第 26 卷第 12 期 无 机 化 学 学 报 Vol.26 No.12
2010 年 12 月 CHINESE JOURNAL OF INORGANIC CHEMISTRY 2251-2258

Cu^{2+}和 Cu^{+}对原代培养的小鼠成骨细胞增殖、分化和钙化的影响

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摘要: 利用噻唑蓝(MTT)法、碱性磷酸酶(ALP)比活性测定、油红 O 染色和矿化结节染色及定量分析,研究了 Cu^{2+}和 Cu^{+}对原代培养的成骨细胞增殖、分化及钙化的影响。结果显示:Cu^{2+}(1×10^{-9}~1×10^{-6} mol·L^{-1})促进成骨细胞增殖,随时间延长,促进作用变弱。Cu^{+}(1×10^{-7}~1×10^{-5} mol·L^{-1})抑制成骨细胞增殖,随时间延长,浓度为 1×10^{-6} mol·L^{-1} 的 Cu^{+}为促进作用,其余浓度则没有影响。对于成骨细胞分化,Cu^{2+}和 Cu^{+}表现出相似的影响,浓度为 1×10^{-9} 和 1×10^{-6} mol·L^{-1} 时均促进成骨细胞分化,而当浓度为 1×10^{-7} 和 1×10^{-5} mol·L^{-1} 时,则抑制成骨细胞分化,随作用时间延长,大多数浓度均表现为促进作用。测试浓度下的 Cu^{2+}和 Cu^{+}均对成骨细胞向脂肪细胞的横向分化表现为促进效应。对矿化功能的影响,1×10^{-5} mol·L^{-1} 的 Cu^{2+}和 Cu^{+}表现出显著的抑制效应,但随浓度降低,抑制效应变弱。1×10^{-7} mol·L^{-1} 的 Cu^{2+}促进成骨细胞矿化结节的形成。结果提示:作用浓度、作用时间及铜离子的价态都是影响 Cu^{2+}和 Cu^{+}生物效应转变(从毒性到活性,从损伤到保护,从下调到上调)的关键因素。

关键词: 铜; 成骨细胞; 增殖; 分化; 钙化
中图分类号: O614.121 **文献标识码**: A **文章编号**: 1001-4861(2010)12-2251-08

Effects of Cu^{2+} and Cu^{+} on the Proliferation, Differentiation and Calcification of Primary Mouse Osteoblasts *in vitro*

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Abstract: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), alkaline phosphatase (ALP) activity, oil red O assays and alizarin red-S (ARS) stain were employed to evaluate the effects of Cu^{2+} and Cu^{+} on proliferation, differentiation and calcification of primary osteoblasts (OBs) *in vitro*. The results showed that Cu^{2+} (1×10^{-9}~1×10^{-6} mol·L^{-1}) promoted the proliferation of OBs for 48 h, and the promotive effect turned to weaken with prolonging incubation time. Cu^{+} (1×10^{-7}~1×10^{-5} mol·L^{-1}) inhibited the proliferation of OBs for 48 h, but turned to promote the proliferation of OBs at a concentration of 1×10^{-6} mol·L^{-1}, had no effect on proliferation of OBs at other concentrations for 72 h. Both Cu^{2+} and Cu^{+} had similar effects on the differentiation of OBs, they promoted the differentiation of OBs at concentrations of 1×10^{-9} and 1×10^{-6} mol·L^{-1} and inhibited the differentiation at concentrations of 1×10^{-7} and 1×10^{-5} mol·L^{-1} for 48 h, promoted the differentiation at most concentrations for 72 h. Both Cu^{2+} and Cu^{+} promoted the adipocytic transdifferentiation of OBs at all tested concentrations. Both Cu^{2+} and Cu^{+} (1×10^{-5} mol·L^{-1}) obviously inhibited the formation of mineralized matrix nodules of OBs, the inhibitory effect turned to weaken with decreasing concentration, Cu^{2+} promoted the formation of mineralized matrix nodules of OBs at a concentration of 1×10^{-7} mol·L^{-1}. The results suggest that concentration, culture time and valence state of copper ion

收稿日期:2010-05-25。收修改稿日期:2010-07-18。
国家自然科学基金(No.20971034),河北省自然科学基金(No.B2009000161),教育部科学技术研究重点项目(No.208018),河北省留学人员科技活动项目和河北大学自然科学基金资助项目。
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are key factors for switching the biological effects of Cu^{2+} and Cu^{+} from toxicity to activity, from damage to protection, or from down-regulation to up-regulation.

Key words: copper; osteoblasts; proliferation; differentiation; calcification

0 Introduction

Osteoporosis is a skeletal disease characterized by loss of bone mass and disruption of bone architecture, resulting in reduced bone strength and increased risk of fracture. The loss of bone mass in osteoporosis is due to an imbalance of the formation and resorption of bone, which, in turn, depends on the interactions of OBs with osteoclasts (OCs)[1].

Osteoporosis is a multifactorial disease with dimensions of genetics, endocrine function, exercise and nutritional considerations. Several trace elements, particularly copper (Cu), manganese (Mn) and zinc (Zn), are essential in bone metabolism as cofactors for specific enzymes[2-3]. Copper, among trace elements, is essential for the normal growth development of the skeleton in humans and animals. Copper, acting as the composition of many metal enzymes, participates in the synthesis of collagen and elastin, and plays some important roles in fracture repair[4]. The role of copper (Cu) deficiency in abnormal bone metabolism has been noted in the human genetic disorder Menkes′ s disease[5], as well as in infants fed diets deficient in this essential element[6]. Strain[7] reported that mild copper deficiency may contribute to the genesis of osteoporosis in humans. Furthermore, Strause et al.[8] demonstrated that supplements of calcium, copper, manganese, and zinc arrested spinal bone loss in postmenopausal women. More recently, the relevance of copper was further supported by a double blind study of 45-56-year old women who received either a copper supplement or a placebo for two years, spinal bone density remained at the same levels in the supplemented group while it declined in the placebo group[9]. Baker et al.[10] reported that markers of bone density and indicators of bone loss such as urinary pyridinium crosslinks were increased in men after 6 weeks on a low copper diet. Strause et al.[11] reported the long-term effects of dietary Mn and Cu deficiencies on osteoblast and osteoclast activities *in vivo* using ectopic models of bone formation and of bone matrix resorption, respectively. The results showed multiple cellular effects of long-term Mn and Cu deficiencies on bone metabolism including decreased osteogenesis and a decrease in osteoclast activity. Previously, we reported that Cu^{2+} at concentrations of 1.00×10^{-6} and 1.00×10^{-7} mol·L^{-1} inhibited osteoclastic activity and caused dose-dependent reduction in the numbers and surface areas of the lacunae. On the other hand, the number of lanunae was increased and osteoclastic bone resorbing function was significantly improved at a concentration of 1.00×10^{-8} mol·L^{-1}[12]. It was reported that valence state of metal ion might be a key factor for switching the biological effects. Zhou et al.[13] reported that the Mn^{2+} and Mn^{3+} could induce apoptosis of SH-SY5Y cell lines, but the latter was much more potent. Zhang et al.[14] studied the effects of Fe^{3+} and Fe^{2+} on proliferation, differentiation and mineralization function of primary OBs in vitro, and found that the valence state of iron ion was a key factor for switching the biological effects of Fe^{3+} and Fe^{2+} from toxicity to activity, from damage to protection, or from down-regulation to up-regulation. Guan[15] reported that Ce^{4+} and Ce^{3+} had different effect on apoptosis of T. cuspidata cells. However, so far no study has been carried out to assess the potential effects of Cu^{2+} and Cu^{+} on the proliferation, differentiation and calcification of primary mouse OBs *in vitro*. The aim of this paper was to investigate the effects of Cu^{2+} and Cu^{+} on the proliferation, differentiation and calcification of primary mouse OBs in order to elucidate the effects of Cu^{2+} and Cu^{+} on bone metabolism.

1 Materials and methods

1.1 Materials and reagents

Kun ming (KM) mice were obtained from Experimental Animal Center of Hebei Medical

University. Dulbecco′s modified Eagle′s medium (DMEM) and trypsin were purchased from GIBCO, USA. MTT, penicillin and streptomycin, β-glycerphosphate, dexamethasone, ascorbic acid, collagenase type II, insulin, oil red O stain, alizarin red-S (ARS) and cetylpyridium chloride were from Sigma, USA. Neonatal bovine serum (NBS) was from Hangzhou Sijiqing Organism Engineering Institute. An ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China), and a micro-protein assay kit was purchased from Beyotime Biotechnology (Haimen, China). Copper chloride and Cuprous chloride (Purity >99.9%, A.R.) were purchased from Fuchen Chemical Reagent Factory (Tianjin, China).

1.2 Methods

1.2.1 Isolation and culture of primary OBs

The mouse OBs were isolated mechanically from newborn mouse skulls by a modification of the method previously reported[16]. Briefly, skulls were dissected from KM mice, endosteum and periosteum were stripped off, and the bone was cut into approximately 1~2 mm^2 pieces and digested with trypsin (2.5 $g \cdot L^{-1}$) for 30 min and the digestion was discarded. Then the bone was digested with collagenase Ⅱ (1.0 $g \cdot L^{-1}$) 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 the experiments.

1.2.2 Cell proliferation assay

The protocol described by Mosmann was followed with some modifications[17]. 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. Cu^{2+} or Cu^{+} was added to the wells at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} $mol \cdot L^{-1}$. Control wells were prepared by addition of DMEM. Wells containing DMEM without cells were used as blanks. Wells containing NaF (1×10^{-6} $mol \cdot L^{-1}$) without Cu^{2+} or Cu^{+} were used as positive control. Upon completion of the incubation, MTT dye solution (20 μL, 5 $mg \cdot mL^{-1}$) was added to each well. After 4 h incubation, the supernatant was removed and DMSO (100 μL) was added to solubilize the MTT. The optical density (OD) of each well was measured on a microplate spectrophotometer (BioRad Model 3550, USA) at a wavelength of 570 nm. The viability rate (%) was calculated according to the formula: $OD_{sample}/OD_{control}\times100$.

1.2.3 Measurement of ALP activity

The OBs were isolated as above. OBs (2×10^4 cells per well) were plated in 48-well culture plates, and treated with Cu^{2+} or Cu^{+} at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} $mol \cdot L^{-1}$ for 48 and 72 h, respectively. Control wells were prepared by addition of DMEM. Wells containing DMEM without cells were used as blanks. Wells containing NaF (1×10^{-6} $mol \cdot L^{-1}$) without Cu^{2+} or Cu^{+} 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 ALP activity(%) was calculated according to the formula: ALP $activity_{sample}$/ALP $activity_{control}\times100$.

1.2.4 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 $\cdot mL^{-1}$ insulin, 10^{-7} $mol \cdot L^{-1}$ dexthamethone) and treated with Cu^{2+} or Cu^{+} at final concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} $mol \cdot L^{-1}$. Control wells were prepared by addition of DMEM. Wells containing DMEM without cells were used as blanks. Wells containing NaF (1×10^{-6} $mol \cdot L^{-1}$) without Cu^{2+} or Cu^{+} were used as positive control. Upon completion of the incubation, fat droplets within differentiated adipocytes from OBs were stained according to the protocol described by Ichiro et al. with some modifications[18]. 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, and recorded by inverted phase contrast microscopy (Olympus IX 51), then isopropyl alcohol was added to the culture plates. After 5 min, the OD of the extract

was measured at 510 nm as above. The adipocytic transdifferentiation promoting rate (%) was calculated according to the formula: $(OD_{sample}/OD_{control}-1)\times100$.

1.2.5 Mineralized matrix formation assay

The OBs were isolated as above. OBs (3×10^4 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, Cu^{2+} or Cu^{+} 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 DMEM. Wells containing DMEM without cells were used as blanks. Wells containing NaF (1×10^{-6} mol·L^{-1}) without Cu^{2+} or Cu^{+} 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 10min at room temperature, then washed by PBS twice and stained with 0.1% alizarin red S 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 as above[19]. The mineralized matrix promoting rate (%) was calculated according to the formula: $(OD_{sample}/OD_{control}-1)\times100$.

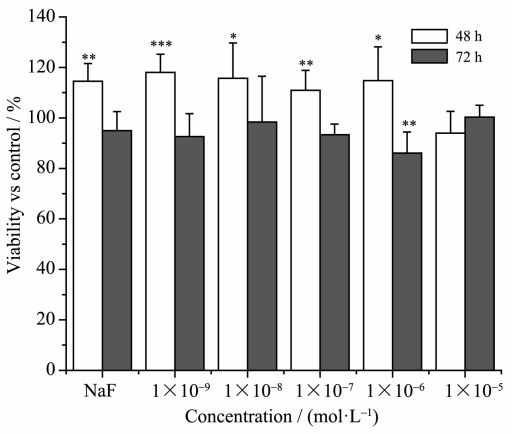
1.2.6 Statistical analysis

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 Effects of Cu^{2+} and Cu^{+} on the proliferation of OBs

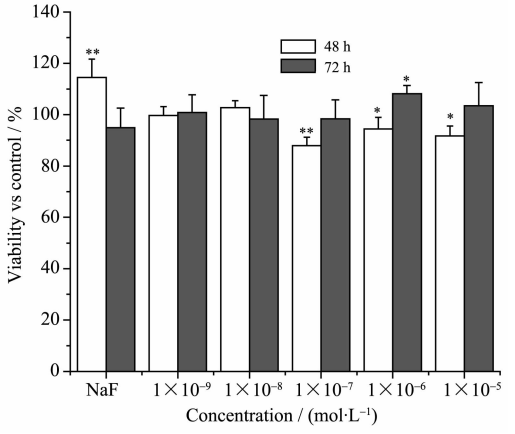
As shown in Fig.1, Cu^{2+} (1×10^{-9}, 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol·L^{-1}) significantly promoted the proliferation of OBs, but had no effect on proliferation of OBs at a higher concentration of 1×10^{-5} mol·L^{-1} for 48 h. Cu^{2+} (1×10^{-6} mol·L^{-1}) turned to inhibit the proliferation of of OBs, but it had no effect on proliferation of OBs at other concentrations for 72 h.



*P<0.05, **P<0.01, ***P<0.001 vs control group, n=5

Fig.1 Effect of Cu^{2+} on the proliferation of OBs

As shown in Fig.2, Cu^{+} (1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}) inhibited the proliferation of OBs, but had no effect on proliferation of OBs at lower concentrations of 1×10^{-9} and 1×10^{-8} mol·L^{-1} for 48 h. Cu^{+} (1×10^{-6} mol·L^{-1}) turned to promote the proliferation of OBs, but had no effect on proliferation of OBs at other concentrations for 72 h.



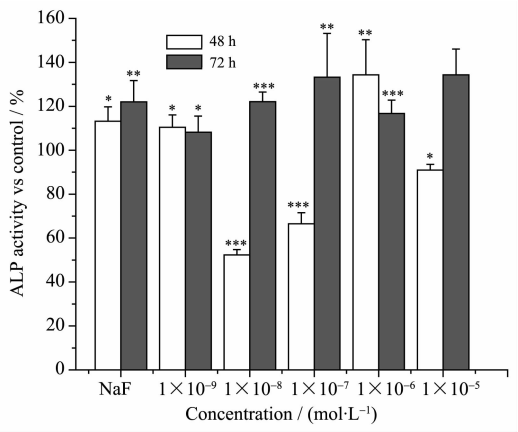
*P<0.05, **P<0.01 vs control group, n=5

Fig.2 Effect of Cu^{+} on the proliferation of OBs

2.2 Effects of Cu^{2+} and Cu^{+} on the differentiation of OBs

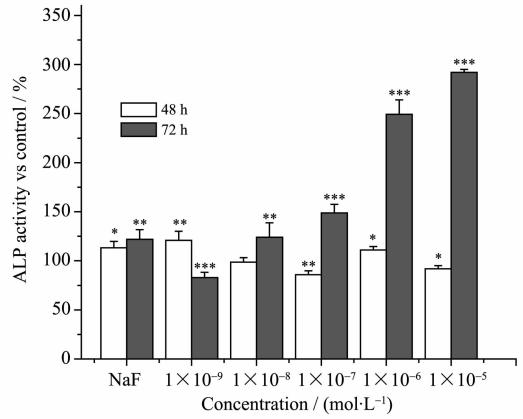
As shown in Fig.3, Cu^{2+} promoted the differentiation of OBs at concentrations of 1×10^{-9} and 1×10^{-6} mol·L^{-1}, but inhibited the differentiation of OBs at concentrations of 1×10^{-8}, 1×10^{-7}, and 1×10^{-5} mol·L^{-1} for 48 h. Cu^{2+} promoted the differentiation of OBs at all tested concentrations for 72 h.

As shown in Fig.4, Cu^{+} promoted the differentia-



*P<0.05, **P<0.01, ***P<0.001 vs control group, n=5

Fig.3　Effect of Cu^{2+} on the differentiation of OBs



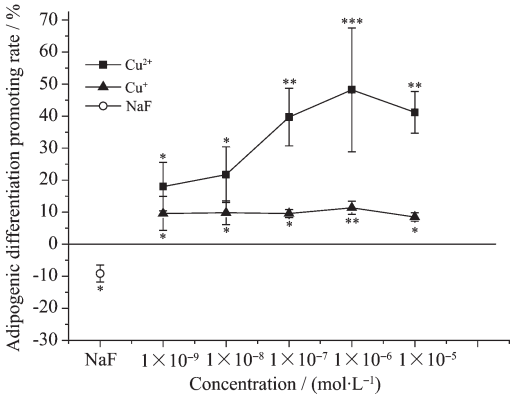
*P<0.05, **P<0.01, ***P<0.001 vs control group, n=5

Fig.4　Effect of Cu^{+} on the differentiation of OBs

tion of OBs at concentrations of 1×10^{-9} and 1×10^{-6} mol·L^{-1}, had no effect at a concentration of 1×10^{-8} mol·L^{-1}, but turned to inhibit the differentiation of OBs at concentrations of 1×10^{-7} and 1×10^{-5} mol·L^{-1} for 48 h. Cu^{+} promoted the differentiation of OBs at concentrations of 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, but inhibited the differentiation of OBs at a lower concentration of 1×10^{-9} mol·L^{-1}.

2.3　Effects of Cu^{2+} and Cu^{+} on the adipocytic transdifferentiation of OBs

As shown in Fig.5, Cu^{2+} and Cu^{+} promoted the adipocytic transdifferentiation of OBs at all tested concentrations, moreover, the effect of Cu^{2+} is stronger than that of Cu^{+} at same concentration. The morphologic observation was in accordance with the results (Fig.6).



*P<0.05, **P<0.01, ***P<0.001 vs control group, n=5

Fig.5　Effects of Cu^{2+} and Cu^{+} on the adipocytic *trans*-differentiation of OBs

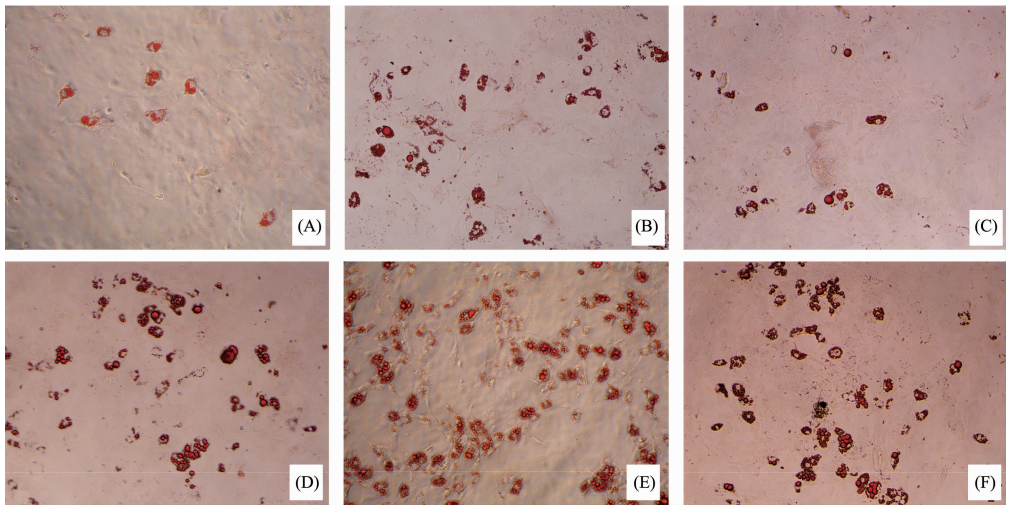
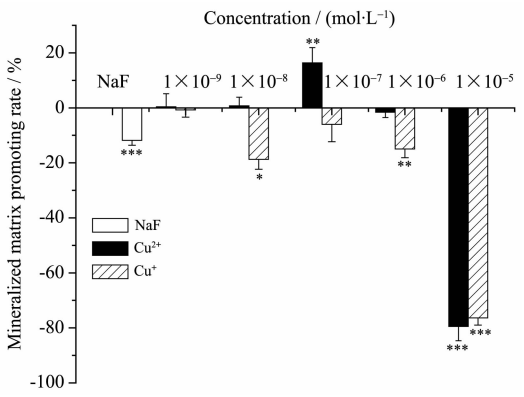


Fig.6　Effects of Cu^{2+} and Cu^{+} on the adipocytic *trans*-differentiation of OBs by Oil Red O stain (×200) (A) Control without adipogenic supplement; (B) Adipogenic supplement; (C) Adipogenic supplement+1×10^{-6} mol·L^{-1} NaF; (D) Adipogenic supplement+1×10^{-5} mol·L^{-1} Cu^{+}; (E) Adipogenic supplement+1×10^{-7} mol·L^{-1} Cu^{2+}; (F) Adipogenic supplement+1×10^{-9} mol·L^{-1} Cu^{2+}

2.4 Effects of Cu^{2+} and Cu^{+} on the formation of mineralized matrix nodules

As shown in Fig.7, Cu^{2+} inhibited the formation of mineralized matrix nodules of OBs at a higher concentration of 1×10^{-5} mol·L^{-1}, turned to promote the formation of mineralized matrix nodules of OBs at a concentration of 1×10^{-7} mol·L^{-1}, and had no significant effect at other tested concentrations. Cu^{+} inhibited the formation of mineralized matrix nodules of OBs at concentrations of 1×10^{-8}, 1×10^{-6} and 1×10^{-5} mol·L^{-1}, had no significant effect at other tested concentrations. The morphologic observation was in accordance with the results (Fig.8).



*P<0.05, **P<0.01, ***P<0.001 vs control group, n=5

Fig.7 Effects of Cu^{2+} and Cu^{+} on the mineralized nodule formation of OBs

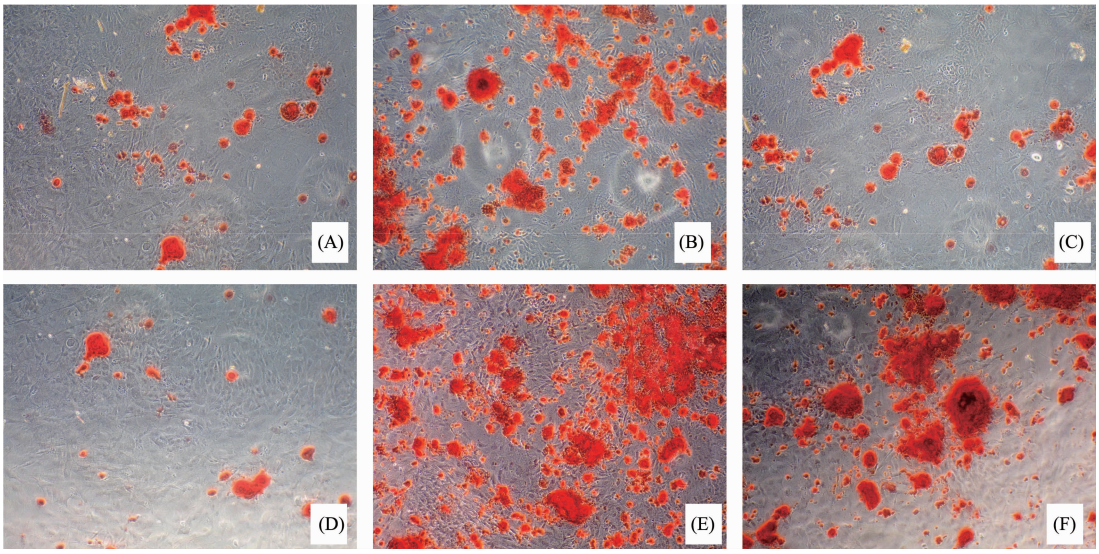


Fig.8 Effects of Cu^{2+} and Cu^{+} on the formation of mineralized matrix nodules of OBs stained by alizarin red S (×100) (A) Control without differentiation medium; (B)Differentiation medium; (C) Differentiation medium+1×10^{-6} mol·L^{-1} NaF; (D) Differentiation medium+1×10^{-5} mol·L^{-1} Cu^{2+}; (E) Differentiation medium+1×10^{-7} mol·L^{-1} Cu^{2+}; (F) Differentiation medium+1×10^{-9} mol·L^{-1} Cu^{+}

3 Discussion

In this work, an *in vitro* model was used to examine the effects of Cu^{2+} and Cu^{+} on the proliferation, differentiation and calcification of primary OBs. The results indicate that the effects of Cu^{2+} and Cu^{+} on proliferation, differentiation and mineralization function of primary OBs depend on concentration, incubation time and valence state of copper ion, but these effects are not dose-dependent. These biology behaviors are smilar to effects of Fe^{3+} and Fe^{2+} on proliferation, differentiation and mineralization function of primary OBs[14]. Reactive oxygen species (ROS) are considered to be responsible for ageing process and in a number of pathological condition such as atherosclerosis, carcinogenesis and infarction[20-21]. The role of ROS in bone metabolism is unique and dual under physiological and pathological conditions[22]. Under physiological conditions, the production of ROS by osteoclasts assists in accelerating destruction of calcified tissue and hence assists in bone remodeling[23-24]. So we deduce that effects of Cu^{2+} and Cu^{+} on primary OBs may be related to the redox reaction of Cu(Ⅰ)→Cu(Ⅱ). In addition, ligands are most often, but not limited to, organic compounds that bind metal ions,

thus modifying the physical and chemical properties of metal ions. Ligands can be introduced into a system to limit the adverse effects of metal ion overload, inhibit selected metalloenzymes, or facilitate metal ion redistribution. In fact, in biological systems, metal ions exist as electron-deficient cations and are hence attracted to electron-rich biological molecules such as proteins and DNA. Biological systems themselves provide innumerable examples of designer ligands that bind metal ions to perform important biological functions[25]. So biological molecules in culture medium may bind Cu^{2+} and Cu^{+} and further form different metal complexes because of the difference of physical and chemical properties between Cu^{2+} and Cu^{+}, this will cause their difference of biology behavior.

It has long been recognized that the p53 tumor suppressor plays a pivotal role in preventing cancer. Lengner et al.[26] and Wang et al.[27] provided genetic evidence that p53 blocked osteoblast differentiation and bone development. Lengner et al.[26] reported that p53 played a critical role in bone organogenesis and homeostasis by negatively regulating bone development and growth and by suppressing bone neoplasia and that Mdm2-mediated inhibition of p53 function is a prerequisite for Runx2 activation, osteoblast differentiation, and proper skeletal formation. Gerard et al.[28] also reported that Wild-type p53 inhibited osteoblast differentiation through the repression of Osterix and Runx2, which play a crucial role in modulating the commitment of mesenchymal stem cells toward the osteoprogenitor lineage Mdm2, which is a negative regulator of p53, can override this response and promote osteoblast differentiation and bone development. Osteoblasts lacking p53 have an enhanced ability to promote osteoclast differentiation and activity. The role of transition metals in the control of p53 has been elucidated[29]. In vitro data show that copper ion binds to p53 and disrupts its native conformation and further suggest that small changes in intracellular transition metals such as zinc or copper may drastically alter p53 protein activity[30]. So we speculated that the effect of copper ion on proliferation and diffenentiation of osteoblasts may be related to p53 signaling pathway.

Adipocytic and osteogenic cells 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[31]. So a reversal of adipogenesis will provide an important therapeutic approach to prevent aged-related and steroids-induced osteoporosis. Rodrignez et al.[32] reported that copper-His complex modified both the differentiation and the proliferative activity of bone marrow stromal cells (MSCs) obtained from postmenopausal women. Copper-His induced a 2-fold increase in osteogenic differentiation of MSCs, and a 1.3-fold increase in the adipogenic differentiation of MSCs. This suggests that copper-His stimulates MSCs differentiation preferentially towards the osteogenic lineage, but the effect of copper-His on adipocytic transdifferentiation of OBs was not studied. In our study, our experimental results indicated that Cu^{2+} and Cu^{+} promoted the adipocytic transdifferentiation of OBs at all tested concentrations. This suggests that Cu^{2+} and Cu^{+} may have negative effect on bone formation.

In conclusion, the effects of Cu^{2+} and Cu^{+} on the proliferation, differentiation and calcification of primary OBs *in vitro* are very complex, but concentration, culture time and valence state of copper ion are key factors for switching the biological effects of Cu^{2+} and Cu^{+} from toxicity to activity, from damage to protection, or from down-regulation to up-regulation. The mechanism of the effects of Cu^{2+} and Cu^{+} on the proliferation, differentiation, adipocytic transdifferentiation and calcification of primary OBs remains to be further studied.

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