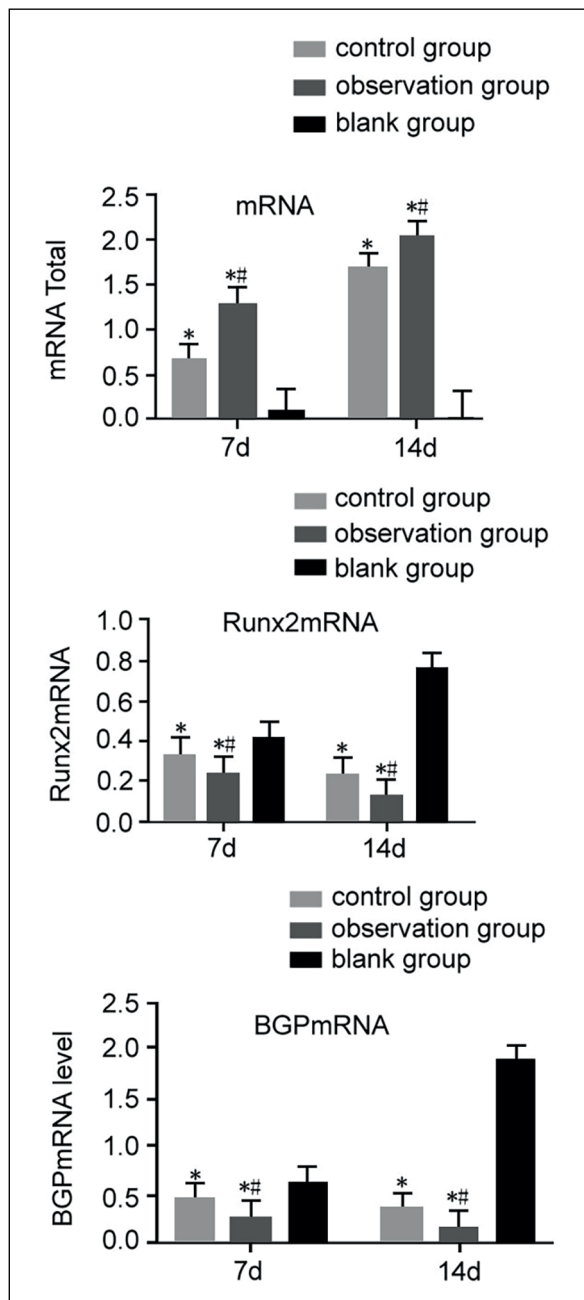


Figure 4. The content of mRNA, Runx2, and BGP. *Compared with blank group, $p < 0.05$; #Compared with control group, $p < 0.05$.

the proliferation of BMSCs transfected with CX-CR4. Related studies confirmed that TiO₂ can cause apoptosis to a certain extent. Apoptosis can activate Bax/Bcl2 and other apoptotic pathways mediated by mitochondria in the cell, and the experimental cells show decreased activity and even a large number of deaths. In order to verify this, in this study, we confirmed through cell ac-

tivity experiments that TiOZ NPs have a certain degree of cytotoxicity which is time-dependent. The longer the time, the more significant the toxic effect. BMSCs proliferation and mineralization are significantly inhibited, and the cytotoxic effects of TiOZ NPs have also been observed in previous studies. LPL, as a glycoprotein that can catalyze the decomposition of TG to provide fatty acids and monoglycerides for tissue oxidation, is mainly synthesized and secreted by bone cells, breast cells and other parenchymal cells. The expression of this substance is correlated with the degree of BMSCs differentiation. The higher the degree of BMSCs differentiation, the higher the secretion of LPL. In the research process, it can be seen that nano-TiO₂ particles have an inhibitory effect on cell differentiation.

Knani et al¹¹ showed that ALP, Runx2 and BGP all appear to have increased expression in the early stage of osteogenesis. Runx2 is the earliest and specific marker in the process of bone formation¹². It is the upstream gene of early osteogenic genes, such as ALP and BGP. To a certain extent, its expression indicates that osteoblasts begin to differentiate, so it is reliable as an early biomarker of bone formation¹³⁻¹⁵. BGP will be secreted in large quantities in the later stage of bone differentiation, because it can inhibit the formation of hydroxyapatite crystals that are easy to appear at this stage, and prevent bone nodules formation,

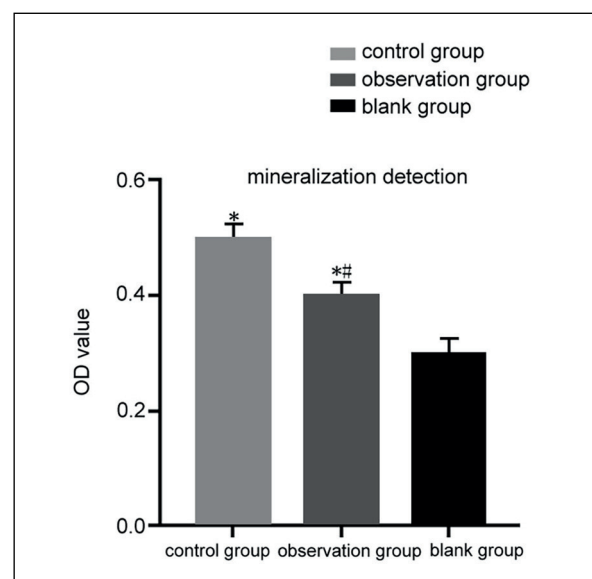


Figure 5. OD value of mineralization detection. *Compared with blank group, $p < 0.05$; #Compared with control group, $p < 0.05$.

so it can be used as an accurate indicator of the activity of bone cells after differentiation^{15,16}. Our results detected that under the condition of 3 nm nano-TiO₂ particles, the osteogenic ability was inhibited to a certain extent. In addition, under the initial conditions of cell differentiation, the influence of 3 nm nano-TiO₂ particles did not appear. As the cell differentiation becomes active and differentiation is vigorous, the amount of 3 nm nano-TiO₂ particles entering the cells gradually accumulates to a certain extent. The specific surface area is becoming larger and larger. With the active differentiation, the 3 nm nano-TiO₂ particles have more frequent contact with the intracellular organs than in the initial stage of differentiation, and the contact area is larger, which will eventually cause inhibited ability of intracellular aggregation. Therefore, its inhibitory effect is negatively related to time. The longer the incubation time, the more prominent the inhibitory effect. At the same time, many scholars at home and abroad have found that nanomaterials have different degrees of inhibitory effects on osteoblast function. Among them, nano-silver can produce a certain amount of silver ions at subtoxic doses, which significantly damages the differentiation potential of stem cells¹⁶⁻¹⁸, the most influential is the osteogenic differentiation ability of BMSCs because of its ability to inhibit the formation of hydroxyapatite crystals that are easy to appear at this stage^{18,19}. In order to remove the 3 nm nano-TiO₂ particles that enter the cell, the cell will produce a large amount of particle aggregation to effectively deposit it. In the late stage of differentiation, cell mineralization will be inhibited to a certain extent. The OD value of our mineralization test in the research indicates that the osteogenic differentiation of BMSC cells in the observation group was low, and the inhibitory effect of nanoparticles on the osteogenic differentiation of BSMC could be seen^{20,21}.

Conclusions

Briefly, our research for the first time demonstrates that TiO₂ nanoparticles have an inhibitory effect on the biological behavior and mineralization of BMSC cells transfected with CXCR4, which is the novelty of our study.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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