第 28 卷第 8 期 无 机 化 学 学 报 Vol.28 No.8
2012 年 8 月 CHINESE JOURNAL OF INORGANIC CHEMISTRY 1749-1754

氯化镨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,研究了不同浓度的氯化镨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: 氯化镨对成骨细胞增殖、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,但没有呈现出时间和剂量依赖性。结果提示, 氯化镨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。 作用浓度和时间是影响氯化镨生物效应转变的关键因素。

关键词: 氯化镨; 成骨细胞; 增殖; 分化; 矿化
中图分类号: O0614.33+4 **文献标识码**: A **文章编号**: 1001-4861(2012)08-1749-06

Effect of Praseodymium Chloride on Proliferation, Differentiation and Mineralization of Primary Mouse Osteoblasts *in vitro*

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Abstract: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), ALP (alkaline phosphatase) activity, oil red O assays, synthesis of type Ⅰ collagen and ARS (alizarin red-S) stain were employed to evaluate the effect of $PrCl_3$ on proliferation, differentiation and mineralization function of primary OBs (osteoblasts) *in vitro*. The results indicate that the effect of $PrCl_3$ on the above functions and the adipocytic transdifferentiation of primary OBs is closely related to the concentration and incubation time, but is not time and dose-dependent. This suggests that the effect of $PrCl_3$ on bone metabolism is complicated; concentration and culture time are the key factors for switching the biological effect of $PrCl_3$ from damage to protection.

Key words: praseodymium chloride; osteoblasts; proliferation; differentiation; mineralization

0 Introduction

Praseodymium, one of the Ln (lanthanides), has been widely used in various fields due to its special physical and chemical natures. For example, salts of praseodymium may be used to color glasses and enamels, when mixed with some other materials, praseodymium produces an intense and unusually clean yellow color in glass. Along with other rare earths, praseodymium is widely used as a core material for carbon arcs used in the motion picture industry for studio lighting and projection. However, these extensive applications increase the chance of human exposure to praseodymium and its compounds, thus raising deep concerns regarding their risks. Ln-incorporated apatite has a series of attractive

收稿日期:2012-01-17。收修改稿日期:2012-03-17。
国家自然科学基金(No.20971034),河北省自然科学基金重点(No.B2009000161)资助项目。
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properties as a potential bioactive bone implant material. However, Ln released may induce favorable/noxious reactions locally or systemically during corrosion of implanted materials. Severe hepatotoxicity has been detected after the administration of praseodymium[1]. Jha et al.[2] reported that praseodymium oxide (Pr_6O_{11}) administered intraperitoneally to Swiss albino mice induced significant increase in the frequency of CAs (chromosomal aberrations) in bone marrow cells. Feyerabend et al.[3] reported that praseodymium showed cytotoxic effects at lower concentrations on MG63 cells.

It was reported that praseodymium was rapidly cleared from the blood and redistributed to tissues, primarily bone after administration[4]. The similarity between Ln^{3+} and Ca^{2+} has been suggested to be responsible for some of their biochemical behaviors. Thus it is likely that the praseodymium intervenes in bone-remodeling process and affects bone cell function. Jha et al.[5] reported that Pr_6O_{11} promoted bone resorption *in vivo*. However, to the best of our knowledge the potential effects of praseodymium on bone metabolism have not been reported at the cell level. In this study, we investigated the effects of $PrCl_3$ on the proliferation, differentiation and mineralization of primary mouse OBs (osteoblasts) *in vitro*.

1 Experimental

1.1 Materials and Reagents

KM (Kunming) mice were purchased from the Laboratory Animal Center of Hebei Province. DMEM (Dulbeccos modified eagles medium) and trypsin were purchased from Gibco. NBS (neonatal bovine serum) was from Hangzhou Sijiqing Organism Engineering Institute. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), penicillin, streptomycin, β-glycerophosphate, ascorbic acid, dexamethasone, collagenase type II, insulin, oil red O, ARS (alizarin red stain), sirius red, and cetylpyridinium chloride were purchased from Sigma (St. Louis, MO, USA). An ALP (alkaline phosphatase) activity kit was purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China) and a micro-protein assay kit was from Beyotime Biotechnology (Haimen, China). Praseodymium chloride ($PrCl_3$, Purity >99.9%) was from Beijing Institute of Rare Earth Sci. & Tech. Co., Ltd. Other chemicals used were of analytical grade.

1.2 Methods

1.2.1 Isolation and culture of primary OBs

Primary OBs were isolated mechanically from skulls of newborn KM mouse by a modification of the method previously reported[6]. Briefly, skulls were dissected, endosteum and periosteum were stripped off, and bone slices were cut into approximately 1~2 mm^2 pieces and digested with 2.5 $g \cdot L^{-1}$ trypsin for 30 min. Then bone slices were collected and digested twice with 1.0 $g \cdot L^{-1}$ collagenase II with 1 h for each time. 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 the experiments.

1.2.2 Cell viability assay

OB viability was determined by MTT assay as described previously[7]. In brief, OBs were seeded in 96-well tissue culture plates at the density of 2×10^4 cells per well and incubated at 37 ℃, in a 5% CO_2 humidified incubator. After the addition of $PrCl_3$ at different concentrations (final concentration 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} $mol \cdot L^{-1}$), 18, 24, 36 and 48 h further incubations were performed. Control wells were prepared by addition of NaCl. Wells containing DMEM without cells were used as blanks. Wells containing NaF (1×10^{-6} $mol \cdot L^{-1}$) were used as positive control. Upon completion of the incubation, 20 μL of MTT (5.0 $mg \cdot mL^{-1}$ in 1×PBS (phosphate buffered saline)) was added and incubated for another 4 h at 37 ℃. The supernatant was removed, and dimethyl sulfoxide (DMSO) was added to each well. The OD (optical density) was measured on a microplate spectrophotometer (MD VersaMax, USA) at a wavelength of 570 nm. The cell viability (%) was calculated according to the formula: ($OD_{treated}$

$-OD_{blank})/(OD_{control}-OD_{blank})\times 100$.

1.2.3　ALP activity

The OBs (2×10^4 cells per well) were plated in 48-well culture plates, and treated with $PrCl_3$ at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol·L^{-1}. Control, blank and positive wells were prepared as above. 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 according to the formula: (ALP activity$_{treated}$ −ALP activity $_{blank}$)/(ALP activity$_{control}$−ALP activity $_{blank}$)×100.

1.2.4　Collagen production analysis

Collagen production analysis was performed as previously described with some modifications [8]. 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. $PrCl_3$ was added at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol·L^{-1}. Control, blank and positive wells were prepared as above. 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. Synthesis of type collagen promotion rate (%) was calculated according to the formula: (type Ⅰ collagen$_{treated}$ −type Ⅰ collagen$_{blank}$)/(type Ⅰ collagen$_{control}$−type Ⅰ collagen $_{blank}$)×100.

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, $PrCl_3$ was added at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol·L^{-1}. Control, blank and positive wells were prepared as above. Upon completion of the incubation, the formation of mineralized matrix nodules was determined by ARS. 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 was performed by elution with 10% (*W*/*V*) cetylpyridium chloride for 10 min at room temperature and the OD was measured at 570 nm [9]. The mineralized function promotion rate (%) was calculated according to the formula: $(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})\times100$.

1.2.6　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 $PrCl_3$ at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} mol·L^{-1}. Control, blank and positive wells were prepared as above. Upon completion of the incubation, fat droplets within differentiated adipocytes from OBs were stained according to the literature [10]. 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: $(OD_{treated}-OD_{blank})/(OD_{control}-OD_{blank})\times100$.

1.2.7　Statistical analysis

Data were collected from at least four separate experiments. Results were expressed as means

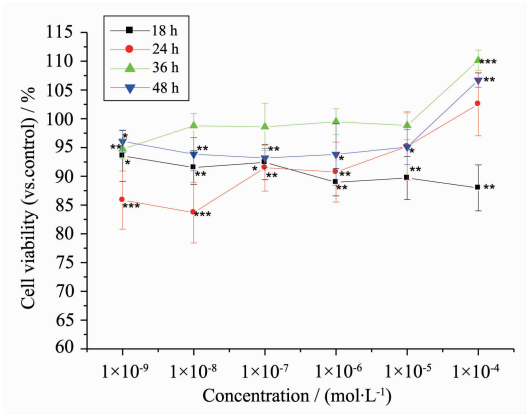
hstandard deviation (SD). Statistical differences were analyzed using SPSS *t*-test. *P* values less than 0.05 were considered to indicate statistical differences.

2 Results and discussion

2.1 Results

2.1.1 Effect of $PrCl_3$ on the viability of OBs

As shown in Fig.1, the results reveal that $PrCl_3$ decreases the viability of OBs at all tested concentrations for 18 h. After 24 and 48 h of $PrCl_3$ treatment, Pr decreases the viability of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol·L^{-1}, but turns to increase the viability of OBs at the highest concentration of 1×10^{-4} mol·L^{-1}. After 36 h of $PrCl_3$ treatment, it shows no significant effect on the viability of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol·L^{-1}, but also turns to increase the viability of OBs at the highest concentration of 1×10^{-4} mol·L^{-1}.



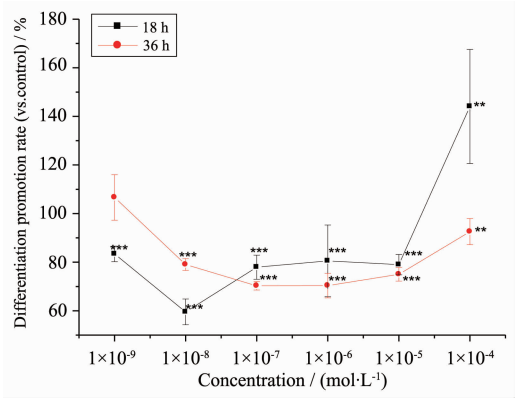
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group, n=6

Fig.1 Effect of $PrCl_3$ on the viability of primary OBs

2.1.2 Effect of $PrCl_3$ on ALP activity of OBs

As shown in Fig.2, after 18 h of $PrCl_3$ treatment, it promotes the differentiation of OBs at the highest concentration of 1×10^{-4} mol·L^{-1}, and inhibits the differentiation of OBs at other tested concentrations. $PrCl_3$ has no significant effect on the differentiation of OBs at the lowest concentration of 1×10^{-9} mol·L^{-1}, and significantly inhibits the differentiation of OBs at other tested concentrations for 36 h.

2.1.3 Effect of $PrCl_3$ on synthesis of type I collagen of OBs



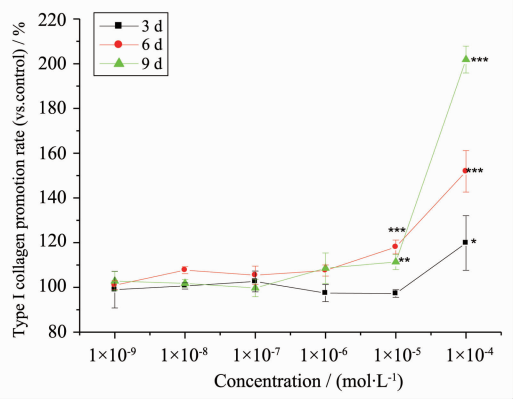
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group, n=6

Fig.2 Effect of $PrCl_3$ on the ALP activity of primary OBs

As shown in Fig.3, on day 3, 6 and 9, $PrCl_3$ has no significant effect on synthesis of type I collagen of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol·L^{-1}. On day 3, $PrCl_3$ has no significant effect on synthesis of type I collagen of OBs, but it turns to promote synthesis of type I collagen of OBs at a concentration of 1×10^{-5} mol·L^{-1} on day 6 and 9. On day 3, 6 and 9, it promotes synthesis of type I collagen of OBs at the highest concentration of 1×10^{-4} mol·L^{-1} in a time-dependent manner.

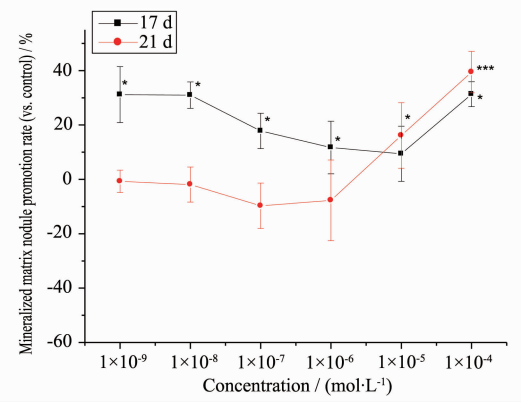
2.1.4 Effect of $PrCl_3$ on the formation of mineralized matrix nodules

As shown in Fig.4, after 17 d of $PrCl_3$ treatment, it significantly promotes the formation of mineralized matrix nodules at all tested concentrations without evident dose-dependence. On 21 d, it has no significant effect on the formation of mineralized



$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control group, n=6

Fig.3 Effect of $PrCl_3$ on the synthesis of type I collagen of primary OBs



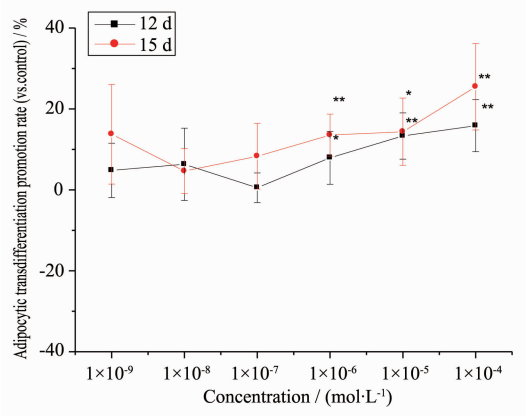
*P<0.05, ***P<0.001 vs control group, n=6

Fig.4　Effect of $PrCl_3$ on the formation of mineralized matrix nodules

matrix nodules at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol·L^{-1}, but turns to promote the formation of mineralized matrix nodules at concentrations of 1×10^{-5} and 1×10^{-4} mol·L^{-1}.

2.1.5　Effect of $PrCl_3$ on the adipocytic transdifferentiation of OBs

As shown in Fig.5, after 12 and 15 d of $PrCl_3$ treatment, it has no significant effect on the adipocytic transdifferentiation of OBs at concentrations of 1×10^{-9}, 1×10^{-8}, and 1×10^{-7} mol·L^{-1}, but turns to promote the adipocytic transdifferentiation of OBs at concentrations of 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} mol·L^{-1}.



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

Fig.5　Effect of $PrCl_3$ on the adipocytic transdifferentiation of OBs

2.2　Discussion

Bone is a specialized and dynamic organ that undergoes a continuous remodeling in an adult life, which include bone formation of OBs and bone resorption of osteoclasts in a coupled manner. 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 to a more mature stage of OB differentiation [11-12]. The formation of mineralized bone nodules is the sign for the final stage of OB differentiation. In addition, both OBs and adipocytes derive from bone marrow stromal cells. There is more and more evidence that suggests the existence of a great degree of plasticity between OBs and adipocytes, and this transdifferentiation is reciprocal [13]. It was reported that adipocytes were secretory cells that might influence hematopoiesis and osteogenesis. Adipocytes synthesized and released a variety of peptide and nonpeptide compounds or secreted cytokines, and the main effect of these cytokines was a stimulation of bone resorption [14]. So there may have protective effect on bone by inhibiting OB transdifferentiation.

In the present study, the model of primary OBs was used to study the effect of $PrCl_3$ on the proliferation, differentiation and mineralization of OBs. The results indicate that the effect of $PrCl_3$ on the proliferation, differentiation and mineralization of OBs is closely related to the concentration and culture time, but has no obvious dose- or time-dependent tendency. These results are accordant with our previous report [15-16]. In addition, our experimental results also suggest that the different species of rare earth ions may behave differently. These differences may relate to the physico-chemical characteristics of the respective cations depending upon features, such as their ionic radii or charge densities.

In addition, in cellular studies of the biological effect of multivalent metal cations, researchers are always concerned about the precipitation paradox. The cations, known as hard Lewis acids tend to precipitate as hydroxides and phosphates/carbonates in cell

culture media and thus complicate the experimental conditions and cause confusion. In fact, numerous pieces of evidence show that the biological effect of lanthanide-based complexes observed is actually in the presence of phosphates and bicarbonates. The particles formed are able to exert the biological effect under physiological conditions[17]. A multi-phase model has been developed and terbium (Tb) speciation in human blood plasma has been studied. At a concentration of 10^{-5} mol·L^{-1}, Tb(Ⅲ) was mostly bound to phosphate to form precipitate of $TbPO_4$. As the concentration of Tb (Ⅲ) increases, phosphate is exceeded and another kind of precipitate of $Tb_2(CO_3)_3$ appears. Among the tiny soluble Tb(Ⅲ) species, Tb(Ⅲ) mainly distributes in [Tb(Tf)] at low concentration and in [Tb(HSA)], [Tb_2(Tf)], [Tb(IgG)], [Tb(Lactate)]$^{2+}$, [Tb (CitArgH)] and free Tb(Ⅲ) at high concentration [18]. Up to now, the working species of praseodymium to exert the effect on the proliferation, differentiation and mineralization of OBs are not well-understood and need to be solved.

In conclusion, the effect of $PrCl_3$ on the bone metabolism is very complicated. The concentration and culture time are key factors for switching its biological effects from damage to protection. The mechanism for the effect of $PrCl_3$ on the proliferation, differentiation, adipocytic transdifferentiation and mineralization function of primary OBs and the working species of praseodymium to exert these effects remain to be further studied.

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