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硝酸锶对原代培养的小鼠成骨细胞增殖、分化和矿化功能的影响

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摘要:采用噻唑蓝(MTT)法、碱性磷酸酶(ALP)比活性测定、油红 O 染色、Ⅰ型胶原测定以及矿化结节染色及定量分析等方法,研究了不同浓度的硝酸锶对原代培养的成骨细胞增殖、分化、矿化功能以及横向分化为脂肪细胞的影响。结果表明:硝酸锶对成骨细胞增殖、分化、矿化功能以及横向分化为脂肪细胞的影响与作用浓度和时间密切相关,但没有呈现出剂量依赖性。结果提示,硝酸锶对骨代谢的影响是复杂的,其具有保护还是损害作用取决于作用浓度和时间,而且它们是影响硝酸锶生物效应(从损伤到保护)转变的关键因素。

关键词:硝酸锶;成骨细胞;增殖;分化;矿化
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Effects of Strontium Nitrate on the Proliferation, Differentiation and Mineralization Function of Primary Mouse Osteoblasts *in vitro*

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Abstract: The effect of $Sr(NO_3)_2$ concentration on proliferation, differentiation and mieralizerzation function of primary osteblasts (OBs) *in vitro* was evaluated by using the 3-(4,5-dimethyl)-2,5-diphenyl tetra- zolium bromide (MTT)method, alkaline phosphatase (ALP) activity, oil red assays, quantitative analysis of promotion rate for type Ⅰ collagen production and Alizarin Red Stain(ARS). The results indicate that the effect of $Sr(NO_3)_2$ on the proliferation, differentiation, mieralizerzation function and adipocytic transdifferentiation of OBs depends on the concentration and incubation time, however, the effect is independent of dosage. This suggests the effect of $Sr(NO_3)_2$ on bone metabolism is a complex issue. Whether the effect of $Sr(NO_3)_2$ is damaging or protection is dependent on two key factors, i.e., the concentration and culture time.

Key words: strontium nitrate; osteoblasts; proliferation; differentiation; mineralization

0 Introduction

Osteoporosis is a systemic skeletal disease characterised by low bone mass and bone matrix deterioration, leading to bone fragility and an increased risk of fracture[1]. As the general population is ageing, osteoporosis is becoming more prevalent, not just in China, but worldwide. It has been recognized as a major public health problem and much attention has been focused on searching powerful medicine and therapeutic strategy[2-3].

The rationale for prevention and treatment of osteoporosis is directed along two basic approaches, namely agents preventing bone resorption and those

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stimulating bone formation[4-5]. In addition, the most frequently used anti-osteoporosis drugs are developed in affluent countries and the costs are too high to benefit a large population in developing or even developed countries for prevention and treatment of osteoporosis. Thus, alternative treatment or prevention regimes for osteoporosis are urgently needed.

A great deal of evidence indicated that certain essential trace elements were reported to be involved in the pathogenesis of osteoporosis[7]. Clinical trails have shown that zinc supplementation inhibits postmenopausal bone loss[8]. Both strontium and calcium belong to the alkaline earth elements, and resemble each other in that >99% of the total amount in the body is localized in bone[9]. A beneficial effect of low doses of stable strontium in the treatment of osteoporosis was reported almost half a century ago[10-11]. Zhang et al. investigated the effect of strontium chloride on the restoration of bone loss in female rats with osteoporosis induced by ovariectomy[12]. The results indicated strontium salt could increase bone mass of rats models of osteoporosis. Strontium ranelate is a newly developed drug that has been shown to be effective in reducing the risk of vertebral and hip fractures in postmenopausal women with osteoporosis. In contrast to other available treatments for osteoporosis, strontium ranelate induced opposite effects on bone resorption and formation. This dual mode of action was demonstrated in pharmacological studies in animals[13]. Until now, the effect of strontium nitrate on the proliferation, differentiation and mineralization function of primary mouse OBs *in vitro* has not been reported. In order to elucidate the effect of strontium nitrate on bone metabolism at cell level, the effects of strontium nitrate on the proliferation, differentiation and mineralization function of primary OBs in vitro were studied in this paper.

1 Experimental

1.1 Materials

Kun ming (KM) mice were obtained from Experimental Animal Center of Hebei Medical University. Dulbecco′s modified Eagle′s medium (DMEM) and fetal bovine serum (FBS) were from Gibco. Benzylpenicillin, streptomycin, MTT, β-glycerophosphate, trypsin, dexamethasone, ascorbic acid, insulin, ARS, oil red O stain and cetylpyridium chloride were obtained from Sigma. Sirius Red was purchased from Solarbio. An ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China), and a micro-protein assay kit was from Beyotime Biotechnology (Haimen, China). $Sr(NO_3)_2$ (Purity > 99.9%, AR) were purchased from Fuchen Chemical Reagent Factory (Tianjin, China).

1.2 Methods

1.2.1 Particle size measurement

A series of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1} dispersion of $Sr(NO_3)_2$ was freshly prepared in DMEM supplemented with 10% FBS or without 10% FBS and the mixture was vortexed for 5 s and then subjected to sonication in a water bath for 60 s at 120 W at room temperature to reduce agglomeration. The size distribution of the particles was measured by a Dynamic Light Scattering (DLS) instrument (Beckman Coulter Inc, USA) with its software. The mean particle diameter was calculated on the basis of the intensity or the number distribution[14].

1.2.2 Isolation and culture of primary OBs

The mouse OBs were isolated mechanically from newborn mouse skulls by a modification of the method previously reported[15]. Briefly, skulls were dissected from KM mice, and the bone was cut into approximately 1~2 mm^2 pieces and digested with trypsin (2.5 g·L^{-1}) for 30 min and the digestion was discarded. Then the bone was digested with collagenase Ⅱ (1.0 g·L^{-1}) twice with 1 h for each, and the cells were collected and cultured in a culture flask. After being incubated overnight in a 5% CO_2 humidified incubator at 37 ℃, following which the used medium was changed. The medium was changed every 3 d in all experiments.

1.2.3 Cell viability assay

The protocol described by Mosmann was followed with some modifications[16]. Briefly, OBs (2×10^4 cells per well) were plated in 96-well culture plates and cultured overnight at 37 ℃, in a 5% CO_2 humidified incubator. Strontium nitrate was added at a final concentration of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1},

respectively. Control wells were prepared by addition of OBs and physiological saline. Wells containing DMEM and physiological saline without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) were used as positive control. Upon completion of the incubation, MTT dye solution (20 μL, 5 mg·mL^{-1}) was added. After 4 h incubation, the supernatant was removed and DMSO (100 μL) was added. The optical density (OD) was measured on a microplate spectrophotometer (MD VersaMax, USA) at a wavelength of 570 nm. The cell viability (%) was calculated according to the formula: $(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})\times100$.

1.2.4 Measurement of ALP activity

The OBs (2×10^4 cells per well) were plated in 48-well culture plates, and treated with strontium nitrate at a final concentration of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, respectively. Control wells were prepared by addition of OBs and physiological saline. Wells containing DMEM and physiological saline without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) were used as positive control. Upon completion of the incubation, the plates were washed thrice with ice-cold PBS and lysed by two cycles of freezing and thawing. Aliquots of supernatants were subjected to ALP activity and protein measurement by an ALP kit and a micro-protein assay kit, respectively. All results were normalized by protein content. The differentiation promotion rate (%) was calculated accor-ding to the formula: $(\text{ALP activity}_{treated}-\text{ALP activity}_{blank})/(\text{ALP activity}_{control}-\text{ALP activity}_{blank})\times100$.

1.2.5 Mineralized matrix formation assay

The OBs (2×10^4 cells per well) were plated in 24-well culture plates and cultured overnight at 37 ℃, in a 5% CO_2 humidified incubator. The medium was then changed to differentiation medium containing 10 mmol·L^{-1} β-glycerophosphate and 50 μg·mL^{-1} ascorbic acid, strontium nitrate was added at a final concentration of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, respectively. Control wells were prepared by addition of OBs and physiological saline. Wells containing DMEM and physiological saline without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) were used as positive control. Upon completion of the incubation, the formation of mineralized matrix nodules was determined by ARS stain. Briefly, Cell monolayers were fixed in 95% ethanol for 10 min at room temperature, then washed by PBS twice and stained with 0.1% ARS for 30 min at room temperature. Quantitation of ARS staining was performed by elution with 10% (*w*/*v*) cetylpyridium chloride for 10 min at room temperature and the OD was measured at 570 nm[17]. The mineralized function promotion rate (%) was calculated according to the formula: $(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})\times100$.

1.2.6 Collagen production analysis

Collagen production analysis was performed as previously described with some modifications[18]. Briefly, OBs (2×10^4 cells per well) were plated in 48-well culture plates and cultured overnight at 37 ℃, in a 5% CO_2 humidified incubator. Strontium nitrate was added at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}. Control wells were prepared by addition of OBs and physiological saline. Wells containing DMEM and physiological saline without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) were used as positive control. Upon completion of the incubation, the plates were washed thrice with ice-cold PBS and fixed for 1 h with 500 μL fixation solution of 10% formaldehyde and then washed with distilled water. 200 μL of dye solution (30 mg Sirius Red in 30 mL saturated solution of picric acid) per well was added with mild agitation for 1 h. After that, they were washed with 0.01 mol·L^{-1} HCl to remove the excess of Sirius Red. The dye fixed collagen was observed and extracted with 200 μL 0.1 mol·L^{-1} NaOH solution per well. The OD was measured at a wavelength of 550 nm. All results were normalized by protein content. The promotion rate for type Ⅰ collagen production (%) was calculated according to the formula: $(\text{type Ⅰ collagen}_{treated}-\text{type Ⅰ collagen}_{blank})/(\text{type Ⅰ collagen}_{control}-\text{type Ⅰ collagen}_{blank})\times100$.

1.2.7 Oil red O stain and measurement

The OBs (2×10^4 cells per well) were plated in 48-well culture plates, after being induced by adipogenic supplement (10 μg·mL^{-1} insulin, 10^{-7} mol·L^{-1}

dexthamethone) and treated with strontium nitrate at a final concentration of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, respectively. Control wells were prepared by addition of OBs and physiological saline. Wells containing DMEM and physiological saline without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) were used as positive control. Upon comp-letion of the incubation, fat droplets within differen-tiated adipocytes from OBs were stained according to the literature[19]. Cells were fixed in 4% formaldehyde, washed in water and stained with a 0.6% (*w*/*v*) oil red O solution (60% isopropanol, 40% water) for 15 min at room temperature. For quantification, cells were washed extensively with water to remove unbound dye, then isopropyl alcohol was added to the culture plates. After 5 min, the OD of the extract was measured at a wavelength 510 nm. The adipocytic transdifferentiation promoting rate (%) was calculated according to the formula: $(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})\times100$.

1.2.8 Statistical analysis

Data were collected from at least four separate experiments. The results were expressed as means ± standard deviation (SD). The statistical differences were analyzed using SPSS′ *t*-test. *P* values less than 0.05 were considered to indicate statistical differences.

2 Results

2.1 Particle size analysis

As shown in Fig.1, no large granule was produced in DMEM with 10% FBS, moreover, no large granule was found in DMEM with 10% FBS and 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, or 1×10^{-5} mol·L^{-1} strontium nitrate. For size and number distribution of the particles, there was no significant change with increasing concentrations, the size of the particles was evaluated to be (3.5±1.2) nm (>90%).

2.2 Effect of strontium nitrate on the viability of OBs

As shown in Fig.2, for 24 h, strontium nitrate (1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}) significicantly increased the viability of OBs, exhibited the strongest effect at a concentration of 1×10^{-9} mol·L^{-1}.

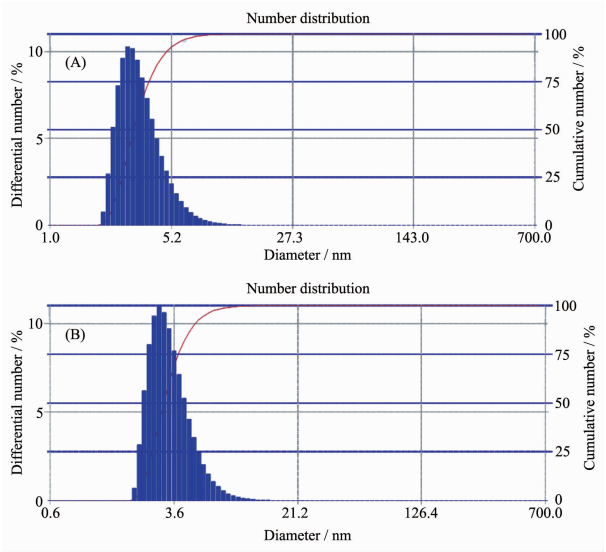
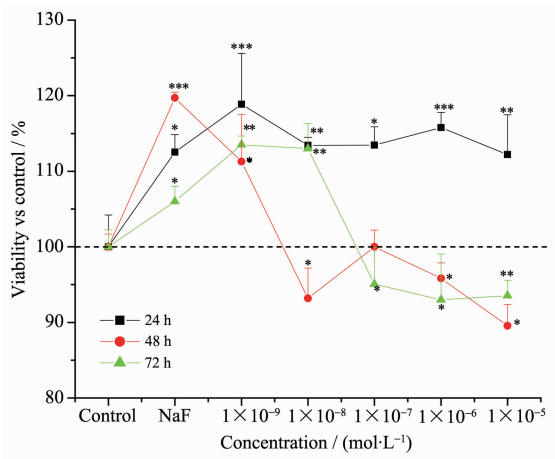


Fig.1 Size distribution of the particles by DLS analysis (A) DMEM with 10% FBS; (B) DMEM with 10% FBS and 1×10^{-5} mol·L^{-1} strontium nitrate



*P<0.05, **P<0.01, ***P<0.001 vs control group, n=6

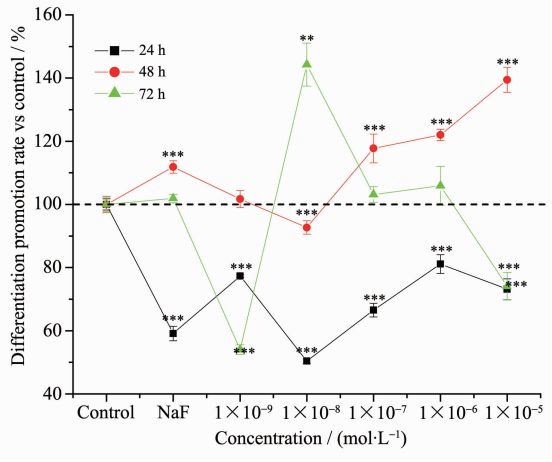
Fig.2 Effect of strontium nitrate on the viability of OBs

Strontium nitrate increased the viability of OBs at the lowest concentration of 1×10^{-9} mol·L^{-1}, decreased the viability of OBs at concentrations of 1×10^{-8}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, but had no effect at a concentration of 1×10^{-7} mol·L^{-1} for 48 h. For 72 h, strontium nitrate increased the viability of OBs at concentrations of 1×10^{-9} and 1×10^{-8} mol·L^{-1}, but turned to decrease the viability of OBs with the increasing concentrations. Strontium nitrate appeared to exhibit greatest promotion effects on the viability of OBs for 24 h.

2.3 Effect of strontium nitrate on the differentiation of OBs

As shown in Fig.3, strontium nitrate inhibited the

differentiation of OBs at all tested concentrations for 24 h. For 48 h, strontium nitrate had no effect on the differentiation of OBs at the lowest concentration of 1×10^{-9} mol·L^{-1}, inhibited the differentiation of OBs at a concentration of 1×10^{-8} mol·L^{-1}, but turned to promote the differentiation of OBs at other concentrations. For 72 h, the effect of strontium nitrate on the differentiation of OBs was complicated. Strontium nitrate inhibited the differentiation of OBs at the lowest concentration of 1×10^{-9} mol·L^{-1}, promoted the differentiation of OBs at a concentration of 1×10^{-8} mol·L^{-1}, but turned to inhibit the differentiation of OBs with increasing concentrations.

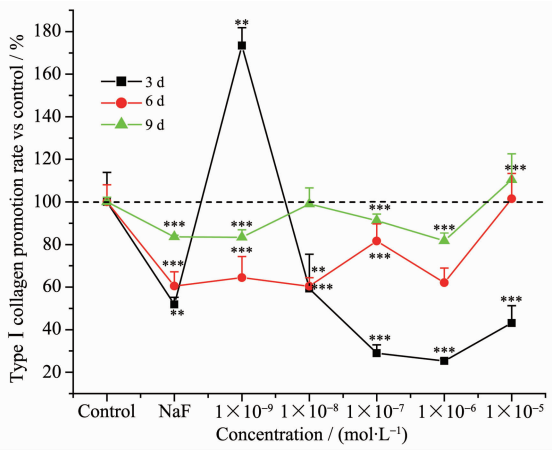


*P<0.05, **P<0.01, ***P<0.001 vs control group, n=6

Fig.3 Effect of strontium nitrate on the differentiation of OBs

2.4 Effect of strontium nitrate on the synthesis of type Ⅰ collagen of OBs

As shown in Fig.4, for 3 d, strontium nitrate promoted the synthesis of type Ⅰ collagen of OBs at the lowest concentration of 1×10^{-9} mol·L^{-1}, but turned to inhibit the synthesis of type Ⅰ collagen of OBs at other concentrations. Strontium nitrate inhibited the synthesis of type Ⅰ collagen of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol·L^{-1}, but turned to have no effect at concentrations of 1×10^{-6} and 1×10^{-5} mol·L^{-1} for 6 d. For 9 d, strontium nitrate promoted the synthesis of type Ⅰ collagen of OBs at the highest concentration of 1×10^{-5} mol·L^{-1}, but inhibited the synthesis of type Ⅰ collagen of OBs at other concentrations.

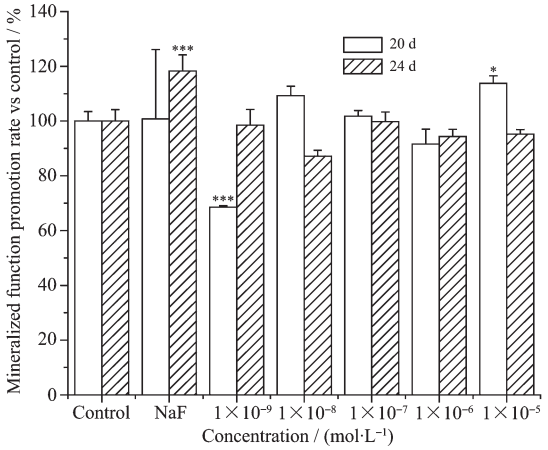


*P<0.05, **P<0.01, ***P<0.001 vs control group, n=6

Fig.4 Effect of strontium nitrate on the synthesis of type Ⅰ collagen of OBs

2.5 Effect of strontium nitrate on the formation of mineralized matrix nodules

As shown in Fig.5, strontium nitrate inhibited the formation of mineralized matrix nodules of OBs at a concentration of 1×10^{-9} mol·L^{-1}, promoted the formation of mineralized matrix nodules of OBs at a concentration of 1×10^{-5}, but had no effect at other concentrations for 20 d. For 24 d, strontium nitrate had no effect on the formation of mineralized matrix nodules of OBs. The morphologic observation was in accordance with the results (Fig.6).

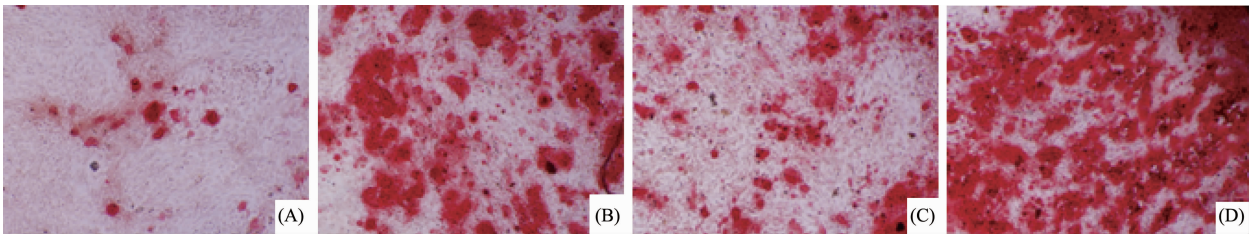


*P<0.05, ***P<0.001 vs control group, n=6

Fig.5 Effect of strontium nitrate on the mineralized nodule formation of OBs

2.6 Effect of strontium nitrate on the adipocytic transdifferentiation of OBs

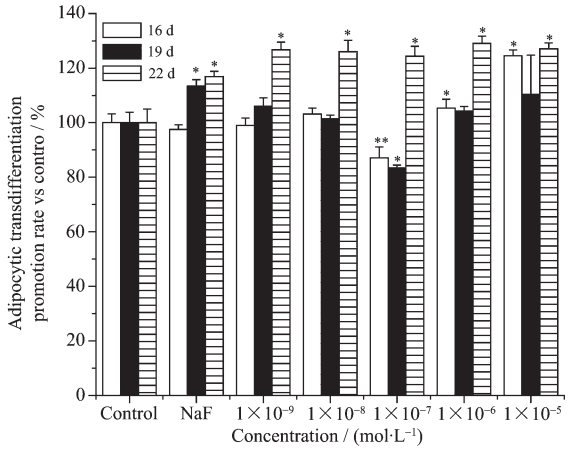
As shown in Fig.7, strontium nitrate promoted the adipocytic transdifferentiation of OBs at concentrations of 1×10^{-6} and 1×10^{-5} mol·L^{-1}, inhibited the adipocytic transdifferentiation of OBs at a concentration of 1×10^{-7}



(A) Control without differentiation medium; (B) Differentiation medium; (C) Differentiation medium+1×10^{-9} mol·L^{-1} strontium nitrate; (D) Differentiation medium+1×10^{-5} mol·L^{-1} strontium nitrate

Fig.6　Effect of strontium nitrate on the formation of mineralized matrix nodules of OBs stained by alizarin red S for 20 d (×100)

mol·L^{-1}, but had no effect at other concentrations for 16 d. Strontium nitrate inhibited the adipocytic transdifferentiation of OBs at a concentration of 1×10^{-7} mol·L^{-1}, but had no effect at other concentrations for 19 d. For 22 d, strontium nitrate promoted the adipocytic transdifferentiation of OBs at the tested concentrations.



*P<0.05, **P<0.01 vs control group, n=6

Fig.7　Effect of strontium nitrate on the adipocytic transdifferentiation of OBs

3　Discussion

OBs progress through a three-stage process of differentiation: proliferation, differentiation and mineralization. OB differentiation is a crucial aspect of bone formation and remodeling. An increase in bone specific ALP activity *in vitro* reflects the maturation from an earlier one stage to a more mature for OB differentiation. ALP hydrolyzes organophosphates to release inorganic phosphate, which appears to be the actual initiator of mineralization[20-21]. The formation of mineralized bone nodules is the sign for the final stage of OB differentiation. Bone nodule formation occurs gradually in several postconfluent osteoblastic cell lines when supplemented with an osteogenic medium over an extended period of time. Mineralization is an ongoing process, therefore it may be necessary to expose the cells for a longer period in order to detect pronounced differences in mineralization in the respective samples. The extent of mineralization depends on the osteoblastic cell type as well as culture conditions[22]. Transdifferentiation is a process whereby a cell type commits to and progresses along a specific developmental lineage by switching into another cell type of a different lineage through genetic reprogramming. Adipocytes and OBs are believed to be derived from multipotential stromal cells in the marrow, and *in vitro* studies have shown an inverse relationship between the differentiation of adipocytic and osteogenic cells[20]. So a reversal of adipogenesis will provide an important therapeutic approach to prevent osteoporosis. Brennan et al. reported that strontium ranelate had positive effect on the replication, differentiation and lifespan of human primary OBs at concentrations of 0.01, 0.1, 1 and 2 mmol·L^{-1}[23]. Verberckmoes et al. reported that the interference of Sr with the bone formation was cell-mediated at lower concentrations, most probably at the level of osteoprogenitor cell differentiation, but Sr had no effect on the OB differentiation at higher concentrations[24]. In present study, the results indicate that the effect of strontium nitrate on the proliferation, differentiation, mineralization function and transdifferentiation of primary OBs is a complicated issue, and it is related to concentration and incubation time.

In fact, numerous pieces of evidence show that the lanthanide-based precipitates formed under physiological conditions may exert some biological effects. Li et al. reported that $GdPO_4$-based particles

formed in cell culture medium acted as a biologically active entity to mediate cell cycle progression in NIH3T3 cells[25]. In our work, the particles also formed in cell culture medium, but there was no significant change for size and number distribution of the particles with increasing concentrations, the size of the particles is (3.5±1.2) nm (>90%). So we deduced that these particles may not play the key role in regulating the proliferation, differentiation and mineralization function of OBs *in vitro*. In addition, in line with its chemical analogy to calcium, Sr is a bone seeking element and 98% of the total body Sr content can be found in the skeleton[26]. So it is possible that the direct action of Sr^{2+} on bone surface and the incorporation in hydroxyapatite. Verberckmoes et al. reported that the effect of Sr on bone formation might result from a direct physicochemical effect of the element on the hydroxyapatite formation[24].

In conclusion, the effect of strontium nitrate on the bone metabolism is very complicated, but the concentration and culture time are key factors for switching the biological effects of strontium nitrate from damage to protection. These findings in primary OBs provide useful information for clinical use.

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