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# Aluminum trichloride inhibits osteoblastic differentiation through inactivation of Wnt/β-catenin signaling pathway in rat osteoblasts

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ABSTRACT

Exposure to aluminum (Al) suppresses bone formation. Osteoblastic differentiation plays a key role in the process of bone formation. However, the effect of Al on osteoblastic differentiation is still controversial, and the mechanism remains unclear. To investigate the effect of Al on osteoblastic differentiation and whether Wnt signaling pathway was involved in it, the primary rat osteoblasts were exposed to 1/40 IC50, 1/20 IC50 and 1/10 IC50 of aluminum trichloride ($AlCl_3$) for 24 h, respectively. The activity analysis of alkaline phosphate, qRT-PCR analysis of type I collagen, alkaline phosphate, Wnt3a and Dkk-1, Western blot analysis of p-GSK3β, GSK3β and β-catenin protein and Immunofluorescence staining for β-catenin suggested that $AlCl_3$ inhibited osteoblastic differentiation and Wnt/β-catenin pathway. Moreover, we found exogenous Wnt3a application reversed the inhibitory effect of $AlCl_3$ on osteoblastic differentiation, accompanied by activating the Wnt/β-catenin pathway. Taken together, these findings suggest that $AlCl_3$ inhibites osteoblastic differentiation through inactivation of Wnt/β-catenin pathway in osteoblasts.



## 1. Introduction

Aluminum (Al) is an accumulative toxic metal for human and can enter body via air, water and foods (Willhite et al., 2014). However, Al is used in various fields, such as water purifiers, food additives, pharmaceuticals, and vaccines; it is also present in ambient and occupational airborne particulatesal (Cao et al., 2010; Ohno et al., 2010; Weinbruch et al., 2010; Boullemant, 2011; Dabeka et al., 2011; Skalny et al., 2015). In addition, the growing prevalence of acid rain and bauxite mines exploitation can result in the discharge of larger amounts of Al salts from insoluble minerals, raising the risk of human contact with Al (Smith, 1996; Borgmann et al., 2007). In daily life, the Al of human absorbed from water and food are 0.005 μg/kg/day and 0.08–0.5 μg/kg/day. But Al absorbed from industrial air and dialysis solution can reach up to 0.6–8 μg/kg/day and 9 μg/kg/day(Yokel and Mcnamara, 2001). Although, only 0.05–2.2% of daily Al intake is absorbed, its elimination is slow and accumulated in deep compartment (e.g., bone) for years under continuous intake conditions(Priest, 2004; Ohno et al., 2010).

Bone is the main target organ of Al accumulation, 58–70% of the total human Al body burden accumulates in bone (Ganrot, 1986; Krewski et al., 2007). Excessive Al accumulation causes toxic effects on bones, thereby induces osteodystrophy, osteomalacia and osteoporosis (Boyce et al., 1992; Jorgetti et al., 1994; Jeffery et al., 1996; Li et al., 2011). In dialyzed patients, as bone Al concentrations increased from 46 ± 7 to 175 ± 22 mg/kg (dry weight), the severity of Al-induced bone disease increased (Hodsman et al., 1982). Al-induced bone disease is characterized by suppressed bone formation (Malluche et al., 1987; Quarles, 1990; Kasai et al., 1991). Osteoblasts (OBs) are main functional cells for bone formation, which can be influenced by proliferation, differentiation, mineralization and apoptosis of OBs (Ducy et al., 2000). Previous studies showed that Al induced osteoblast apoptosis and inhibited proliferation and mineralization of osteoblast (Sedman et al., 1987; Blair et al., 1989; Bellows et al., 1995; Li et al., 2012; Cao et al., 2015). However, the effect of Al on the osteoblastic differentiation is controversial. Some research showed that Al inhibited the osteoblastic differentiation (Lieberherr et al., 1987; Lau et al., 1991), while others presented the opposite results (Karlsson et al., 2003; Song et al., 2013). Thus, it is necessary to clarify the effect of Al on osteoblastic differentiation.

The Wnt/β-catenin pathway is an important modulator of OBs function and bone formation (Glass and Karsenty, 2007; Deschaseaux et al., 2009; Baron and Kneissel, 2013). This pathway is triggered by binding Wnt glycoprotein family members (such as Wnt-1 and 3) to a co-receptor complex including Frizzled and low density receptor-like proteins 5 and 6 (Mao et al., 2001b). This is followed by phosphorylation (inactivation) of glycogen

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synthase kinase (GSK3β) to stabilize of β-catenin. The β-catenin then translocates into the nucleus, where it forms a complex with a T-cell factor to induce the transcription of osteoblastic genes (Bennett et al., 2005; Gaur et al., 2005; Sato et al., 2009). Many studies showed that the Wnt/β-catenin pathway is involved in the osteoblastic differentiation (Chen et al., 2010; Guo et al., 2011; Park et al., 2011; Tian et al., 2011; López-Herradón et al., 2013; Pan et al., 2014). However, it remains unknown whether Wnt/β-catenin pathway is involved in the effect of Al on osteoblastic differentiation.

To examine the effect of $AlCl_3$ on osteoblastic differentiation and whether Wnt/β-catenin pathway was involved in it, the primary rat OBs were treated with $AlCl_3$. Then the effects of $AlCl_3$ on the osteoblastic differentiation markers (ALP, CoL-Iand Runx2) and key components of Wnt/β-catenin signaling were determined in OBs. We found $AlCl_3$ suppressed osteoblastic differentiation and inactivated the Wnt/β-catenin pathway. Moreover, we observed the inhibitory effect of $AlCl_3$ on osteoblastic differentiation and Wnt/β-catenin pathway were both reversed by exogenous Wnt3a. Our results suggest $AlCl_3$ inhibits osteoblastic differentiation through inactivation of Wnt/β-catenin pathway in rat OBs.

## 2. Materials and methods

### 2.1. OBs culture and treatment

The experimental designs and procedures were approved by the Animal Ethics Committee of the Northeast Agricultural University (Harbin, CHN). The primary OBs were derived from calvarium of 1-day-old Sprague–Dawley rats as previously described (Pan et al., 2014). The rat calvarium was cut into 1–2 $mm^2$ pieces and consecutively digested using trypsin (2.5 g/L; Gibco, USA) for 10 min and collagenase II (1.0 g/L; Gibco, USA) for three sequential digestion periods of 15, 30 and 60 min at 37 °C. The supernatant of 15 min and 30 min digestions were discarded, and cells obtained from the 60 min digestions were in presence of DMEM(Gibco, USA) supplemented with 10% FBS(Gibco, USA), 2 mM-glutamine (Gibco, USA), 1% penicillin/streptomycin (Gibco, USA) in a humidified 5% $CO_2$ incubator at 37 °C. The cells between 2th and 3th passage were harvested after reaching 90% confluence, and then were plated in medium supplemented using 50 μg/mL ascorbic acid (Sigma, USA) and 10 mM β-glycerophosphate (Sigma,USA) for 12 days, and the medium was changed every 2 days. The treatments of OBs were divided into two parts, one part was treated with 0 (control group, CG), 1/40 IC50 (Low-dose group, LG), 1/20 IC50 (Mid-dose group, MG) and 1/10 IC50 (High-dose group, HG) of $AlCl_3$ (Aladdin, CHN), the other part was treated with $AlCl_3$ (1/10 IC50), $AlCl_3$ (1/10 IC50) +Wnt3a (100 ng/mL; PeproTech, USA) and without $AlCl_3$ and Wnt3a (control group, CG) at 37 °C in 5% $CO_2$ for 24 h, respectively. Our previous work had demonstrated that the IC50 of $AlCl_3$ on OBs was 8.16 mmol/L (Li et al., 2012). The final concentration of Wnt3a was 100 ng/mL, according to the previous research (Guo et al., 2011; Arioka et al., 2014).

### 2.2. ALP activity

The intracellular ALP activity was quantitated using commercially available kits (Beyotime, CHN). OBs were rinsed twice using ice-cold PBS, followed by homogenization in alkaline lysis buffer. After centrifugation, the resulting cell homogenate was incubated with *p*-nitrophenyl phosphate at 37 °C for 30 min. The results were normalized by the total intracellular protein content determined by the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, CHN) and expressed in nanomoles of produced *p*-nitrophenol per min per mg of protein (nmol/min/mg protein).

### 2.3. qRT-PCR analysis

OBs were harvested and rinsed twice using ice-cold PBS. The total RNA was extracted using Trizol Reagent (Invitrogen, USA), and was analyzed using spectrophotometry at 260 and 280 nm (Pharmacia Biotech, UK). Only samples with an optical density ratio at 260/280 nm > 1.8 were used for further analyses. Then each sample was reversely transcribed into cDNA using a reverse transcription kit (Trans Script First-Strand cDNA Synthesis Super Mix, Trans Gen Blotech, CHN). The primers of the genes are shown in Table 1. Gene expressions were examined using SYBR Green/Fluoresce in qPCR Master Mix via 7000 real-time PCR detection system (ABI, USA).

### 2.4. Western blot analysis

The OBs were harvested in cell lysis buffer, and incubated for 15 min at 0 °C. Then they were centrifuged for 5 min at 600 × *g* at 4 °C, and the supernatant was used to detect the object protein. The protein concentration of the supernatant was determined by BCA assay (Beyotime, CHN). The protein was separated using polyacrylamide gels, electro-transferred onto PVDF membranes, and blocked with 5% non-fat milk in TBST buffer for 2 h. Then the membranes were incubated using anti-GSK3β, anti-p-GSK3β and anti-β-catenin (Santa, USA) at dilutions of 1:400 in 5% non-fat milk overnight at 4 °C, and washed three times using TBST, for 20 min each time. After that, the membranes were incubated using the secondary antibodies at dilutions of 1:4000 for 1 h at room temperature, and then washed three times using TBST. Finally, the object protein was detected using the enhanced chemiluminescent (ECL) reagent (Beyotime, CHN). To assess the presence of comparable amount of proteins in each lane, the membranes were stripped finally to detect the β-actin. Quantitative analysis was carried out using Gel-Pro analyzer 4 image analysis system.

### 2.5. Immunofluorescence localization of β-catenin

The OBs growing on coverslips were exposed to $AlCl_3$ for 24 h, and then rinsed using cold PBS twice, flexed using ice-cold methanol and permeabilized using 0.5% Triton X-100 for 15 min. After blocked with 0.5% bovine serum albumin for 1 h at room temperature, OBs were incubated using mouse monoclonal anti-β-catenin (1:200) overnight at 4 °C followed by FITC-conjugated secondary antibody and DAPI. The fluorescence signal of β-catenin was visualized using confocal fluorescence microscopy (Olympus, Japan).

### 2.6. Statistical analysis

Data were presented as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance followed by Student's t-test (SPSS 17.0 software; SPSS Inc., Chicago, IL, USA) and drawn histograms by Graphpad Prism 6.0. Values are means ± SD of at least three independent measurements in triplicate. Values of $p < 0.05$ (*or#) were considered statistically significant and values of $p < 0.01$ (**or##) were considered highly significant.

## 3. Results

### 3.1. $AlCl_3$ inhibited osteoblastic differentiation

To investigate the effect of $AlCl_3$ on osteoblastic differentiation, ALP activity, the ALP and COL-I mRNA levels were examined using commercially available kits and qRT-PCR. The ALP activity and the mRNA levels of ALP and COL-I were lower in $AlCl_3$-treated group than those in control group (Fig. 1). These results suggest that $AlCl_3$ inhibits osteoblastic differentiation in OBs.