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Decreased Autophagy, Enhanced Apoptosis and Raised Intracellular Calcium in Astrocytes Exposed to Excessive Fluoride as well as the Attenuated Effect of Ifenprodil on the Neurotoxicity

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ABSTRACT

Purpose: The neurotoxic influence of fluoride on the levels of autophagy, apoptosis and intracellular calcium in astrocytes, as well as the neuroprotective effect of Ifenprodil, an inhibitor of N-methyl-D-aspartate receptor (NMDAR), were investigated.

Methods: The primary astrocytes identified by immunofluorescence staining were divided randomly into 6 groups, i.e., control (no treatment), low dose fluoride (exposed with 0.1 mmol/l F^-, prepared with NaF, in medium), high dose fluoride (1 mmol/l F^-), Ifenprodil treatment (10 μmol/l), low fluoride plus Ifenprodil, and high fluoride plus Ifenprodil. The subcellular structure of astrocytes was observed under transmission electron microscope (TEM); the expressions of autophagy-related proteins detected by Western Blotting; the distributions of intracellular calcium by laser confocal scanning; and the level of apoptosis by flow cytometry.

Results: The results showed that in the astrocytes with the increased exposure of fluoride, the swelling or expansion of mitochondria and endoplasmic reticulum under TEM were observed; the expressions of autophagy-related proteins ULK1 (autophagy-activating UNC-51-like kinase 1), Beclin1 (BECN1) and LC3 (microtubule-associated protein 1 light chain 3) decreased, but p62 increased; the levels of late apoptosis increased; and the content of intracellular calcium raised. Interestingly, Ifenprodil treatment attenuated these changes occurred in astrocytes resulted from fluoride exposure.

Conclusions: The results indicated that excessive fluoride induced the modified autophagy and neurotoxic alterations of astrocytes, whereas Ifenprodil played a neuroprotective effect, suggesting that the neuropathological changes in astrocytes resulted from fluorosis may in mechanism involve NMDAR.

Key-words: *Fluoride; Astrocytes; Autophagy; Apoptosis; N-Methyl-D-aspartate receptor*

INTRODUCTION

Long-term exposure to high dose of fluoride induces adverse effects on the body, especially on the central nervous system (CNS).[1,2] At present, the neuronal damages caused by fluorosis have attracted more and more attention.[3] It has been confirmed that excessive fluoride intake will lead to varying degrees of atrophy, deep staining and number reduction of nerve cells, decline of intracellular Nissl bodies and ultrastructural changes such as endoplasmic reticulum (ER) swelling and mitochondria lysis, resulting in neurofunctional damage.[4-6] However, little is known about the effects of fluoride on glial cells.

Astrocytes are the largest one of the glial cells and most widely distributed in the mammalian brain with unique structure and function. Astrocytes are involved in the formation of the blood-brain barrier, providing nutrition, support and protection for the neuronal network,[7,8] and play a key role in the dynamic balance of physiological functions of the CNS. The cells can secrete a variety of regulatory factors as well, including platelet protein, glycine, cysteine, tumor necrosis factor and so on, which are concerned in neuronal synaptic formation and maintenance of structure and function.[9] In addition, astrocytes also have a variety of neurotransmitter receptors, such as glutamate-, γ-aminobutyric acid-, glycine- and acetylcholine-receptors, which play an important role in the activation and transmission of synaptic signal pathways in neurons.[10] In addition, astrocytes are closely related to the pathogenesis of many diseases, such as neurodegeneration, brain injury and infection.[11,12]

Autophagy refers to the process in which misfolded macromolecular proteins or damaged organelles are transported to lysosomes in a special way, and then degraded into usable small molecules by lysosome enzymes and then reused.[13] Through this process, cells achieve the dual functions of waste removal and energy recovery, and achieve the effective use of energy and material quality control.[14] Astrocytes can not only serve multiple functions in maintaining cellular homeostasis of the CNS, but also is considered to play a vital role in autophagy correlated with the pathogenesis of aging and neurodegenerative diseases.[15] Immunohistochemical analysis showed that fluoride exposure induced autophagy in rat hippocampal neurons, which might involve the impaired learning and memory ability.[16]

Apoptosis is a kind of conserved and programmed cell death, which is an orderly cell death controlled by intrinsic genes to maintain a stable internal environment under certain physiological or pathological conditions.[17] Apoptosis is divided into the endogenous apoptotic and the exogenous apoptotic pathway.[18] Our previous study indicated that exposure to excessive fluoride resulted in the increase of apoptosis in rat brains and SH-SY5Y cells.[19]

In addition, calcium regulates programmed cell death processes such as apoptosis and autophagy.[20] Importantly, with the increased concentrations of fluoride, calcium ion concentration, and the expressions of calcium/calmodulin-dependent protein kinase II (CaMKII) and catus proto-oncogene protein c-fos (c-fos) all tend to increase in the brains of rats with chronic fluorosis.[21]

Glutamate is the most important excitatory amino acid in the brain. N-methyl-D-aspartate receptor (NMDAR), the most important glutamate ionoreceptor, is permeable to Na^{2+}, K^{+} and Ca^{2+}, especially to Ca^{2+}, and participates physiological processes in CNS such as excitatory synaptic transmission, synaptic plasticity and long-term potentiation induction.[22] Previously, We found that in the brains of adult rats and pups as well as in primary neurons exposed to high fluoride, the expressions of NMDAR subunits were significantly altered with the reduced level of phosphor-CaMKII, and the enhanced levels of Ca^{2+} influx and apoptosis, which might be important effects involved in brain damage induced by chronic fluorosis.[23]

However, it is not clear that whether fluoride can influence the levels such as autophagy, apoptosis and Ca^{2+} influx in astrocytes. Therefore, in the study we exposed the primary astrocytes with different concentrations of fluoride or/and the treatment of Ifenprodil, an NMDAR inhibitor, to understand the effect of fluoride on astrocytes in the aspect.

MATERIAL AND METHODS

Materials:

Sodium fluoride (NaF, analytical reagent) and Ifenprodil (Sigma Aldrich, USA); anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Denmark); anti-LC3 (microtubule-associated protein 1 light chain 3) A/B and -rabbit IgG conjugated with horseradish peroxidase antibodies (Cell Signaling Technology, Inc., USA); anti-p62, -ULK1 (autophagy-activating UNC-51-like kinase 1), -Beclin1 (BECN1) and -GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibodies (Abcam, UK); Hyper Performance Chemiluminescence film and electrochemiluminescence (ECL) plus reagent (Amersham, Sweden); Apoptosis kit (CST, USA); Cell Counting Kit-8 (CCK8) (Dojindo, Japan); and all other general chemicals (Sigma Aldrich, USA) were purchased from the sources indicated.

Identification and incubation of primary astrocytes:

Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center in Guizhou, China. Brain tissues were taken from neonatal SD rats and the intact cerebral cortex was put into the precooled D-hanks solution. After carefully removing the meninges and blood vessels from the surface of the cortex, astrocytes were shredded from the tissues, digested with trypsin, filtered with 200 mesh sieve and implanted in the cell culture bottle at the density of 1x106/ml. The astrocytes were purified and identified by immunofluorescence staining of GFAP, a specific marker of astrocytes, and cells within 5 generations were selected for subsequent experiments. The study protocol was pre-approved by the Animal Ethics Committee of Guizhou Medical University, China (Approval No. 2000868).

Selection of suitable concentration of fluoride exposure by CCK8:

The purified astrocytes were inoculated on 96-well plates with suitable density. After being respectively treated with 0-4 mmol/l of fluoride (F^-, prepared with NaF) or/and 0-500 µmol/l of Ifenprodil for 24 h, the astrocytes were incubated in the complete medium containing 10 µl CCK8 for 2 h. The absorbance of the cells at 450 nm was measured on the enzyme meter, and the survival rate of each concentration calculated according to the formula of survival rate = (experimental group-blank group)/(control group-blank group).

Subcellular structure of primary astrocytes observed under transmission electron microscope (TEM):

The cultured cells were discarded from the medium and fixed with 3 ml 2.5% glutaraldehyde fixative solution at room temperature away from light for about 5 min. The cells were gently scraped off in one direction with a clean cell scraper, the mixture was sucked into the centrifuge tube with a pasteurized pipette and centrifuged for 2500 r/min for 2 min. By discarding the supernatant, a new fixing solution was added and fixed at room temperature for 2-4 h away from light. After dehydration, infiltration and embedding, the 60-80 nm ultrathin sections were sliced by ultra-thin microtome, stained with uranium lead, dried overnight at room temperature, observed under TEM, and the images were collected and analyzed.

Expressions of autophagy related proteins detected by Western blotting:

The astrocytes in different experimental groups were washed twice with sterile pre-cooled PBS and then fully cracked on ice with 100 µl RIPA lysate. After centrifugation, the supernatant was taken for use. The total protein contents were determined by BCA protein quantification method. After denaturation at 100°C for 10 min, the same amount of protein (40 µg) was taken for polyacrylamide gel electrophoresis. The protein was transferred to PVDF membrane by electric transfer, and sealed with skim milk at room temperature for 2 h. The primary antibodies for ULK1 (1:1000), Beclin1 (1:1000), LC3 (1:1000), P62 (1:1000) and GAPDH (1:500) with the samples were incubated, respectively, at 4°C overnight. After washing, the films were incubated with second antibody for 2 h. Finally, the samples were treated with ECL Plus reagent for 5 min and the resulting signal detected by High-Performance Chemiluminescence. The quantitative analysis was performed by ImageJ gray scale analysis software.

Rates of Apoptosis detected by Flow cytometry:

The treated cells of each group were washed twice with sterile pre-cooled PBS, digested by pancreatic enzymes, and then centrifuged. Thereafter, the cells were suspended in 1×binding buffer at 1×106/ml, added 5 µl AnnexinV-FITC and 5 µl PI staining solution, and cultured of 10 min under the condition of avoiding light at room temperature. After adding 400 µl 1xbuffer solution, the apoptosis rate in the cells was detected by Flow cytometry.

Intracellular calcium concentration measured by laser confocal microscope:

An equal volume of 20% Pluronic F-127 solution was added to the Rhod-2AM+DMSO storage solution so that the final concentration of Pluronic F-127 was 0.02%. The cultured astrocytes were mixed with Rhod-2AM working solution (2 M) prepared with the dilution

of HBSS and incubated in 37°C for 20 min, and then added with 5 times of the volume HBSS containing 1% fetal bovine serum. By incubation for 40 min, the cells were washed with HEPES buffer for 3 times. After incubation at 37°C for 10 min, the intracellular calcium concentration was observed under confocal laser microscope, and the average fluorescence intensity calculated by Image J software.

Statistical analysis:

All values are presented as means±SD. Multiple comparisons between the control and treated groups were made by one-way analysis of variance (ANOVA). LSD-t test was used for multiple comparison of variance among groups. Dunnett's ST3 test was used for inconsistent variance. P-values lower than 0.05 were considered statistically significant. All statistical analyses were performed with SPSS 26.00 software (SPSS Inc., USA).

RESULTS

Identification of primary astrocytes:

On the third day during the incubation, the cell body was enlarged and tapered with several elongated protrusions (Figure 1A). On the 5th day, the cell body further enlarged, the protrusions increased (Figure 1B). On the 7th day, after purification and subculture, the cells showed irregular shape, long synapse and staggered distribution, showing a typical "stone road" (Figure 1C). After staining by the immunoreaction with astrocyte specific antibody GFAP and DAPI, the astrocyte cytoplasm exhibited bright green fluorescence and the nucleus showed blue one, which indicates that the high purity of the primary astrocytes were obtained (Figure 1D-F).

Suitable concentrations of fluoride exposure and Ifenprodil treatment screened by CCK8:

As shown in Figure 2A, with the increase of fluoride concentration, the cell survival rates were increased slightly at 0.1 mmol/l F^- and then significantly decreased at 1 mmol/l F^-. According to the results of CCK8, the follow-up experiment adopted the experimental scheme of control group (no treatment), low dose fluoride (0.1 mmol/l F^-) and high dose fluoride (1 mmol/l F^-) exposures were selected with the treatment period of 24 h. The effect of NMDA receptor inhibitor Ifenprodil on the cell survival rates was shown in Figure 2B, and 10 μmol /l Ifenprodil was selected as the dose of treatment for the subsequent experiment.

Subcellular structure of the cells from different groups under TEM:

As shown in Figure 3A, no swelling of organelles and the abnormal nuclear structure were observed in the astrocytes from the control group under the ETM. Compared with the control group, the cells in the low dose fluoride group (Figure B) showed slight swelling of mitochondria and ER, as well as presenting primary and secondary lysosomes and a few of autophagic lysosome, and no significant change of nucleus. In the cells from the high dose fluoride group (Figure C), there were significant swelling, disordered or partially disappeared ridge in mitochondria, and enlarged and vacuolated rough ER with ribosome lost. In addition, enlarged autophagic lysosome, expanded Golgi cyst pool, and hypertrophic and depressed nuclear, and locally widened perinuclear space were observed in the cells exposed to high dose fluoride.

The protein expressions of autophagy related ULK1, Beclin1, LC3 and P62 of the cells from different groups:

As shown in Figure 4, the expressions of autophagy-related proteins ULK1, Beclin1 and LC3 were significantly declined in the high dose fluoride group than those in the control and the low dose fluoride groups, and whereas the expression of p62 protein was higher in the low fluoride and high fluoride groups than that of controls.

The levels of apoptosis of the cells from different groups:

As shown in Figure 5, there was no significant difference in the proportion of early apoptosis (quadrant 4) and late apoptosis (quadrant 2) between the low dose fluoride and the control groups. However, the proportions of early and late apoptosis were significantly increased in the high dose fluoride group as compared to the low dose fluoride and control groups.

Level of intracellular calcium of the cells from different groups:

As shown in Figure 6, compared with the control group, no obvious change of the level of intracellular calcium was detected in the low dose fluoride group. While, a large amount of calcium aggregation were found in the cells exposed to high dose fluoride.

The effect of Ifenprodil treatment on the cells from different groups:

The treatments of Ifenprodil significantly attenuated the changed protein expressions of ULK1, Beclin1 and P62, but not influenced on LC3; the enhanced level of late apoptosis rate; and the raised content of intracellular calcium in the cells resulted from the exposure to high dose fluoride (Figures 4-6).

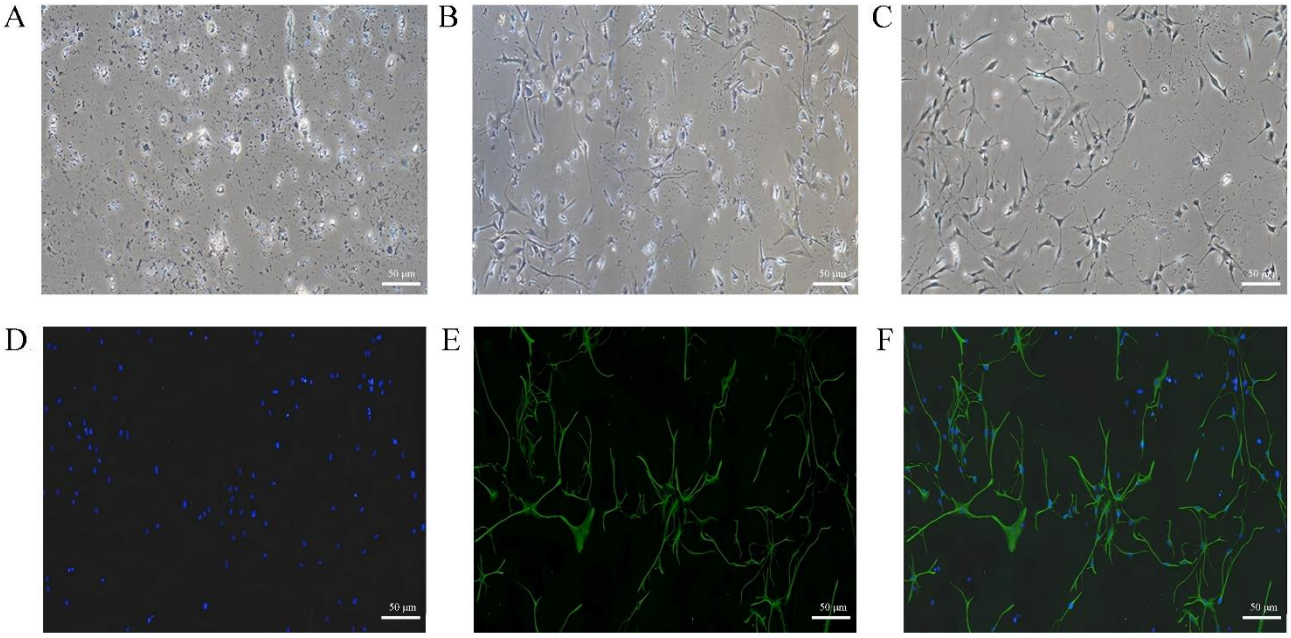

Figure 1. Morphology and identification by GFAP immunofluorescence of primary astrocytes (x 200). Morphology of cultured astrocytes: A, the first day; B, the fifth day; and C, the seventh day. Identification by GFAP immunofluorescence shown as D: DAPI; E: GFAP; and F: merge of D and E.

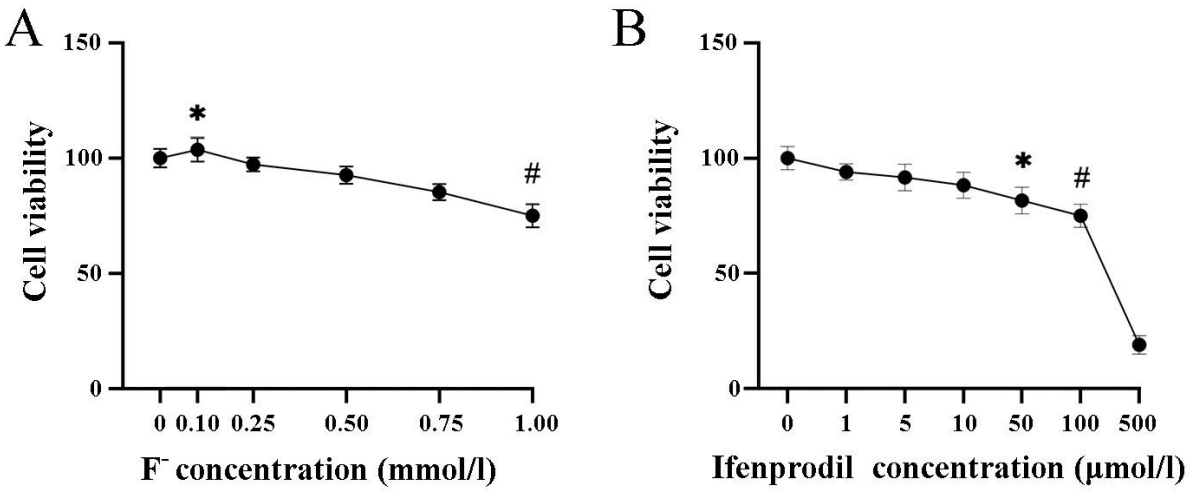

Figure 2. Survival rates of primary astrocytes exposed to fluoride or Ifenprodil. Survival rates of astrocytes screened by CCK8 detected kit. A: astrocytes exposed with 0-1 mmol/l F^-, respectively, for 24 h; B: astrocytes treated with 0-500 μmol/l Ifenprodil, respectively, for 24 h. *$P < 0.05$ and $^{\#}$ $P < 0.01$ as compared to controls.

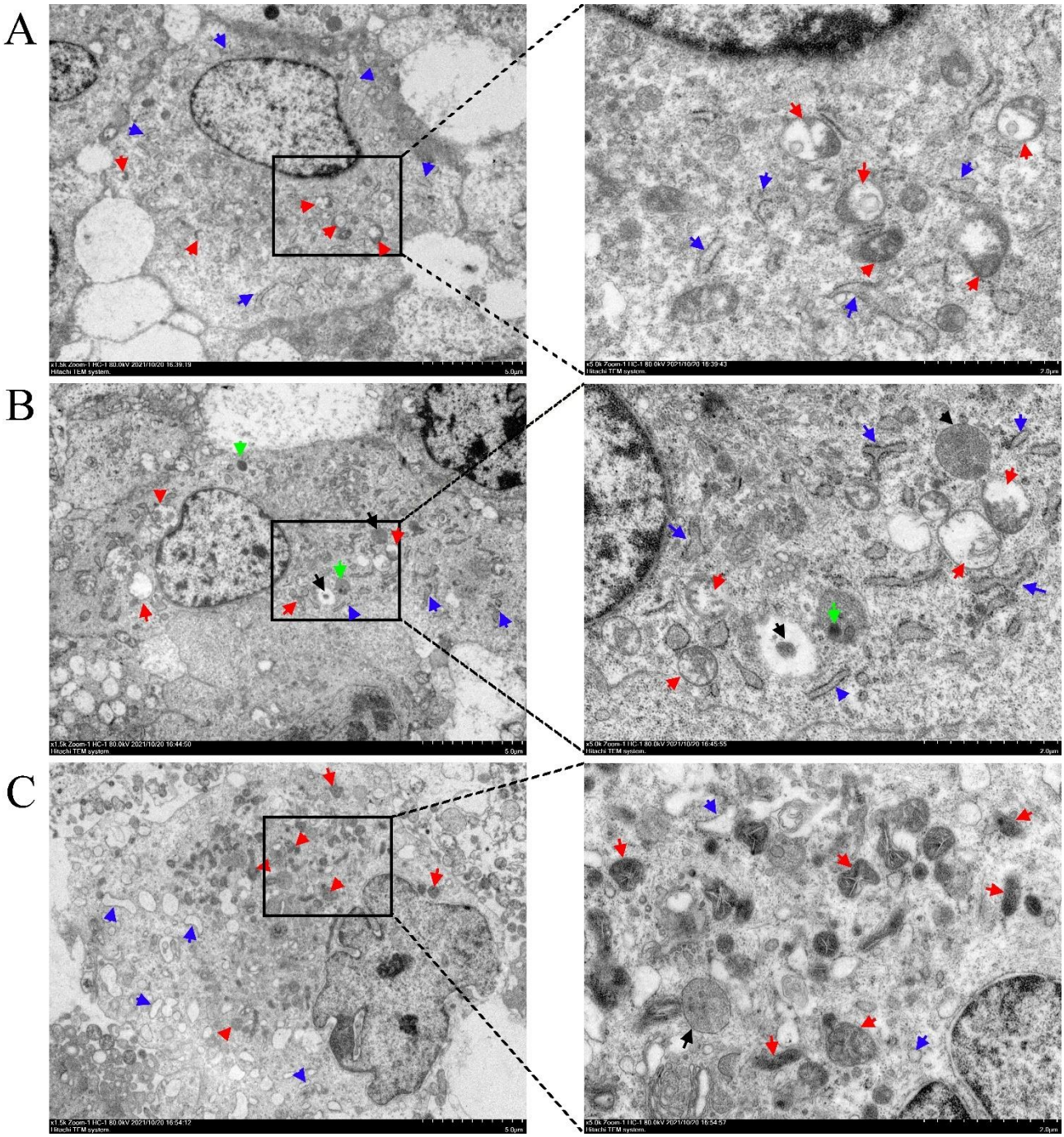

Figure 3. Effect of fluoride exposure on the subcellular structures of primary astrocytes (x 15000/50000). A: controls; B: low dose fluoride; C: high dose fluoride. Red arrow indicates mitochondria; blue arrow thick endoplasmic reticulum; green arrow primary and secondary lysosomes; and black arrow autophagy lysosomes.

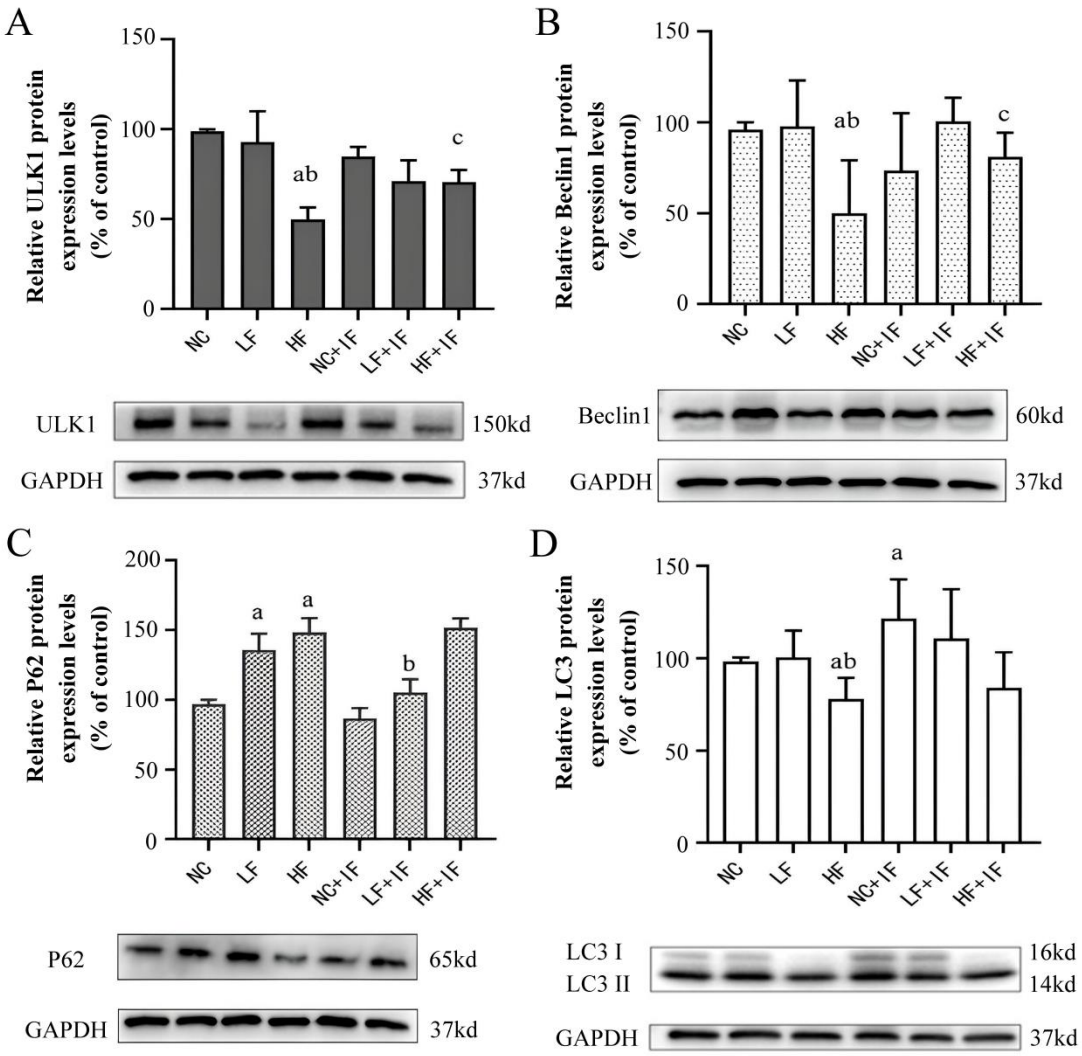

Figure 4. Expressions of autophagy related proteins in astrocytes from different groups. A. The relative expression of ULK. B. Beclin1. C. P62. D. LC3. NC: no treatment controls; LF: low dose fluoride; HF: high dose fluoride; IF: Ifenprodil. Lowercase "a" indicates $P < 0.05$ compared to controls; "b" means $P < 0.05$ compared to low dose fluoride; "c" means $P < 0.05$ compared to high dose fluoride.

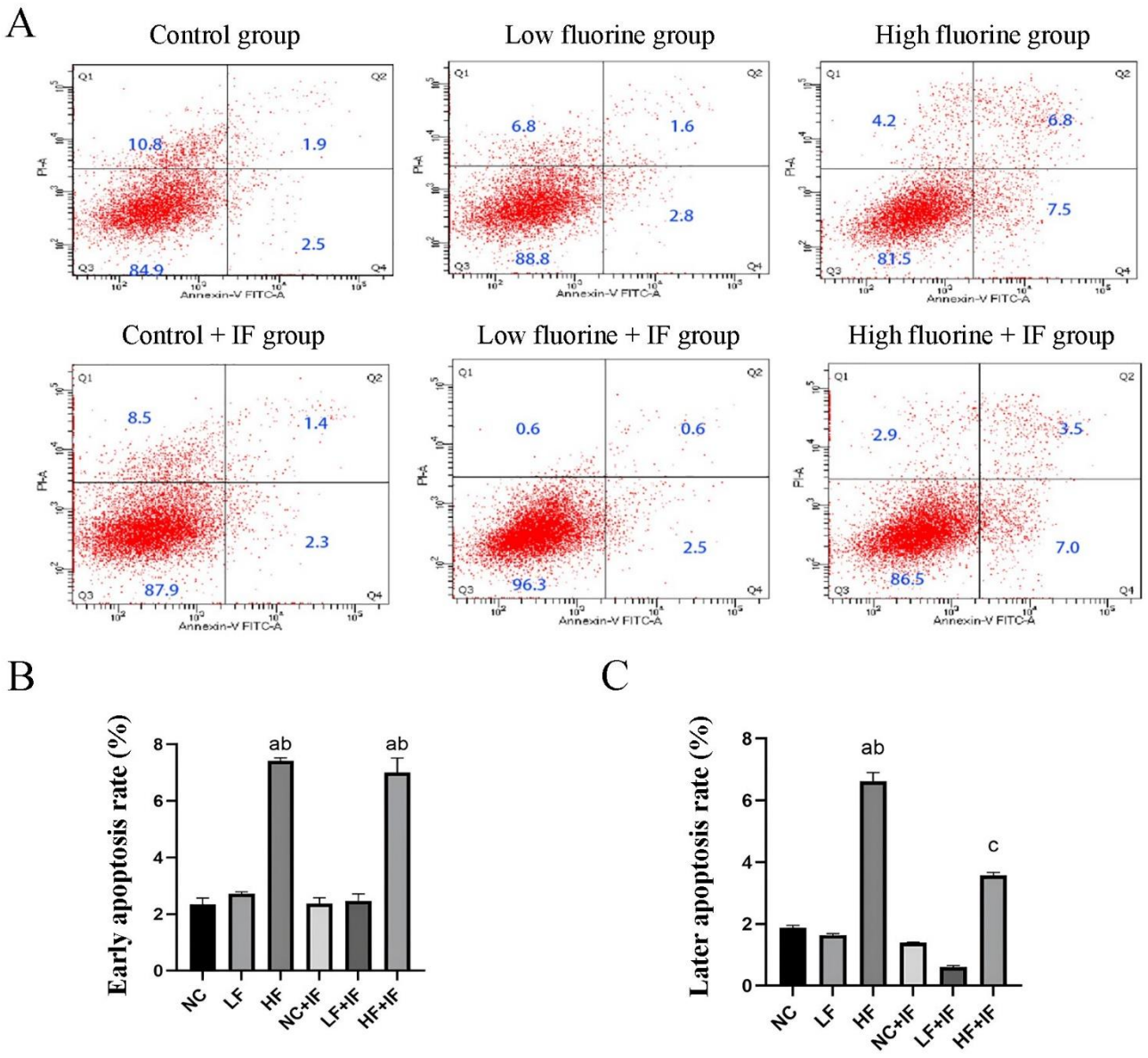

Figure 5. Apoptosis rates of primary astrocytes from different groups. A: the apoptosis rates of primary astrocytes from diferrent groups. From left to right in the first row in Figure 5A: controls, low dose and high dose fluoride; in the second row in A: control+Ifenprodil, low dose fluoride+Ifenprodil and high dose fluoride+Ifenprodil. In A, Q1 indicates mechanically damaged cells; Q2 late apoptotic or necrotic cells; Q3 normal test cells; Q4 early apoptotic cells. B: the proportion of early apoptosis (Q4) and C: late apoptosis (Q2). NC: no treatment controls. LF: low dose fluoride; HF: high dose fluoride; IF: Ifenprodil. Lowercase “a”indicates $P < 0.05$ compared to controls; “b” means $P < 0.05$ compared to low dose fluoride; “c” means $P < 0.05$ compared to high dose fluoride.

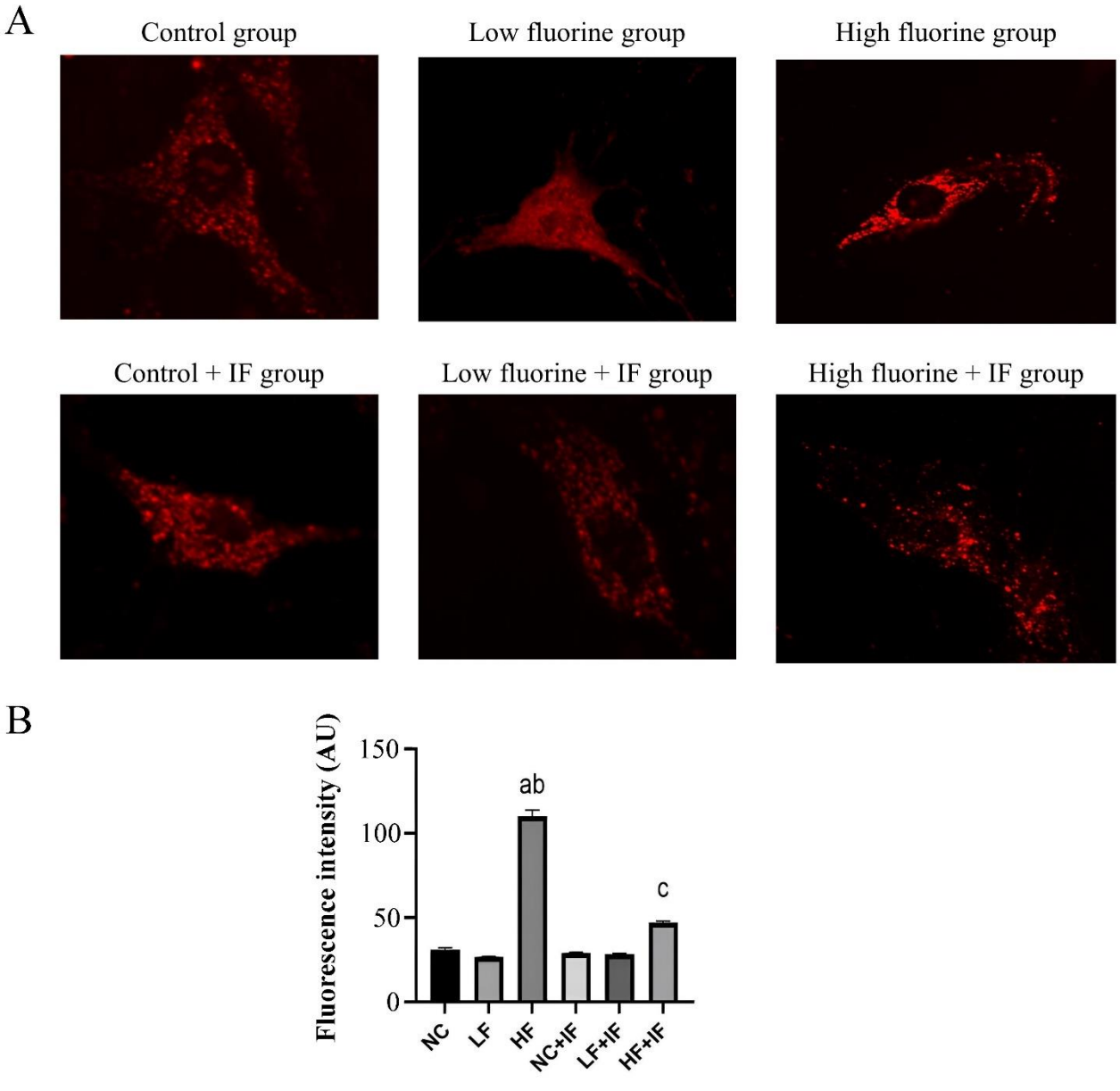

Figure 6. Levels of intracellular calcium in primary astrocytes from different groups. A. Astrocytes exposed to fluoride and controls (from left to right site: the control, low dose fluoride and high dose fluoride). B. Astrocytes treated with fluoride and Ifenprodil (from left to right site: the control+Ifenprodil, low dose fluoride+Ifenprodil and high dose fluoride+Ifenprodil). NC: no treatment controls; LF: low dose fluoride; HF: high dose fluoride; IF: Ifenprodil. AU: the average fluorescence intensity. Lowercase "a" indicates $P < 0.05$ compared to controls; "b" means $P < 0.05$ compared to low dose fluoride; "c" means $P < 0.05$ compared to high dose fluoride.

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DISCUSSION

GFAP is a kind of cytoskeleton, which belongs to type III intermediate filament and can be used as a specific marker of astrocytes in the CNS.[24] Therefore, we identified the astrocytes by immunofluorescence staining with GFAP and obtained high purity of the astrocytes.

Although many studies exhibit morphological change of neurons influenced by fluoride exposure, but little is known about the histological modification of glial cells effected by the ion. In our experiment presented here, the changes of subcellular structure of astrocytes exposed to fluoride were observed under TEM, showing obviously swollen of mitochondria and ER and abnormal exhibition of other organelles in the astrocytes exposed to excessive fluoride, giving evidence of the damaged ultrastructure and changed autophagy fluxes in astrocytes resulted from fluorosis, indicating a disturbance of cell energy production and protein synthesis.[25]

Autophagy is a conserved metabolism and recycling mechanism in cells, achieving effective utilization of energy and quality control of substances by removing damaged or excess protein molecules and organelles, promoting cell survival or accelerating cell death.[26] It was indicated that fluoride exposure induced autophagy, which may involve the impaired learning ability.[27] Whereas, another report showed that fluoride impaired the learning and memory abilities of offspring rats with decreased neuronal number, suppressed autophagy and enhanced apoptosis in hippocampus.[28] In our study here, we found that with the increased exposure of fluoride, the expressions of autophagy related proteins such as ULK1, Beclin1 and LC3 decreased, suggesting a weakened autophagy in astrocytes influenced by fluoride. In addition, the raised level of P62, a regulatory protein to guide the substrate protein to the degradation of autophagy, was increased, suggesting its regulatory effect on declining of autophagy. Change of autophagy associated proteins in astrocyte may be a compensatory response to neurotoxic effects.

The increased rate of apoptosis in CNS induced by fluorosis has been confirmed by our early investigation and many other studies.[19,23] Here, we determined that high level of fluoride increased the early and late apoptosis in astrocytes, which is consistent with the neuronal results influenced by fluorosis. Interestingly, both autophagy and apoptosis are involved in the regulation of the procedure of fluorosis, in which inhibition of autophagy can reduce fluoride-induced cell death and apoptosis.[29] The reduced levels of autophagy we found in astrocytes exposed to fluoride may be related to the regulation for reducing apoptosis.

In the study, the raising content of intracellular calcium in astrocytes exposed to high dose fluoride was observed. In our previous results, the enhanced levels of Ca^{2+} influx and apoptosis as well as the reduced phosphor-CaMKII were found in the brains of rats and cultured neurons exposed to high fluoride, which of such modifications may involve brain damage induced by chronic fluorosis.[23] Intracellular Ca^{2+} concentrations are strictly controlled by plasma membrane transporters, the ER, and mitochondria, in which Ca^{2+} uptake is mediated by the mitochondrial calcium uniporter complex, while efflux occurs mainly through the mitochondrial Na^{+}/Ca^{2+} exchanger.[30] The changed intracellular calcium in astrocytes may be correlated with the damaged mitochondria and ER induced by fluorosis as observed under TEM. Furthermore, the elevated concentration of intracellular calcium in CNS may indicate the initiating factor of neuronal apoptosis induced by fluoride.[21] On the other hand, calcium has been implicated in autophagic signaling pathways by triggering autophagy as a pro-autophagic signal.[31] However, cellular calcium signals can exert anti-autophagic actions too. For example, Ca^{2+} channel blockers induce autophagy due to the loss of autophagy-suppressing Ca^{2+} signals.

The excessive activation of NMDAR can lead to an increase in the influx of calcium ions in cells, which will further affect calcium-related organelles such as mitochondria and ER, and activate a series of signal pathways.[32] In our previous study, in the brains of adult rats and pups as well as in primary neurons exposed to high fluoride, the mRNAs encoding GluN1 and GluN2B subunits and the corresponding proteins were elevated, the level of phosphor-CaMKII was reduced, and Ca^{2+} influx and apoptosis enhanced, indicating that such modifications may involve excitotoxicity and increasing neuronal apoptosis induced by chronic fluorosis.[24] In astrocytes, NMDAR generates ionic signals and is linked to several astroglial homoeostatic molecular cascades.[33]

Ifenprodil is a novel NMDAR antagonist that selectively inhibits the receptors containing the NR2B subunit.[34] In the study, we used Ifenprodil to treat the astrocytes with the exposure of fluoride in order to reveal whether the NMDAR inhibitor could interference the neurotoxic effect of fluoride on astrocytes. Interestingly, the treatments of Ifenprodil significantly attenuated the neurotoxicity in astrocytes resulted from high dose fluoride, alleviating the modified protein expressions of autophagy, the enhanced late apoptosis and intracellular calcium. Since Ifenprodil inhibits NMDAR, it is speculated that the changes concerning autophagy, apoptosis and

intracellular calcium retention in astrocytes may be involved in modification of NMDAR. In our further plan, the expressions of NMDAR in astrocytes will be investigated.

CONCLUSIONS

Excessive exposure of fluoride induced the neurotoxic alterations of astrocytes, including the modified autophagy, the enhanced apoptosis and the raised accumulation of intracellular calcium, as well as the pathological changes of subcellular structures. Furthermore, Ifenprodil attenuated these changes as a neuroprotective factor, indicating that the neurotoxic changes of astrocytes induced by fluoride in mechanism may be involved in NMDAR.

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CONFLICT OF INTERESTS

None.

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