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Autophagy Protects MC3T3-E1 Cells upon Aluminum-Induced Apoptosis

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Abstract
Aluminum (Al) exposure has adverse effects on osteoblasts, and the effect might be through autophagy-associated apoptosis. In this study, we showed that aluminum trichloride ($AlCl_3$) could induce autophagy in MC3T3-E1 cells, as demonstrated by monodansylcadaverine (MDC) staining and the expressions of the ATG3, ATG5, and ATG9 genes. We found $AlCl_3$ inhibited MC3T3-E1 cell survival rate and caused apoptosis, as evidenced by CCK-8 assay, Annexin V/PI double staining, and increased expressions of Bcl-2, Bax, and Caspase-3 genes. In addition, increased autophagy induced by rapamycin further attenuated the MC3T3-E1 cell apoptosis rate after $AlCl_3$ exposure. These results support the hypothesis that autophagy plays a protective role in impeding apoptosis caused by $AlCl_3$. Activating autophagy may be a strategy for treatment of Al-induced bone disease.

Keywords Aluminum · MC3T3-E1 cell · Autophagy · Apoptosis

Introduction

Aluminum (Al) is a metal that is widespread in food additives, water purification reagents, antacids, cosmetics, and cookware [1]. Human beings could inevitable exposure to Al through their diets, skin, medicine, and simply breathing [2, 3]. Once being absorbed, 70% of Al accumulates in the body and is retained within bones with a half-life of 10–20 years [4]. Excessive Al accumulation suppresses bone formation and causes bone diseases, which defined as "Al-induced bone diseases" (AIBD), including osteomalacia and osteoporosis [5]. Sun et al. reported that aluminum trichloride ($AlCl_3$) could cause bone impairment through oxidative stress and the inhibition of bone formation [6]. Bone formation is a process of laying down new materials by osteoblasts (OBs). Once OBs are injured, bone formation is decreased, resulting in bone loss and bone diseases [7]. Our laboratory has found that Al exposure inhibits OB proliferation, differentiation, and mineralization [1, 8, 9], and causes OB apoptosis through activating oxidative stress and disrupting calcium homeostasis [10, 11].

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Autophagy is major intracellular degradation process that delivers old organelles, misfolded proteins, or damaged molecules to the lysosome [12]. At the base level, autophagy plays a housekeeping role in maintaining cell homeostasis against various cytotoxic stimuli [13]. On the contrary, excessive autophagy can trigger cell death, which is called type II programmed cell death and differs from apoptosis [14]. Some evidence has demonstrated that autophagy can protect OBs against mental-induced toxicity [15]. Liu et al. found that autophagic response plays a protective role in impeding cadmium-induced apoptosis in primary rat OBs [16]. Lv et al. reported that activating autophagy could reduce lead chloride-induced OBs apoptosis [17]. In contrast, inhibiting autophagy also aggravates the inhibitory effects of high glucose levels on the viability and function of OBs [18]. Until now, there has been no report to Al-induced OB autophagy.

It has been reported that Al can induce primary rat astrocyte apoptosis via over-activating autophagy [19]. In SH-SY5Y cells, Al increased LC3 protein expression, a protein marker for autophagy [20]. Both Al-induced SH-SY5Y cell apoptosis and LC3 protein expression could be reduced by necrostatin-1, a specific inhibitor of necroptosis, indicating that autophagy participates in Al-induced SH-SY5Y apoptosis [21]. However, no study has been conducted to investigate the effect of autophagy on OBs treated with Al. In the current study, we explored whether Al can induce autophagy in MC3T3-E1 cells (OB cell line) and which role of autophagy was acting. These results may illustrate a novel toxic mechanism of Al.

Materials and Methods

Cell Culture and Reagents

MC3T3-E1 cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in α-minimum essential medium (Hyclone) supplemented with 15% fetal bovine serum (FBS) (Hyclone) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; Gibco, Grand Island, New York, USA) in a humidified 5% CO_2 atmosphere at 37 °C. The medium was renewed every other day.

Annexin V-FITC apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Nantong, China). The autophagy inducer rapamycin (RAP) and $AlCl_3$ were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). Standard solution of Al (100 μg/mL) was provided by the National Institute of Metrology (Beijing, China).

Cell Survival Rate

The MC3T3-E1 cell survival rate was evaluated by cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Briefly, MC3T3-E1 cells were seeded onto 96-well plates (10^4 cells/well) and cultured for 24 h under 5% CO_2 at 37 °C. Then the cells were respectively treated with 2, 4, 6, 8, 10, or 12 mM $AlCl_3$ for 24 h. After the $AlCl_3$ treatment, a mixed solution containing the culture medium (90 μL) and CCK-8 reactant (10 μL) was added to each well. Then the plate was incubated at 37 °C for 2 h in dark. Finally, the absorbance at 490 nm was recorded in a Tecan Sunrise microplate reader. Each Al treatment had six replicated wells on a plate and repeated three times.

Determination of Autophagic Vacuoles

Autophagic vacuoles were detected by monodansylcadaverine (MDC) staining, according to Zhang et al. [22]. Briefly, cells were treated with or without 8 mM $AlCl_3$ for 24 h. Then, cells were incubated with 50 μM MDC for 45 min in dark. After staining, the cells were washed three times with PBS and then fixed in 4% paraformaldehyde. The stained cells were immediately viewed using by fluorescent microscopy (Eclipse-Ti; Niko, Japan). Autophagy in MC3T3-E1 cell was analyzed and quantified by the fluorescence intensity of MDC.

Apoptosis Analysis

MC3T3-E1 cell apoptosis was measured using an Annexin V-FITC/propidium iodide (AV/PI) apoptosis detection kit according to the manufacturer's instruction [23]. Briefly, the cells were treated with 8 mM $AlCl_3$ with or without RAP (100 nM) for 24 h. Following the treatment, MC3T3-E1 cells were harvested and washed twice with ice-cold PBS. Then, the cells were incubated with Annexin V-FITC and PI at room temperature in dark for 30 min. The apoptosis rate was detected by flow cytometry (FACS-caliber, Becton Dickinson, San Jose, CA, USA) and all samples were analyzed by Mod Fit software. The apoptotic rate was calculated as a percentage of Q2 + Q4 quadrants.

Quantitative RT-PCR

The expressions of Bcl-2, Bax, Caspase-3, ATG3, ATG5, and ATG9 mRNA were determined by qRT-PCR [24, 25]. Total mRNA was extracted by Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNA was synthesized using First-Strand cDNA Synthesis kit (TransScript First-Strand cDNA Synthesis SuperMix, TransGen Biotech, China). qRT-PCR was performed using SYBR Green/Fluorescein qPCR Master Mix on 7000 real-time PCR detection system (ABI, USA). Each sample was analyzed in triplicates, and the mean value was calculated. Relative mRNA expression was normalized to the β-actin level. Gene-specific primer pairs were shown in Table 1.

Statistical Analysis

Data are presented as the mean ± SD. The data were analyzed by SPSS 22.0 package programmer (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to check the normal distribution of data. Levene's test was used to assess the variance homogeneity. One-way ANOVA with LSD and Bonferroni's method were used to conduct multiple comparisons. Unpaired Student's t test was used to compare differences between two groups. The values of $P > 0.05$ was considered no significant differences, values of $P < 0.05$ was considered statistically significant, and values of $P < 0.01$ was considered highly significant.

Results

$AlCl_3$ Inhibits MC3T3-E1 Cell Survival Rate

The cell survival rate was evaluated by CCK-8 assay. The CCK-8 assay showed that the cell survival rate did not decrease with the doses of $AlCl_3 \leq 6$ mM ($P > 0.05$). When $AlCl_3$ concentrations were ≥8 mM $AlCl_3$, the cell survival rates were significantly decreased ($P <$

Table 1 Primer sequences and amplification lengths of destination fragments

Gene	Primer sequence	Primer length (bp)	Product length (bp)
Bcl-2	UP 5′ CTCGTCGCTACCGTCGTGACTTCG 3′ LOW 5′ CAGATGCCGGTTCAGGTACTCAGTC 3′	24 25	242
Bax	UP 5′ GAGCTGCAGAGGATGATTGCT 3′ LOW 5′ TGATCAGCTCGGGCACTTTA 3′	21 20	178
Caspase-3	UP 5′ GCTTGGAACGGTACGCTAAGA 3′ LOW 5′ CCCAGAGTCCACTGACTTGC 3′	21 20	120
ATG3	UP 5′ AGCTGGAGATCACTTAGTCCACC 3′ LOW 5′ TTGTCTGTCGGAAGATATGCCT 3′	23 22	90
ATG5	UP 5′ TTGAATATGAAGGCACACCCC 3′ LOW 5′ ATAAAGTGAGCCTCAACCGCATC 3′	21 23	169
ATG9	UP 5′ ACTATGACATCCTCTTTGCCAAC 3′ LOW 5′ CAGAAGACACCAGCAATGACCA 3′	23 22	166
β-actin	UP 5′ CAAGAGAGGTATCCTGACCT 3′ LOW 5′ TGATCTGGGTCATCTTTTCAC 3′	20 21	188

0.01) (Fig. 1a). Thus, 8 mM $AlCl_3$ was chosen for subsequent tests. Based on the dose-response curve, IC50 value of $AlCl_3$ was 10.02 ± 0.22 mM when MC3T3-E1 cells were treated for 24 h.

$AlCl_3$ Causes MC3T3-E1 Cell Apoptosis

As shown in Fig. 1b, the mRNA expressions of Bax and Caspase-3 were higher in the $AlCl_3$ treatment for 24 h, as

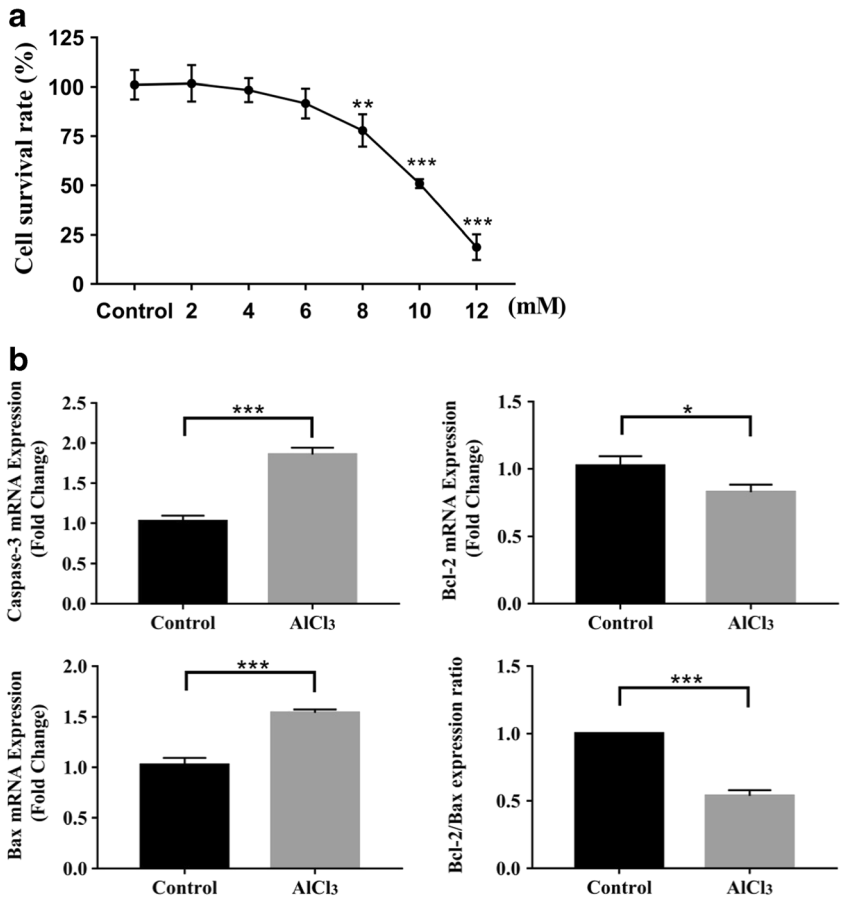

Fig. 1 **a** Effects of $AlCl_3$ on the survival rate of MC3T3-E1 cells. MC3T3-E1 cells were treated with the indicated concentrations (2, 4, 6, 8, 10, and 12 mM) of $AlCl_3$ for 24 h. **b** Caspase-3, Bcl-2, and Bax mRNA expressions were analyzed by qRT-PCR. MC3T3-E1 cells were treated with 8 mM $AlCl_3$ for 24 h. Data are expressed as mean ± SD (n = 3, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

compared with the control ($P < 0.01$). The mRNA expression of Bcl-2 and the Bcl-2/Bax ratio were lower in the $AlCl_3$ treatment than the control ($P < 0.05$). Consistently, the apoptotic rate of MC3T3-E1 cells was higher in $AlCl_3$ treatment than the control (Fig. 3). Thus, MC3T3-E1 cell apoptosis was induced by $AlCl_3$ treatment.

$AlCl_3$ Triggers Autophagy in MC3T3-E1 Cell

MDC is a specific label for autophagic vacuoles and was used to identify $AlCl_3$-induced autophagy. As shown in Fig. 2a, $AlCl_3$ (8 mM) exposure increased the MDC fluorescence intense compared to the control ($P < 0.01$), indicating that $AlCl_3$ caused MC3T3-E1 cell autophagy. To further validate the autophagy level in MC3T3-E1 cells in response to $AlCl_3$ treatment, the mRNA expressions of ATG3, ATG5, and ATG9 were examined, and their expressions were all increased, as compared with those in the control ($P < 0.05$) (Fig. 2b). Taken together, these data supported that $AlCl_3$ exposure activated autophagy in MC3T3-E1 cells.

Autophagy Inhibits $AlCl_3$-Induced Apoptosis in MC3T3-E1 Cell

To investigate the role of autophagy in MC3T3-E1 cell apoptosis under $AlCl_3$ exposure, the apoptosis rate in MC3T3-E1 cells was detected after 8 mM $AlCl_3$ exposure with or without RAP. As shown in Fig. 3, the apoptosis rate was increased to 24.5% after 8 mM $AlCl_3$ treatment alone; While MC3T3-E1 cells were co-treated with $AlCl_3$ and RAP (100 nM), the apoptosis rate was reduced to 18.2%. RAP alone had no effect on the apoptosis incidence in MC3T3-E1 cells. These results proved that autophagy could protect MC3T3-E1 cells from apoptosis under $AlCl_3$ exposure.

Discussion

The MC3T3-E1 cell line has an ability to differentiate into premature and mature OB, and has been extensively used as a model system for studies on OB function [26]. Therefore, we used the MC3T3-E1 cells as the OB model to investigate the role of autophagy under $AlCl_3$ exposure. Despite numerous

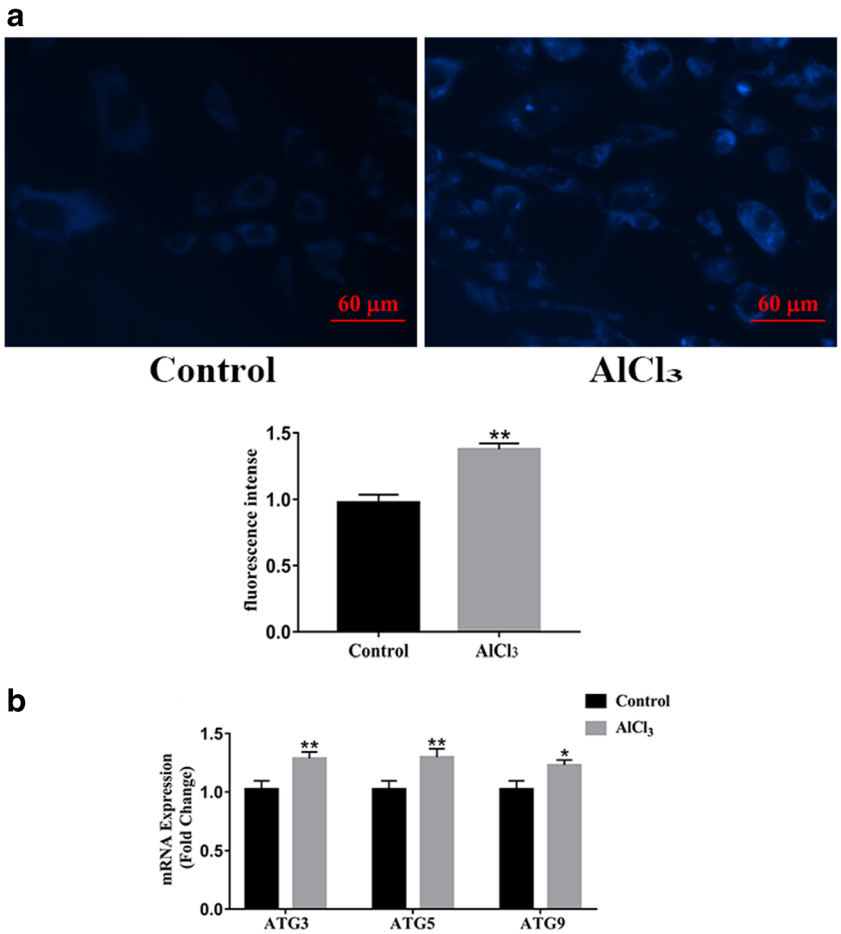

Fig. 2 MC3T3-E1 cells were cultured in the absence or presence of 8 mM $AlCl_3$ for 24 h. **a** Autophagy in MC3T3-E1 cells were analyzed and quantified by MDC staining (× 400). **b** The mRNA expressions of ATG3, ATG5, and ATG9 were analyzed by qRT-PCR. The data are expressed as mean ± SD ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$)

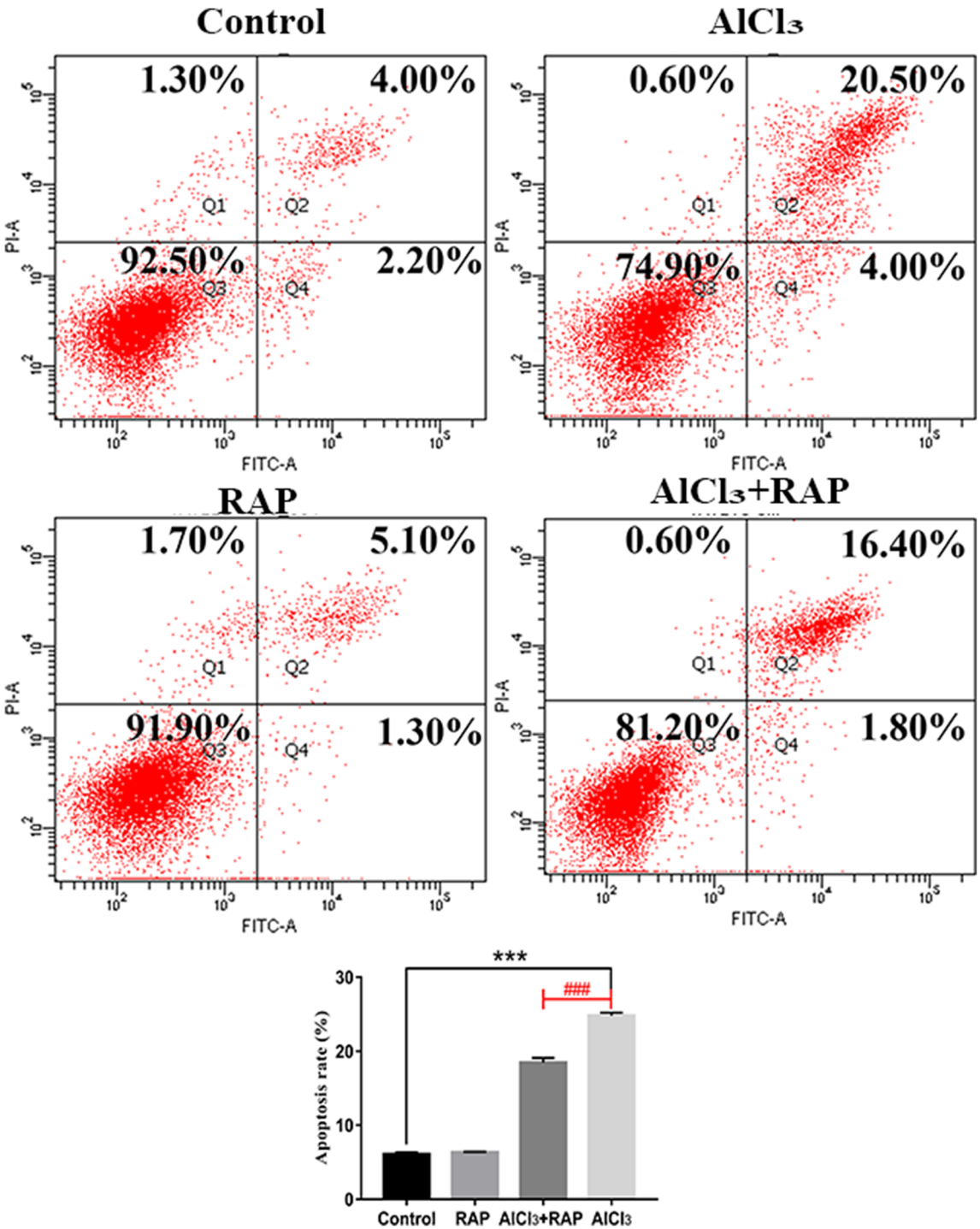

Fig. 3 MC3T3-E1 cells were cultured in the absence or presence of 8 mM $AlCl_3$ for 24 h with or without 100 nM RAP. Apoptosis incidence in MC3T3-E1 cell was detected with flow cytometry. The data are expressed as mean ± SD ($n = 3$, $^{***}P < 0.001$, $^{###}P < 0.001$)

reports that Al exposure has adverse effects on bone tissue and OB, the exact molecular mechanisms remain unclear [1, 6, 8, 10, 11]. In this study, we found that the cell survival rate gradually decreased with the increment of $AlCl_3$ dose. Other studies confirmed that $AlCl_3$ exposure also caused apoptosis of primary OBs by regulating the expression of the apoptosis-related factors Bcl-2, Bax, and Caspase-3 [11, 27]. As cell survival rate is closely related to apoptosis [28], we hence assumed that the decreased survival rate of MC3T3-E1 cells was attributed to increased apoptosis.

The Bcl-2 family proteins (Bcl-2 and Bax) play vital roles in the control of OB fate [23]. The anti-apoptotic molecule Bcl-2 is important for maintaining OB survival and function. On the contrary, pro-apoptotic gene Bax plays a causal role in promoting OB apoptosis. A decreased Bcl-2/Bax ratio means that the cell is undergoing apoptosis [29, 30]. Xu et al. reported that $AlCl_3$ exposure inhibited the Bcl-2 protein expression while increasing the expression of Bax, indicating that Bax and Bcl-2 participate in OB apoptosis induced by $AlCl_3$. In this study, the expressions of Bax and Bcl-2 mRNA in MC3T3-E1 cells after the $AlCl_3$ treatment were consistent with Xu's results and the expression of Bcl-2/Bax mRNA ratio was decreased, suggesting that $AlCl_3$ induced MC3T3-E1 cell apoptosis. This was reinforced by the changes in Caspase-3 in the current study. Caspase-3 is a key executor of apoptosis [23, 31]. Our previous study showed that the increased enzymatic activity and mRNA expression of Caspase-3 mediated primary OB apoptosis caused by $AlCl_3$ [27], and in this study, the expression of Caspase-3 mRNA was increased after $AlCl_3$ treatment, demonstrating that apoptosis occurred actually.

Autophagy is a catabolic process in which cell components are delivered to the lysosomal compartment for degradation [32]. Autophagy is involved in the formation of autophagosome and autophagolysosome. *ATG3*, *ATG5*, and *ATG9* are key genes involved in the initiation of autophagosome formation and are used as markers for autophagy. In the present study, we found the expressions of ATG3, ATG5, and ATG9 mRNA were all increased following $AlCl_3$ exposure, confirming that $AlCl_3$ activated autophagy. This was further supported by the MDC staining results that the increase in fluorescence intensity in the cells treated with $AlCl_3$ indicates that $AlCl_3$ increased the level of autophagy.

The link between apoptosis and autophagy is complicated and difficult to elucidate. Many studies have demonstrated that autophagy protects cells from apoptosis and various stress challenges via degradation of damaged proteins and organelles [17]. Zheng et al. reported that TNF-α induced both autophagy and apoptosis in OBs, and up-regulated autophagy protected cells by inhibiting TNF-α-induced apoptosis [33]. Impairing autophagy aggravates the inhibitory effects of high glucose levels on OB viability and function [18]. Our results of the MC3T3-E1 cell apoptosis rate after the treatment with $AlCl_3$ in the absence or presence of RAP confirmed that the apoptosis rate was decreased after induction of autophagy, so autophagy could alleviate apoptosis caused by $AlCl_3$ in MC3T3-E1 cells.

By contrast, Zeng et al. found that aluminum maltolate induced primary rat astrocyte apoptosis via over-activating of autophagy, which indicated that autophagy also has a harmful effect [19]. According to the current study, this might be attributed to the different roles that autophagy plays in astrocytes and MC3T3-E1 cells under $AlCl_3$ stimulation, as well as the differences in the doses of $AlCl_3$.

In conclusion, these results demonstrated that $AlCl_3$ exposure could trigger autophagy in MC3T3-E1 cells. The enhancement of autophagy could relieve MC3T3-E1 cells from apoptosis upon $AlCl_3$ exposure. These findings suggest that autophagy in MC3T3-E1 cells might be an important process to rescue the detrimental effects of $AlCl_3$ exposure and increasing the level of autophagy might provide potential therapeutic strategies to mitigate Al-induced bone diseases. However, which pathway mediates $AlCl_3$ triggered autophagy in MC3T3-E1 cell remains unclear. In the light of this, we will further investigate the exact molecular mechanism that is involved in $AlCl_3$-induced autophagy.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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