Original Research

Fluoride induces osteoblast autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway *in vivo* and *in vitro*

Yan Linghu[1,2], Chao-Nan Deng[2], Li He[2], Qi Wu[2], Lin Xu[3] and Yan-Ni Yu[2]

[1]Department of Human Anatomy, School of Basic Medicine, Guizhou Medical University, Guiyang 550025, China; [2]Department of Pathology, The Affiliated Hospital of Guizhou Medical University, Guiyang 550004, China; [3]Department of Obstetrics and Gynecology, Guiyang Maternal and Child Health Care Hospital, Guiyang 550004, China
Corresponding author: Yan-Ni Yu. Email: yuyannigmubl@163.com

Impact statement

Fluorosis, which is widespread globally, is a type of chronic poisoning caused by excessive fluorine intake and manifests mainly as bone damage in the form of dental fluorosis and skeletal fluorosis. However, the pathological mechanism remains unclear. This study found that fluoride induces autophagy in osteoblasts by inhibiting the PI3K/AKT/mTOR signaling pathway, which is followed by bone formation. Therefore, fluoride-induced autophagy is likely a mechanism of bone damage. This study provides certain new insights into skeletal fluorosis.

Abstract

Fluorosis primarily manifests as bone damage in the form of dental fluorosis and skeletal fluorosis and represents a critical global public health challenge. However, few studies have examined autophagy-related signaling pathways in skeletal fluorosis. This study aimed to investigate the effect of fluoride on autophagy in osteoblasts using comprehensive methods and to explore the role of the PI3K/AKT/mTOR signaling pathway in regulating fluoride-induced autophagy in osteoblasts. Sprague–Dawley (SD) rats were exposed to different concentrations of fluoride (NaF: 5, 50, and 100 mg/L) for six months. Primary osteoblasts were treated with 0.5, 1.0, or 3.0 mM NaF. Hematoxylin and eosin (H&E) staining, transmission electron microscopy (TEM), immunohistochemistry (IHC), immunofluorescence staining, and western blotting were performed to evaluate morphological changes in bone tissues and autophagosomes and to detect the protein expression of autophagy-related markers and PI3K/AKT/mTOR signaling pathway-related molecules both *in vivo* and *in vitro*. The bone tissues of fluoride-exposed rats showed osteosclerosis, autophagosomes and autolysosomes. LC3B immunofluorescence staining revealed an increase in autophagosomes in the primary osteoblasts treated with fluoride. The LC3 Ⅱ/Ⅰ ratio and levels of autophagy-related markers (Beclin 1 and Atg7) were increased, whereas P62 levels were decreased in bone tissues and primary osteoblasts in the fluoride groups. Simultaneously, p-AKT and p-mTOR levels were reduced in bone tissues and primary osteoblasts in the fluoride groups. Moreover, a PI3K inhibitor (LY294002) further downregulated p-AKT and p-mTOR protein expression but slightly increased the LC3 Ⅱ/Ⅰ ratio in primary osteoblasts. These results demonstrate that fluoride induces autophagy in osteoblasts by inhibiting the PI3K/AKT/mTOR signaling pathway, which deepens our understanding of the molecular mechanisms underlying fluoride-induced bone damage and provides a theoretical basis for the prevention and treatment of skeletal fluorosis.

Keywords: Fluorosis, autophagy, PI3K/AKT/mTOR, bone damage, osteoblast, rat

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Introduction

Fluoride is a trace element essential for bone metabolism[1] that is found throughout the environment. Long-term consumption of excessive fluorine, such as that in drinking water, air, food, and other sources, causes fluorosis, which is a serious public health challenge.[2,3] Previous studies have confirmed the high fluoride sensitivity of bone.[4] Excessive fluoride accumulates mainly in bone, resulting in bone damage, dental fluorosis, and skeletal fluorosis.[5]

Bone damage caused by fluoride mainly manifests as disordered bone metabolism, affecting bone formation and absorption. Osteoblasts are crucial cells in bone metabolism that synthesize and secrete bone matrix and participate in bone mineralization during bone formation. Autophagy is a highly conserved intracellular lysosomal degradative process that plays important roles in various physiological and pathological processes.[6,7] Accumulating evidence indicates that autophagy plays an essential role in regulating bone homeostasis.[8–11] Osteoblast mineralization is accompanied by autophagy, and autophagy deficiency leads to a decrease in bone mineralization and trabecular bone mass *in vitro* and *in vivo*.[12] The targeted deletion of FIP200 (focal adhesion kinase family interacting protein of 200 kD), an

essential autophagy gene, significantly decreased autophagy and bone mass in rats, and reduced osteoblast differentiation, mineralization, and new bone matrix synthesis.[13] Mice with osteoblast-specific Atg7 conditional knockout showed a reduced bone mass during development and adulthood.[14]

Fluoride induces autophagy in various cells and organs, such as neurons, ameloblasts, testes, livers, and kidneys, and is shown by increased LC3, P62, and Beclin1 levels and autophagosomes.[15–20] Notably, previous studies have shown that fluoride also induces autophagy in osteoblasts, which may be involved in fluoride-induced bone damage.[21–24]

PI3K/AKT/mTOR is an important upstream signaling pathway for autophagy regulation.[25] Several studies have demonstrated that fluoride decreases PI3K and AKT levels, inhibits mTOR phosphorylation, and increases the levels of autophagy markers such as LC3, Beclin1, and Atg5 in leydig cells, splenocytes, and chondrocytes.[26–28]

The PI3K/AKT/mTOR signaling pathway plays a vital role in bone metabolism and autophagy.[29,30] However, knowledge regarding the mechanism by which the PI3K/AKT/mTOR signaling pathway regulates autophagy in bone damage caused by fluoride is limited. This study investigated the effects of fluoride on autophagy and explored the regulatory role of the PI3K/AKT/mTOR signaling pathway in rat bone tissues and primary osteoblasts. The results demonstrated that fluoride induced autophagy in the bone tissues of rats and primary osteoblasts, and that autophagy was regulated by inhibiting PI3K/AKT/mTOR signaling pathway activation. These findings contribute to a better understanding of the molecular mechanisms underlying skeletal fluorosis and provide a theoretical basis for its prevention and treatment.

Materials and methods

Animals and treatment

Forty-eight healthy Sprague–Dawley (SD) rats weighing 90 to 100 g were provided by the Animal Experimental Center of Guizhou Medical University. The rats were randomly divided into four groups (12 rats in each group, with 6 males and 6 females). The control group was given normal tap water containing less than 0.5 mg/L fluoride (F-). The chronic fluoride exposure groups were administered different concentrations of fluoride (5, 50, and 100 mg/L) for six months. Fluorine was obtained from sodium fluoride (NaF, Sigma, USA, S7920) and dissolved in tap water. Throughout the experimental period, the rats had free access to water. The Animal Care Welfare Committee of Guizhou Medical University approved all animal experimental procedures.

Dental fluorosis and measurement of the fluoride contents in bone and urine

After six months of fluoride exposure, dental fluorosis was diagnosed using a modified Dean's fluorosis classification system.[31] Teeth with white or pigmented bands were classified as I°, those with gray enamel as II°, and those that were cracked or had lost tooth structure as III°. In addition, 24-h urine samples were collected from all rats, and bone tissues were harvested, ground to a powder, and dissolved in hydrochloric acid. Following established protocols,[32] the fluoride content in the urine and bones was detected using the fluoride ion-selective electrode method.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining

The proximal tibia was fixed in 4% paraformaldehyde for 48 h, decalcified in a 10% ethylenediaminetetraacetic acid solution (EDTA, Solarbio, China) for approximately six weeks, and washed with running tap water for 24 h. Subsequently, the samples were dehydrated in gradient ethanol and embedded in paraffin wax. The resulting paraffin-embedded tibia was sliced in the sagittal plane into 4-μm sections, which were then dewaxed and rehydrated. H&E staining was performed with some sections, while other sections were treated with 3% hydrogen peroxide for 15 min to eliminate endogenous peroxidase. Antigen retrieval was carried out, and the sections were blocked with 10% goat serum (Bioss, China) at 37°C. The sections were then incubated overnight with a primary antibody against mTOR (phospho-Ser2448) (1:100, Assay Biotechnology, USA) at 4°C. The next day, the sections were incubated with peroxidase-conjugated secondary antibody (Bioss, China) and diaminobenzidine substrate (Boster, China). Hematoxylin was used to counterstain the slices. Negative controls were treated in the same way as the experimental samples, except that the primary antibody was replaced with phosphate-buffered saline (PBS). The slices were examined using a light microscope (Nikon, Japan) and photographed.

Transmission electron microscopy

Rat femurs were rapidly harvested and cut into pieces of approximately 1 mm^3, which were then fixed in 2.5% glutaraldehyde at 4°C for 24 h, and decalcified in a 10% EDTA solution for approximately 10 days. Then, the bone tissues were fixed in 1% OsO_4 for 1 h, dehydrated in gradient ethanol, and embedded in epoxy resin. Ultrathin sections were sliced, placed on copper grids, and then stained with 1% uranyl acetate and lead citrate. The sections were analyzed and photographed with a transmission electron microscope (Hitachi, Japan).

Isolation, culture and identification of primary osteoblasts

Primary osteoblasts were isolated and cultured as described previously with minor modifications.[24,33] Briefly, calvaria were dissected from neonatal SD rats within 24 h of birth, and the connective tissues were removed, cut into pieces under sterile conditions, rinsed twice in cold PBS, and sequentially digested in 0.25% trypsin (Solarbio, China) for 20 min followed by 1 mg/mL collagenase type I (Solarbio, China) for 90 min at 37°C. The digested cells were harvested, counted, and cultured in complete growth medium supplemented with 10% fetal bovine serum (Tianhang Biotechnology, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio, China) at 37°C in a humidified atmosphere containing 5% CO_2. Osteoblasts from the third passage were used for

all experiments. A chromogenic alkaline phosphatase assay (BCIP/NBT chromogenic kit: Solarbio, China) and Alizarin Red S staining (Solarbio, China) were performed to identify osteoblasts. Osteoblasts with a purity greater than 90% were used for further experiments and treated with 0.5, 1.0, or 3.0 mM fluoride (NaF solution: Sigma, USA).

Immunofluorescence staining

Immunofluorescence staining was performed to detect the effect of fluoride on autophagy in osteoblasts. In summary, osteoblasts were fixed with 4% paraformaldehyde at room temperature for 15 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 for 15 min. Nonspecific antigen-binding sites were blocked with 5% bovine serum albumin (BSA) at room temperature for 30 min. The osteoblasts were incubated with an anti-LC3B antibody (1:100, Cell Signaling Technology, USA) for 2 h and then with secondary antibodies conjugated to Alexa Fluor 488 (Beyotime, China) for 1 h in a dark chamber, and the nuclei were counterstained with 0.1% DAPI (Solarbio, China) for 5 min at room temperature. The immunofluorescence signals were analyzed with a fluorescence microscope (Olympus, Japan).

Western blotting

Bone powder from triturated femurs and primary osteoblasts was prepared in RIPA lysis buffer (Solarbio, China) supplemented with protease and phosphatase inhibitors (Kangchen, China) on ice. Proteins were quantitated with a BCA protein assay kit (Beyotime, China), separated by SDS–PAGE in gels of different concentrations, and then transferred to PVDF membranes. After blocking in 5% skimmed dry milk for 2 h, the membranes were incubated with specific primary antibodies overnight at 4°C. The next day, the membranes were incubated with horseradish peroxidase-conjugated (HRP)-conjugated secondary antibodies for 1 h. The proteins on the blots were visualized with an enhanced chemiluminescence detection system (Millipore, USA) and exposed to film. The intensity of each protein band was analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA), and β-actin was used as an internal reference to evaluate the relative expression levels of the proteins. The primary antibodies used were as follows: anti-P62 (1:1000, Abcam, UK), anti-Beclin1, anti-Atg7, anti-PI3K, anti-AKT, anti-mTOR (1:1000, Cell Signaling Technology, USA), anti-LC3, anti-p-AKT, anti-p-mTOR (1:500, Cell Signaling Technology, USA), and anti-β-actin (1:10,000, Affinity Biosciences, USA).

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) was used for multiple comparisons when the data followed a normal distribution, and the Kruskal–Wallis test was used when the data did not follow a normal distribution. Statistical significance was considered at a P value less than 0.05. All experiments were repeated at least three times.

Table 1. The incidence of dental fluorosis was evaluated in rats exposed to fluoride.

Groups	Number	Dental fluorosis (degree)				Incidence (%)
		Normal	Ⅰ°	Ⅱ°	Ⅲ°	
Control	12	12	0	0	0	0
5 mg/L Fluoride	12	3	8	1	0	75.0%
50 mg/L Fluoride	12	1	5	6	0	91.7%
100 mg/L Fluoride	12	0	3	6	3	100.0%

Results

Establishment of the chronic fluorosis rat model

After six months of fluoride exposure, the fluoride treatment groups showed dose-dependent damage, as presented in Table 1. Furthermore, the fluoride concentrations in the urine and bone of the rats were significantly and dose-dependently increased (Figure 1(A) and (B)). These results suggest that the rat model of chronic fluorosis was successfully established.

Pathological changes in bone tissue of the rats

Analysis of the bone tissue revealed pathological changes, with thicker bone trabeculae and an increased trabecular area, indicating osteosclerosis (Figure 2(A) to (C)).

Effects of fluoride on the protein expression levels of autophagy-related markers and autophagosomes in bone tissues

To quantitatively assess autophagy, the protein levels of autophagy-related markers were examined by western blotting (Figure 3(A) to (E)). Fluoride exposure significantly increased the LC3 Ⅱ/Ⅰ ratio and the protein expression levels of Beclin1 and Atg7 in bone tissues compared to those in the control group. However, the protein level of P62 was significantly reduced, indicating that fluoride exposure triggered autophagy in the bone tissues of rats.

To find morphological evidence of fluorine-induced autophagy in bone tissues, autophagosomes and autolysosomes were observed by transmission electron microscopy (TEM). Ultrastructural analysis revealed autophagosomes and autolysosomes in the fluoride groups (Figure 4(A) to (D)). This result further demonstrated that fluoride induced autophagy in the bone tissues of rats.

Identification of primary osteoblasts in rat calvaria

To confirm the identity of the isolated cells as osteoblasts, the morphological characteristics of second-generation primary osteoblasts from rat calvaria were observed. The cells were approximately triangular, polygonal or irregular in shape, with abundant cytoplasm containing black particles and a large nucleus (Figure 5(A)). Furthermore, the cells were stained with an alkaline phosphatase substrate for a chromogenic assay (BCIP/NBT) and Alizarin Red S staining. The cells exhibited high alkaline phosphatase activity, and blue granules were observed in the cytoplasm (Figure 5(B)), indicating that more than 90% of the cells were osteoblasts.

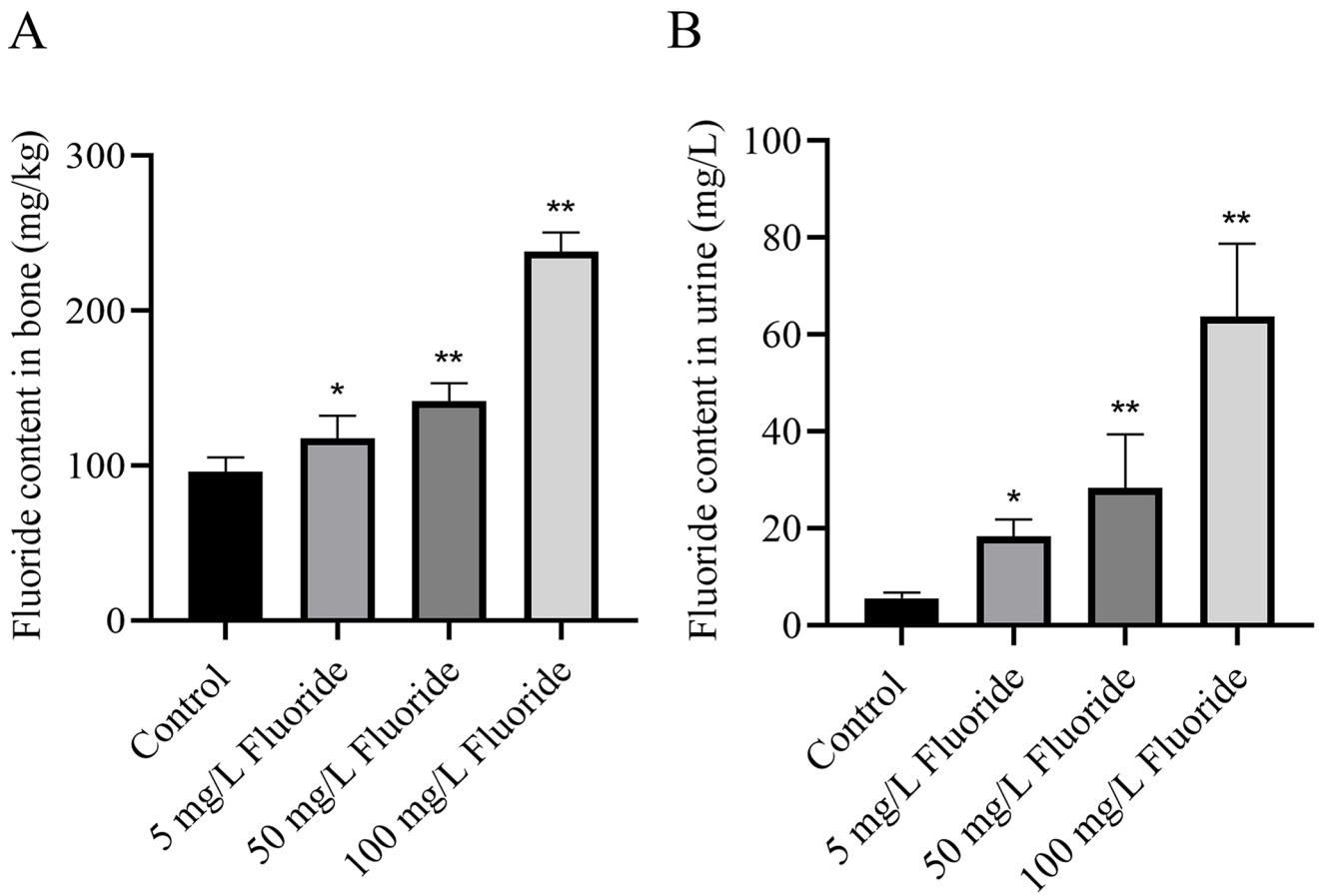

Figure 1. The levels of fluoride in the bone and urine of both control and fluoride-exposed rats. (A) Fluoride content in bone. (B) Fluoride content in urine. Data are presented as the means ± SDs.
*$P < 0.05$, **$P < 0.01$ in comparison to the corresponding control groups.

The cultured osteoblasts exhibited osteogenic characteristics, and opaque orange–red calcified nodules were observed by Alizarin Red S staining after 28 days of culture (Figure 5(C)).

Effects of fluoride on autophagy in primary osteoblasts

To verify the induction of autophagy by fluoride exposure, primary osteoblasts were treated with 0.5, 1.0, or 3.0 mM fluoride for 24 h, and the resulting autophagosomes (LC3B puncta) were visualized by immunofluorescence staining. The results showed a significant increase in LC3B puncta in the primary osteoblasts treated with 1.0 mM and 3.0 mM fluoride compared to the control group (Figure 6(A) and (B)). Western blotting also revealed a marked increase in the ratio of LC3II/I, as well as in the protein expression levels of Beclin1 and Atg7, and a significant reduction in P62 protein expression in the groups exposed to 0.5, 1.0, and 3.0 mM fluoride (Figure 7(A) to (E)). The changes in the protein expression of autophagy-related markers in the primary osteoblasts were similar to those observed in bone tissues, confirming that fluoride exposure induced autophagy in osteoblasts *in vitro*.

Effects of fluoride on the protein expression levels of p-mTOR *in vivo* and *in vitro*

mTOR is known to inhibit autophagy, and its expression was assessed in both bone tissues and primary osteoblasts through immunohistochemical staining and western blotting. Immunohistochemical staining allowed for direct observation of the location and level of p-mTOR expression. In osteoblasts obtained from rats with chronic fluorosis, the protein expression level of p-mTOR decreased with increasing fluoride concentration compared to that in the control group *in vivo* (Figure 8(A) and (B)). In addition, western blotting revealed a significant decrease in the ratio of p-mTOR (Ser2448) to total mTOR in primary osteoblasts treated with 1.0 and 3.0 mM fluoride compared to the control group *in vitro* (Figure 8(C) and (D)).

Effects of fluoride on the protein expression of related regulatory molecules upstream of mTOR

The PI3K/AKT pathway is a key upstream regulator of mTOR, and the expression of its members was assessed in primary osteoblasts treated with 0.5, 1.0, or 3.0 mM fluoride for 24 h by western blotting. No significant difference in total PI3K protein expression was found between the fluoride groups and the control group (Figure 9(A) and (B)). However, the ratio of p-AKT (Thr308) to total AKT was significantly decreased in the 1.0 and 3.0 mM fluoride groups compared to the control group (Figure 9(A) and (C)). In addition, the decrease in the p-mTOR/mTOR ratio was more pronounced than that in the p-AKT/AKT ratio in primary osteoblasts treated with fluoride (Figures 8 and 9).

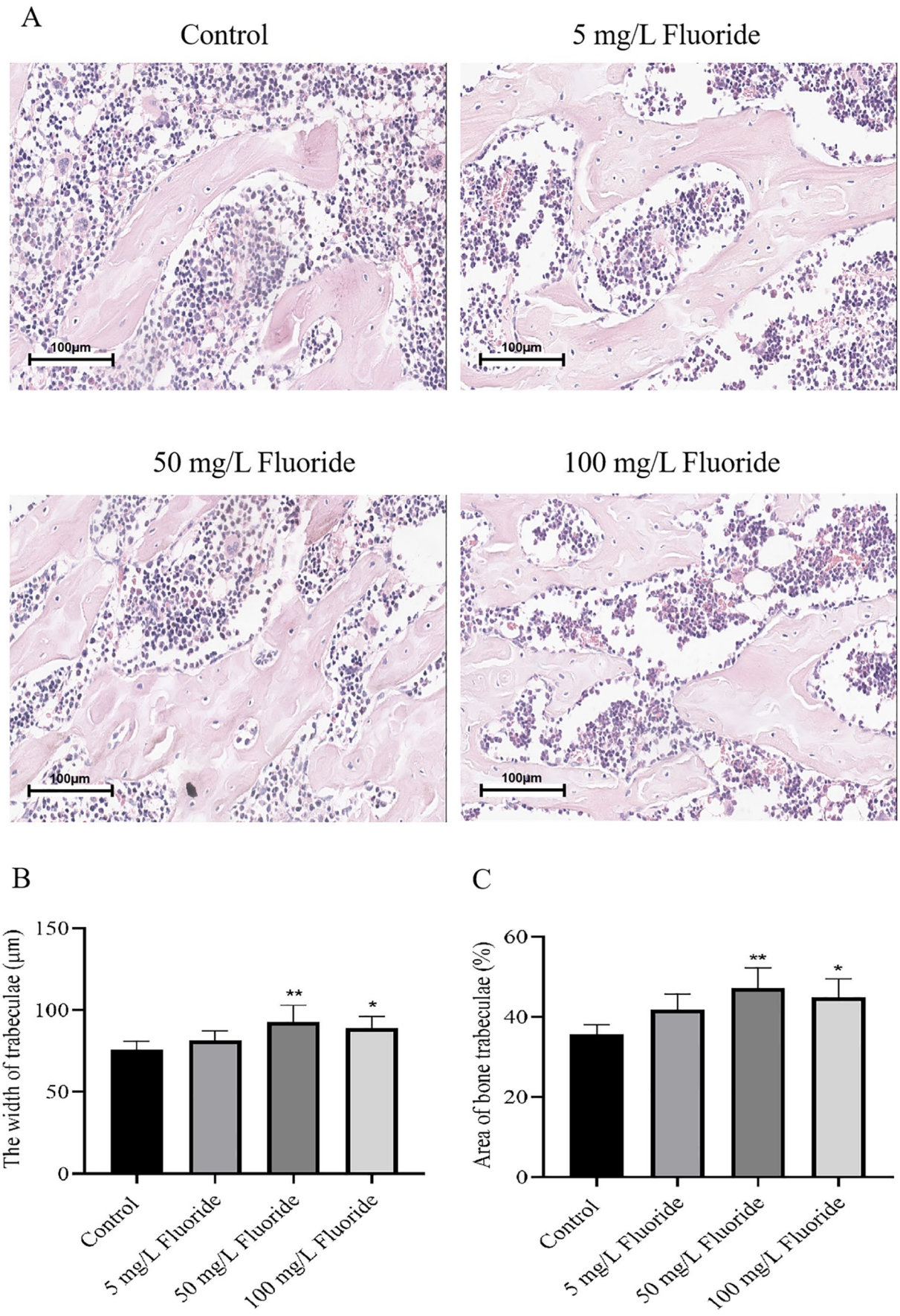

Figure 2. The pathological changes in bone tissues of the control and fluoride-exposed rats, revealed by H&E staining. (A) Representative images of bone tissues. Scale bar, 100 μm. (B) The width of the bone trabeculae. (C) The area of the bone trabeculae. Data are presented as the means ± SDs. H&E: hematoxylin & eosin. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to the corresponding control groups.

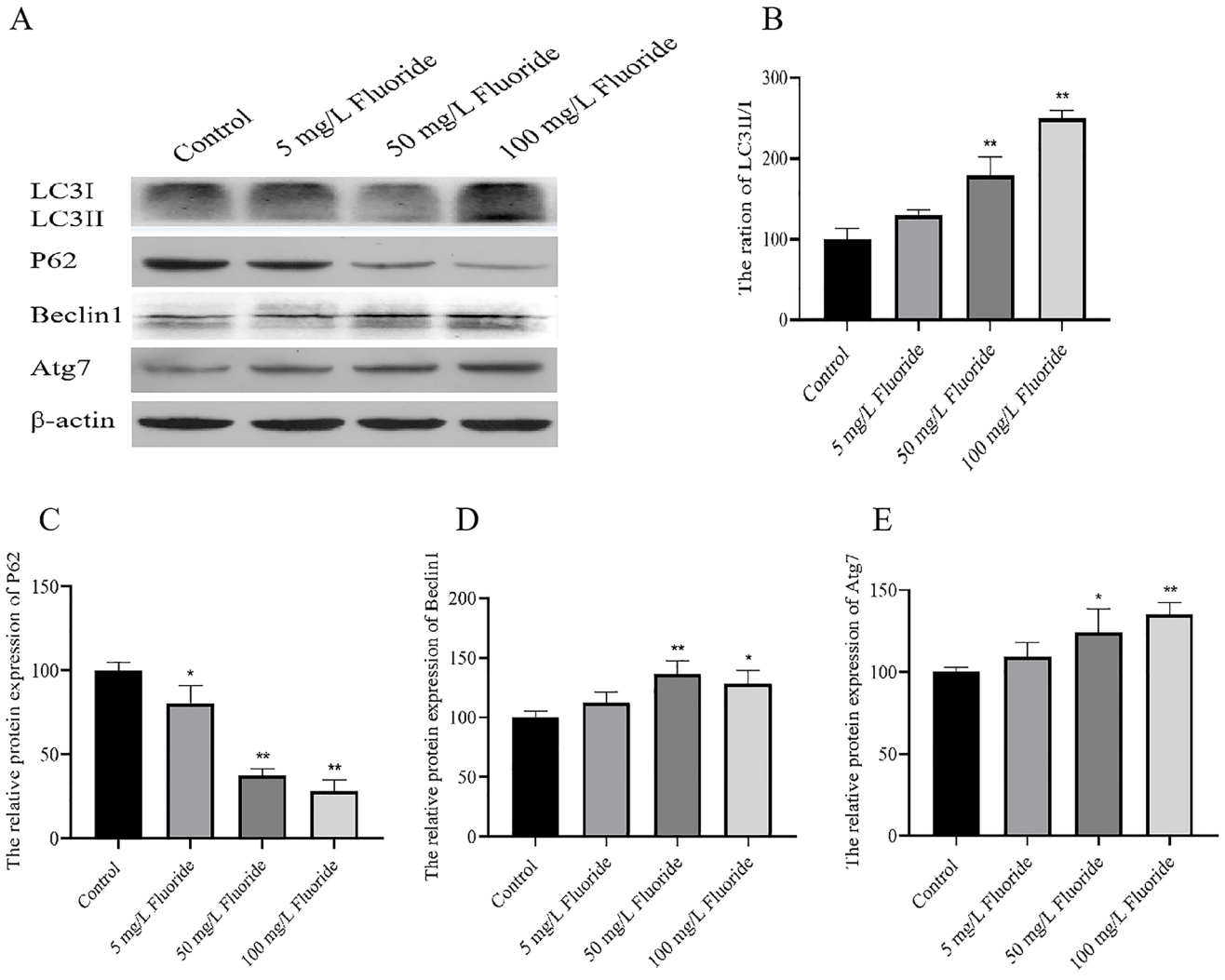

Figure 3. Effects of fluoride on the protein expression levels of autophagy-related markers in bone tissues of rats exposed to fluoride for six months. The ratio of LC3II/I (A and B) and the protein expression of the autophagy-associated markers P62 (A and C), Beclin1 (A and D), and Atg7 (A and E) were determined by western blotting. Data are presented as the means ± SDs. Immunoblot images are shown with bar graphs.
$P < 0.05$,$P < 0.01$ in comparison to the corresponding control groups.

To further investigate the role of the PI3K/AKT/mTOR pathway in fluoride-induced autophagy, primary osteoblasts were pretreated with the PI3K inhibitor LY294002 and then with 3.0 mM fluoride, and the levels of phosphorylated AKT and mTOR and the LC3 Ⅱ/I ratio were assessed through western blotting. The results showed a significant decrease in the ratios of p-AKT (Thr308) to total AKT and p-mTOR (Ser2448) to total mTOR in both the fluoride group and the fluoride with LY294002 group compared to the control group (Figure 10(A) to (C)). Correspondingly, the LC3 Ⅱ/I ratio was significantly increased in both the fluoride group and the fluoride with LY group compared to the control group (Figure 10(D) and (E)). Notably, combined treatment with fluoride and LY294002 further reduced the levels of phosphorylated AKT and mTOR, increased the ratio of the autophagy marker proteins LC3 Ⅱ/I, and induced autophagy to some extent.

Discussion

Fluoride toxicity primarily affects bone.[28] The most severe form of damage caused by fluoride is skeletal fluorosis. However, the molecular mechanisms underlying bone damage due to excessive fluoride exposure remain unclear. To shed light on this issue, we conducted a study using a chronic fluorosis rat model and primary osteoblasts isolated from rat calvaria. Our objective was to investigate the effects of fluoride on osteoblast autophagy and identify the possible regulatory mechanisms. Our results revealed that fluoride inhibits activation of the PI3K/AKT/mTOR signaling pathway in osteoblasts, thereby subsequently promoting autophagy.

In this study, we successfully established a rat model of chronic fluorosis by administering fluoride water to rats for six months. The fluoride-exposed rats exhibited dose-dependent dental fluorosis and increased fluoride levels in their bone and urine. In addition, the observed pathological changes in the bone tissue were consistent with previous reports, showing increased bone mass and osteosclerosis.[34]

Autophagy is responsible for degrading and eliminating damaged organelles and macromolecules in eukaryotic cells and is essential for maintaining intracellular homeostasis.[35] Moderate autophagy is a protective mechanism against

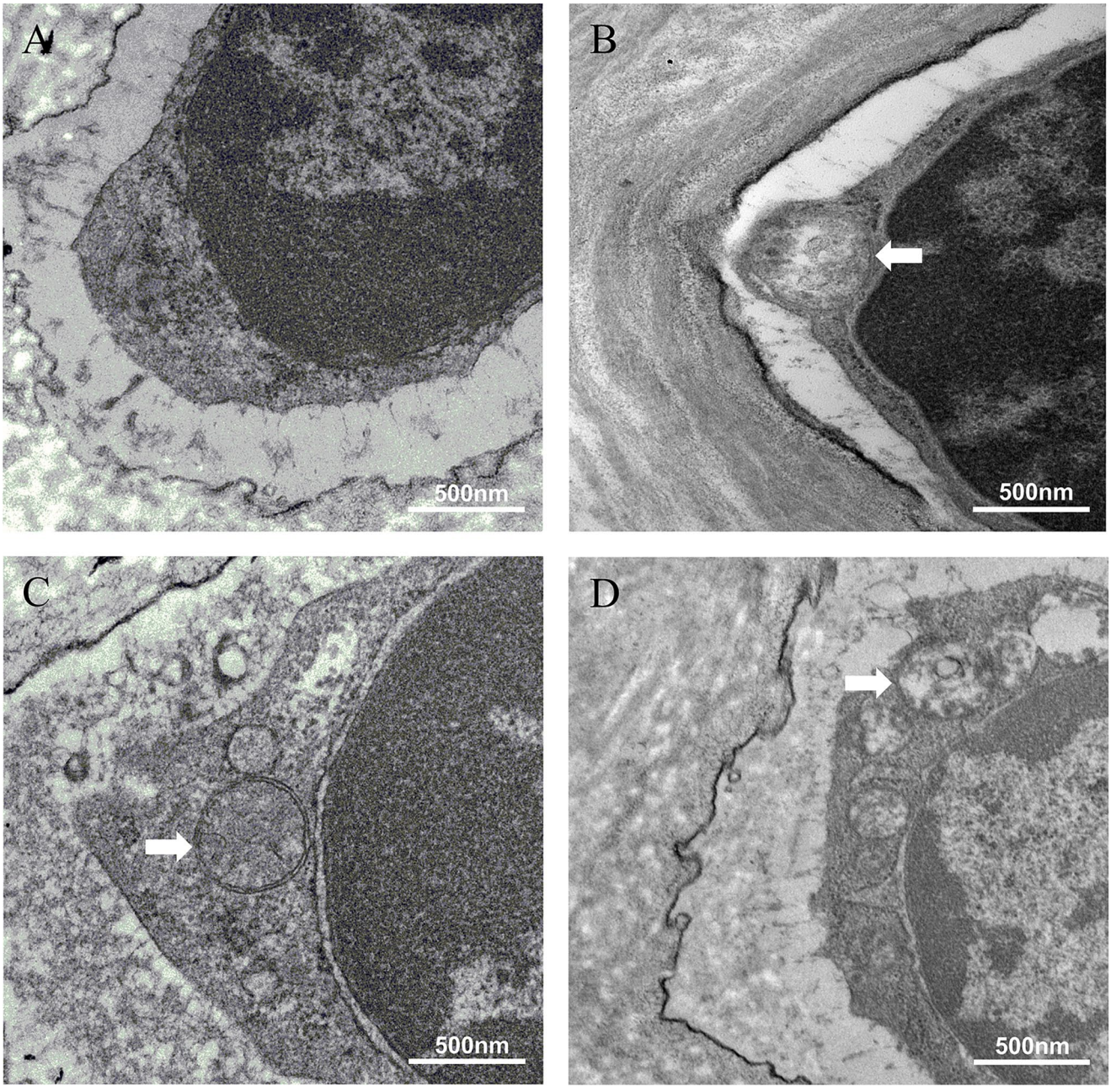

Figure 4. Transmission electron microscopy (TEM) revealed autophagosomes and autolysosomes in the bone tissues of rats exposed to fluoride for six months. (A) Control, (B) 5 mg/L fluoride group, (C) 50 mg/L fluoride group, and (D) 100 mg/L fluoride group. The white arrow indicates a typical autophagosome and autolysosome. Scale bar, 500 nm.

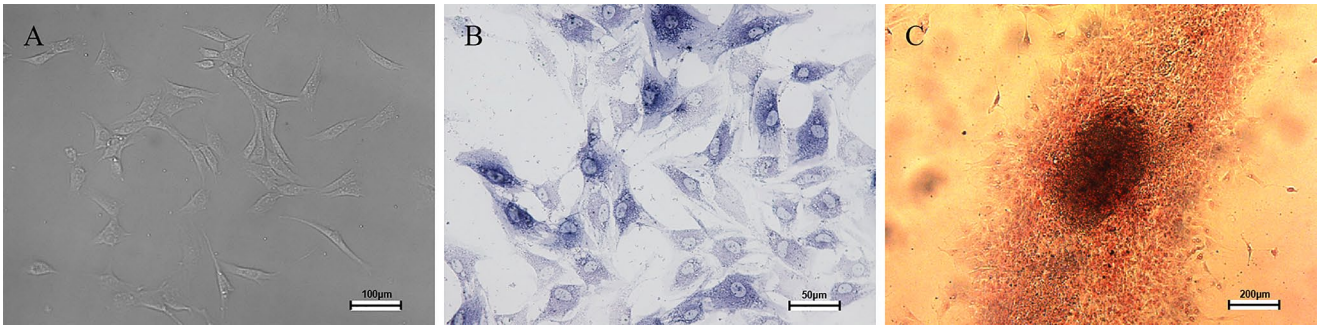

Figure 5. Identification of osteoblasts by morphological assessment, the chromogenic alkaline phosphatase assay (BCIP/NBT) and Alizarin Red S staining. Second-generation osteoblasts are shown in (A) at scale bar of 100 μm. These cells displayed high alkaline phosphatase activity and blue granules in the cytoplasm, as demonstrated in (B) at a scale bar of 50 μm. Orange–red calcified nodules were observed, as demonstrated in (C) at a scale bar of 200 μm.

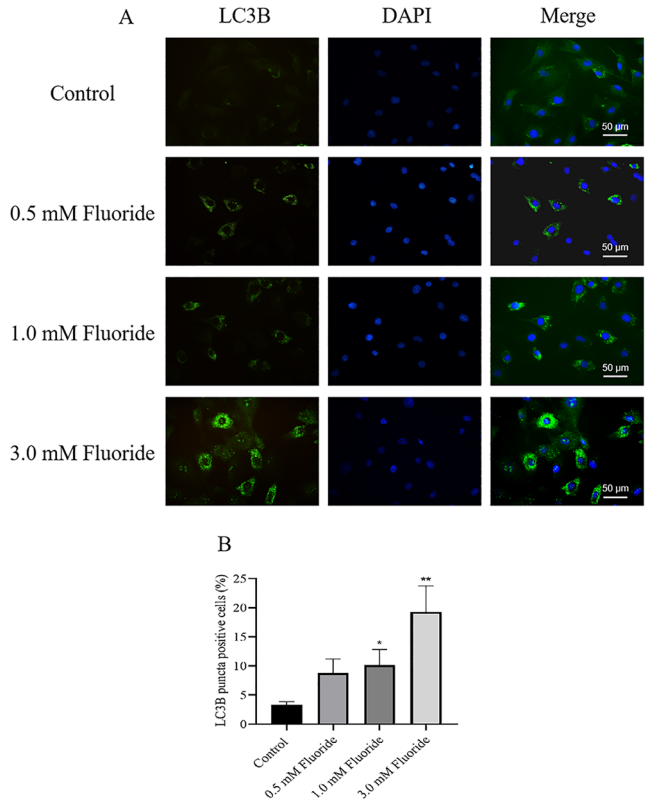

Figure 6. Immunofluorescence staining was used to detect LC3B puncta, a marker of autophagosomes, in primary osteoblasts treated with different concentrations of fluoride. (A) The images show LC3B puncta in osteoblasts at a scale bar of 50 μm. (B) The number of LC3B puncta-positive cells was determined by counting microscopic fields under higher magnification. Data are presented as the means ± SDs.
*$P < 0.05$, **$P < 0.01$ in comparison to the corresponding control groups.

various stress stimuli.[36] However, excessive autophagy or insufficient autophagy can result in cell dysfunction and even cell death.[7,25] Autophagy can be induced by several environmental factors that cause oxidative stress.[27,37] Studies have shown that fluoride exposure can trigger autophagy as it causes toxic damage. Excessive fluoride exposure has been shown to impair autophagy, leading to the accumulation of autophagosomes and blockade of autophagic flux, which has been linked to fluoride-induced neurotoxicity in rats and SH-SY5Y cells.[15,16] Similarly, abnormal autophagy has been observed in rat testicular cells,[18] suggesting that autophagy is a crucial mechanism involved in fluoride-induced organ toxicity. Interestingly, in the context of fluoride-induced bone damage, autophagy may play a protective role.[21,24] Upregulation of autophagy in MC3T3-E1 cells was indirectly observed through an increase in SIRT1 expression in response to fluoride exposure, leading to the significant attenuation of apoptosis.[22] Moreover, the inhibition of fluoride-induced autophagy resulted in increased apoptosis.[23]

Autophagy is regulated by autophagy-associated proteins such as LC3, Beclin1, Atg7, and P62, which are commonly used as markers to monitor autophagy in various studies.[25] During autophagy, the precursor of LC3 is converted to the cytosolic form, LC3I, which is then converted into the membrane-bound form, LC3II, by conjugation with phosphatidylethanolamine (PE) on the autophagosome membrane, promoting autophagosome formation.[38,39] LC3II is the most important marker of autophagosomes, and an increase in the LC3II/I ratio is considered a sign of autophagic activity. P62, a ubiquitin-binding protein, mediates the binding of ubiquitinated proteins to autophagosomes, which is eventually degraded in autolysosomes. When autophagy is activated, the P62 protein level is normally decreased.[40] However, when P62 degradation is blocked, the P62 protein accumulates and increases with the LC3 level, resulting in autophagy dysfunction.[17] Therefore, LC3 and P62 are routinely detected and simultaneously evaluated as indicators of autophagic flux. Beclin1 is a well-characterized and crucial regulator of autophagy and is specifically involved in the initiation of autophagy and the formation of autophagosomes.[41,42] Atg7 plays crucial roles in regulating autophagy and participates in two conjugated systems, Atg12-Atg5 and LC3-PE, to regulate the elongation of autophagosomal membranes and the maturation of autophagosomes.[43–45] Previous reports have shown that fluoride induces autophagy by increasing the LC3II/I ratio and Beclin l expression, as well as by downregulating P62 expression in osteoblasts.[21,33] Consistent with previous studies, this study shows that the protein expression levels of the autophagic markers LC3II, Beclin1, and Atg7 were significantly increased, while the expression level of P62 was decreased in bone tissue and primary osteoblasts treated with fluoride, as determined by western blotting. Autophagosomes are the gold standard for confirming autophagy. In this study, autophagosomes with a bilayer membrane and autolysosomes with a monolayer membrane in the bone tissues of rats in the fluoride group were observed by TEM. In primary osteoblasts treated with fluoride, LC3B puncta, a marker of autophagosome formation, was widely detected by immunofluorescence staining. These results indicated that fluoride induced autophagy in both bone tissue and primary osteoblasts.

A previous study demonstrated that at higher concentrations, fluoride can induce excessive autophagy and cause damage to MC3T3-E1 osteoblastic cells.[23] Based on the biphasic role of fluoride and the histopathological changes in skeletal fluorosis, it has been hypothesized that high concentrations of fluoride may induce excessive autophagy and apoptosis in osteoblasts, ultimately leading to osteoporosis. Conversely, low concentrations of fluoride may induce moderate autophagy in osteoblasts, promoting osteoblast proliferation and differentiation and resulting in osteosclerosis. However, as autophagy plays a dual role, understanding its effect on fluoride-induced bone damage still requires further research.

The PI3K/AKT/mTOR signaling pathway is involved in various pathophysiological processes.[46] Three isoforms of PI3K are expressed in mammalian cells. PI3K type I activates AKT through phosphorylation. The activation of AKT initiates a series of events to trigger mTOR activation and then regulate a variety of biological processes. The PI3K/AKT signaling pathway is associated with bone metabolism. Calcium alleviates fluoride-induced bone damage via

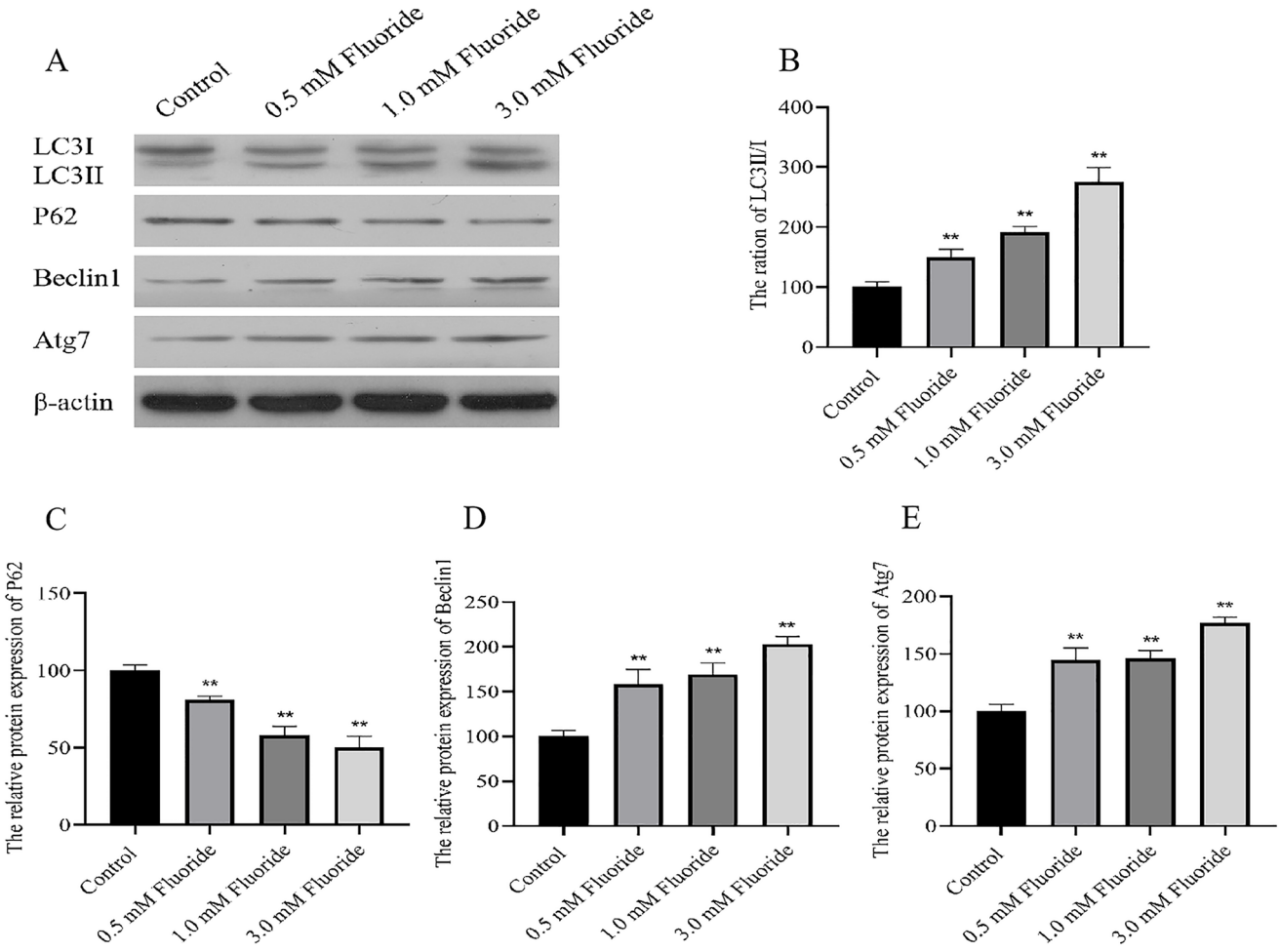

Figure 7. Effects of fluoride on the protein expression levels of autophagy-related markers in primary osteoblasts. The ratio of LC3II/I (A and B) and the protein expression of the autophagy-associated markers P62 (A and C), Beclin1 (A and D), and Atg7 (A and E) were determined by western blotting. Data are presented as the means ± SDs. Immunoblot images are shown with bar graphs.
$^{**}P < 0.01$ in comparison to the corresponding control groups.

the PI3K/AKT signaling pathway.[47] The PI3K/AKT signaling pathway may be involved in the excessive proliferation and differentiation of osteoblasts in rats with chronic fluorosis, resulting in excessive bone mass and osteosclerosis. Pinocembrin alleviated glucocorticoid-induced cell viability injury and apoptosis, inhibited the PI3K/AKT/mTOR pathway, and activated autophagy by increasing Beclin1 and LC3 levels and decreasing P62 levels in mouse long bone cells Y4 (MLO-Y4).[29] Furthermore, excessive fluoride exposure decreased the mRNA expression of PI3K and AKT, inhibited phosphorylation of the mTOR protein, and increased the mRNA and protein expression of the autophagy markers LC3, Beclin1 and Atg5 in mouse leydig cells and splenocytes and increased the number of autophagosomes.[26,27] Similarly, fluoride significantly downregulated PI3K, AKT, mTOR, 4EBP1, and S6K1 expression in the mouse ATDC5 chondrogenic cell line and facilitated autophagy. MHY1485, a small-molecule mTOR activator, completely reversed the fluoride-induced increase in autophagy activity.[28] These studies revealed that the PI3K/AKT/mTOR signaling pathway negatively regulates autophagy induced by excessive fluoride to some extent. Based on the aforementioned findings, it was hypothesized that fluoride induces autophagy via the PI3K/AKT/mTOR signaling pathway in osteoblasts. In this study, changes in the expression and phosphorylation of key proteins in the PI3K/AKT/mTOR signaling pathway in bone tissues from rats with fluorosis and primary osteoblasts were detected by immunohistochemical staining and western blotting, respectively. The levels of the phosphorylated AKT and mTOR proteins decreased with increasing fluoride concentration, which is in agreement with previous reports. Moreover, in primary osteoblasts, the decrease in phosphorylation of the AKT and mTOR proteins with increasing fluoride concentration is consistent with the fluoride-induced increase in autophagy activity, which demonstrated that fluoride may induce autophagy in osteoblasts by inhibiting the PI3K/AKT/mTOR signaling pathway.

LY294002 is a PI3K type I inhibitor that targets the catalytic subunit of PI3K and blocks the activation of AKT. This compound is widely used in studies of the PI3K/AKT signaling pathway. In this study, LY294002 was used to explore the relationship between the PI3K/AKT/mTOR signaling

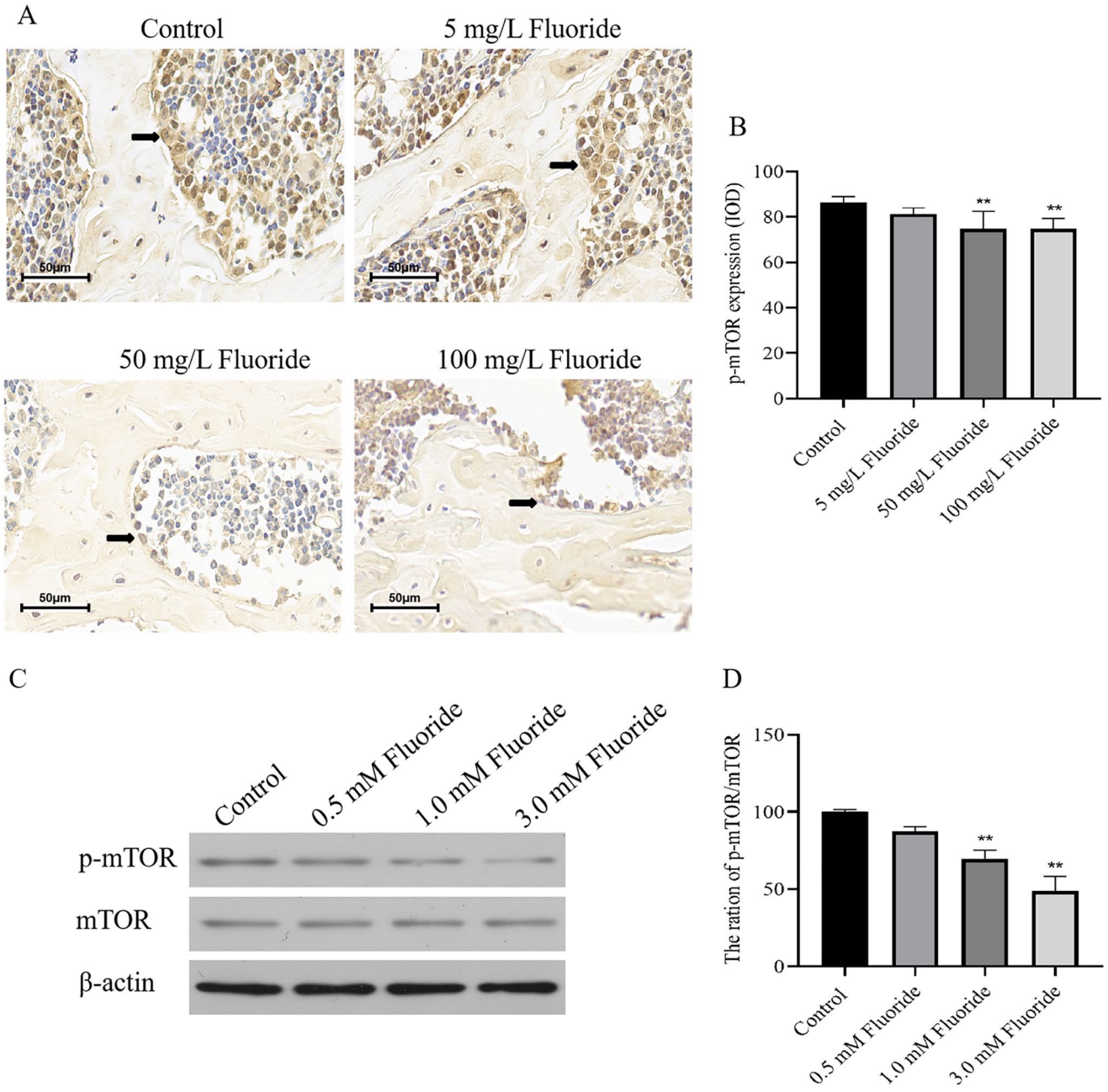

Figure 8. Effects of fluoride on p-mTOR protein expression in bone tissues and primary osteoblasts. (A) p-mTOR expression was determined by immunohistochemical staining. Black arrows indicate p-mTOR-positive osteoblasts. Scale bar, 50 μm. (B) Integrated optical density (IOD) of p-mTOR-positive osteoblasts in the bone tissue of rats exposed to fluoride. (C and D) The expression of p-mTOR was determined by western blotting in primary osteoblasts. Data are presented as the means ± SDs. Immunoblot images are shown with bar graphs.
**$P<0.01$ in comparison to the corresponding control groups.

pathway and autophagy. LY294002 increased fluoride-induced autophagy in primary osteoblasts, indicating that fluoride promotes autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway.

Further investigations into the role of the PI3K/AKT/mTOR signaling pathway in skeletal fluorosis could provide valuable insights into the pathogenesis of this disease and lead to the development of new therapeutic strategies. It is possible that targeted inhibition of this pathway could prevent or ameliorate fluoride-induced bone damage by regulating autophagy and other downstream effector molecules, as evidenced by a recent study in which targeted drugs were used to interfere with this signaling pathway in the treatment of a variety of tumors.[48] However, it is important to note that any potential therapeutic interventions must be carefully evaluated for safety and efficacy before they can be used in clinical settings. Thus, future studies should continue to explore the molecular mechanisms underlying skeletal fluorosis and identify new targets for therapeutic intervention.

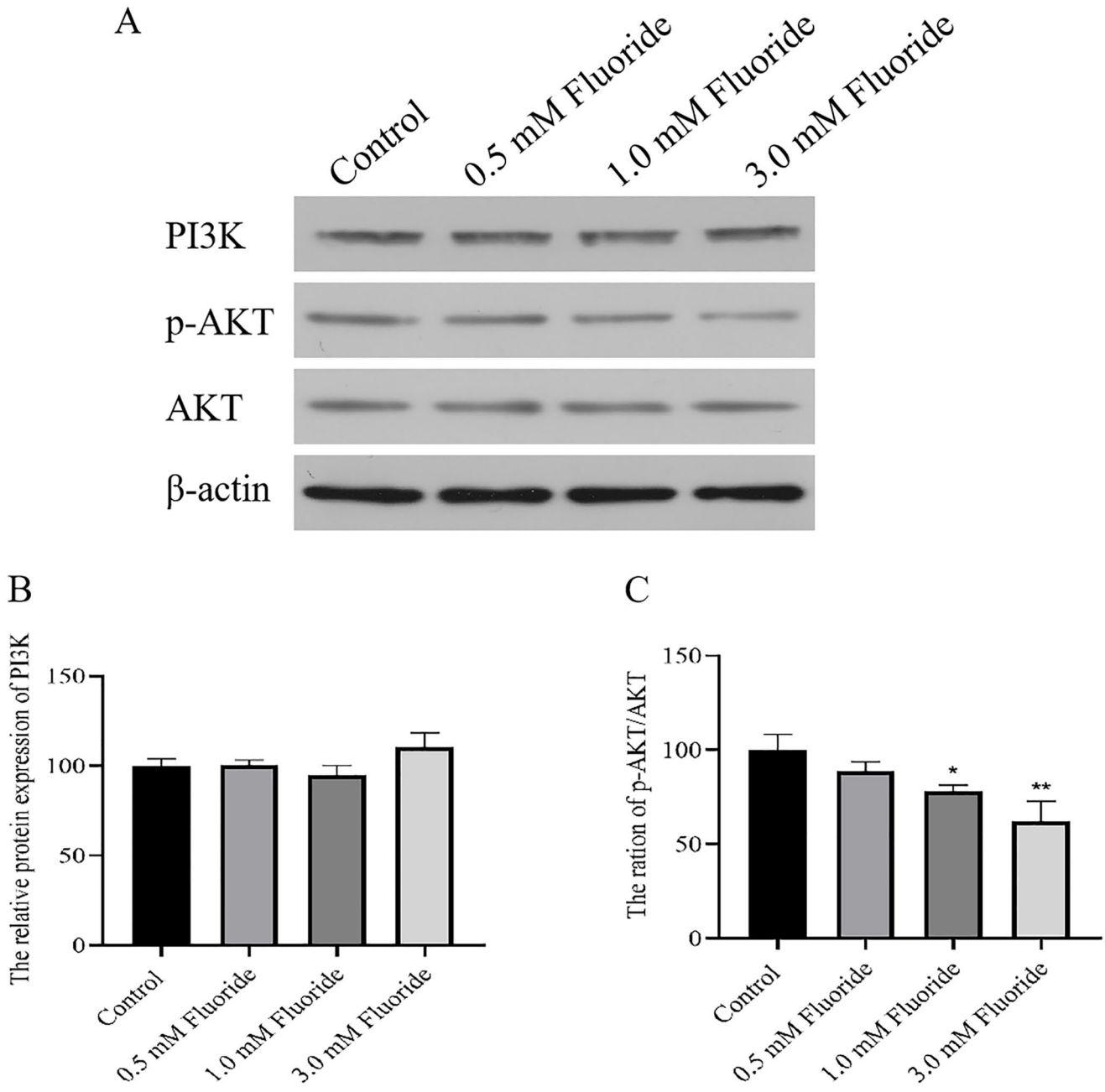

Figure 9. Effects of fluoride on the protein expression of PI3K and AKT in primary osteoblasts. Western blotting was performed to detect the PI3K protein level and the protein expression of p-AKT. Data are presented as the means ± SDs. Immunoblot images are shown with bar graphs.
$P < 0.05$,$P < 0.01$ in comparison to the corresponding control groups.

The PI3K/AKT/mTOR signaling pathway is a complex and interconnected network. mTOR is considered to be a central link in the regulation of autophagy, which is regulated by various upstream and downstream signaling molecules. Fluoride may simultaneously activate multiple autophagy-related signaling pathways, which jointly modulate mTOR expression, to ultimately determine autophagy activity. The bone damage caused by fluoride involves complex molecular regulation. Therefore, further investigations should be designed to fully elucidate the precise molecular mechanisms underlying fluoride-induced autophagy and its impact on bone health.

Conclusions

The study suggests that excessive exposure to fluoride can cause osteosclerosis, increase the expression levels of autophagy-related proteins, and decrease the protein expression of p-AKT and p-mTOR. These results suggest that fluoride induces autophagy in osteoblasts by inhibiting the PI3K/AKT/mTOR signaling pathway. The findings of this study deepen our understanding of the molecular mechanisms underlying fluoride-induced bone damage and provide a theoretical basis for the prevention and treatment of skeletal fluorosis.

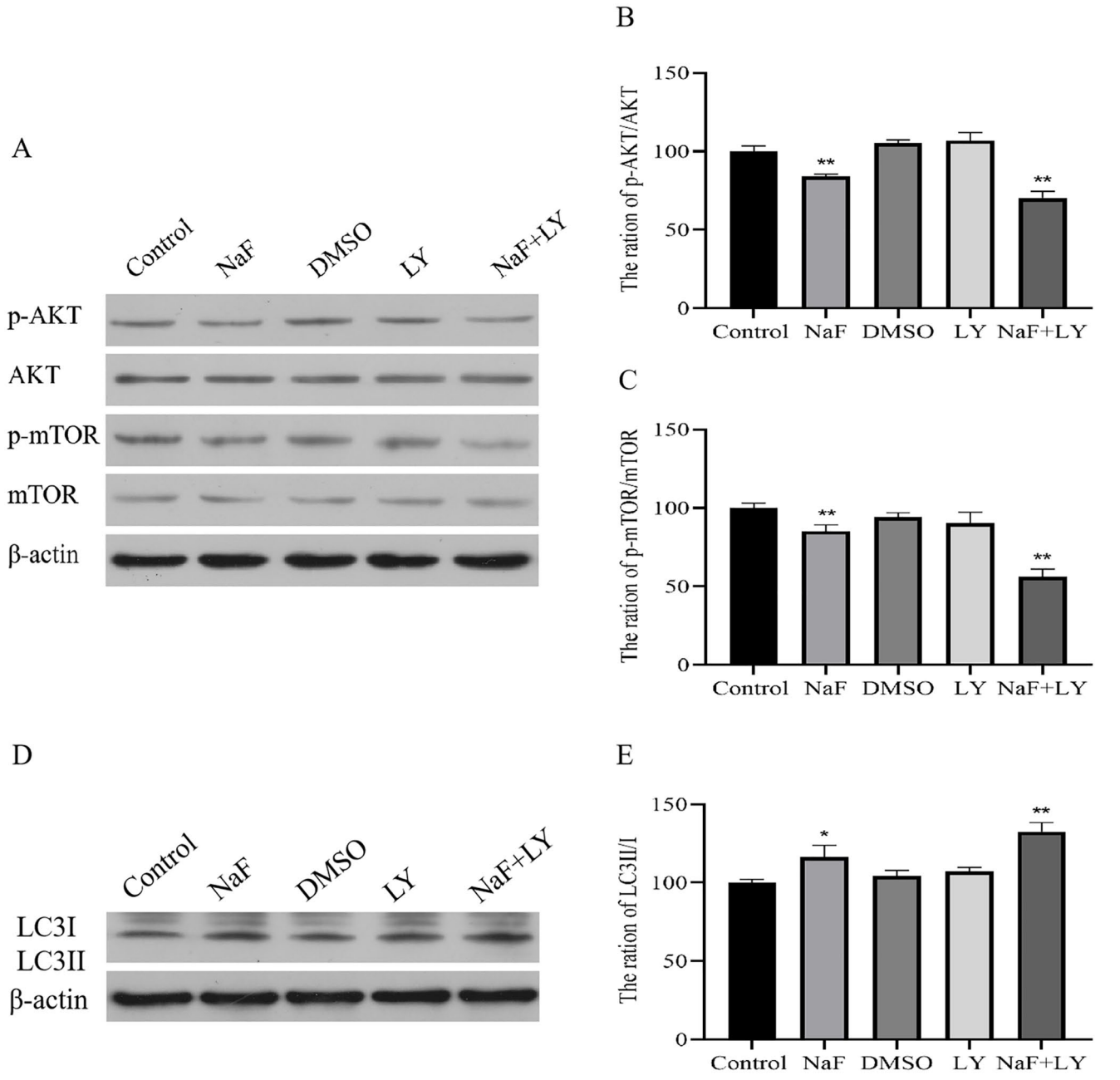

Figure 10. The effects of the autophagy inhibitor LY294002 (LY) on the levels of p-AKT (A and B), p-mTOR (A and C), and the autophagy marker LC3 (D and E) in primary osteoblasts are presented as the means ± SDs. The statistical significance of the data is denoted by asterisks (*$P < 0.05$, **$P < 0.01$) when compared to the corresponding control groups. The bar graphs are accompanied by western blotting images.

AUTHORS' CONTRIBUTIONS

YLH and YNY conceived of and designed the research. YLH, LH, QW, and LX performed the experiments. CND analyzed and interpreted the data. YLH drafted and revised the manuscript. YNY reviewed the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID ID

Yan-Ni Yu https://orcid.org/0000-0001-8338-4158

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