Oral toxicity to high level sodium fluoride causes impairment of autophagy

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Abstract

Background: Gingival recession and concomitant alveolar bone resorption are hallmarks of periodontal diseases and excessive fluoride intake has some deleterious effects on teeth, bone and soft tissues. Autophagy is a highly conserved intracellular digestion process that degrades damaged proteins and organelles but the biological roles of autophagy in pathological aspects of oral tissues remain largely unknown. We sought to elucidate the function of autophagy, especially its interplay with apoptosis and oxidative stress, in the oral toxicity induced by exposure to 5 mM sodium fluoride (NaF).

Methods: HCEM2 cementoblast cells in culture were exposed to 5 mM NaF for 5 min, after which cell viability and cell apoptosis were assessed using the MTS assay and an Annexin V-FITC/PI apoptosis detection kit, respectively. Real-time quantitative RT-PCR and Western blotting were performed to characterize the mRNA and protein expression levels of markers for autophagy, apoptosis, and oxidative stress. C57BL/6 mice were exposed to 5 mM NaF in their drinking water from 12 to 58 weeks. Micro-computed tomography was used to measure changes in their alveolar bone while immunohistochemistry and immunofluorescence staining was used to evaluate protein expression levels of autophagy, apoptosis, and oxidative stress markers.

Results: Cementoblasts exposed to 5 mM NaF had decreased levels of autophagy, as shown by reduced expression levels of ATG5, Beclin1 and LC3-II, elicited excessive apoptosis, which in turn induced oxidative stress and inflammation, as manifested by elevated levels of Bax, cleaved caspase-3, SOD1 and phospho NF-κB. Consistently, the *in vivo* results verified the findings of the *in vitro* study and treatment of mice with 5 mM NaF resulted in histological abnormalities in periodontal tissues, induced excessive oxidative stress and apoptosis, and reduced autophagy. These results correlated with the immunofluorescence observations, thus confirming the pivotal role of autophagic flux dysfunction in 5 mM NaF-induced cell death. Micro-computed tomography analysis demonstrated that 5 mM NaF caused a decrease in bone areas of mice compared with controls. Exposure to 5 mM NaF induced RANKL (receptor activator of nuclear factor κB ligand) and cathepsin K expression in periodontal tissues, while ATG5 and Beclin1 expression was abrogated by 5 mM NaF.

Conclusion: Taken together, our findings suggest that 5 mM NaF elicits oral toxicity that contributes to excessive apoptosis, oxidative stress, and defective autophagy, which aggravates periodontal tissue damage.

Background

The dual nature of sodium fluoride (NaF) has been well established with positive or negative effects on the developmental processes of calcified structures such as bone and teeth [1, 2]. An optimum level of fluoride is known to provide protective effects against dental caries. However, skeletal, and dental fluorosis have been reported due to long term and/or excessive exposure to fluoride [3, 4]. High levels of fluoride can also cause problems in nervous, gastrointestinal, genitourinary, and excretory systems [5]

and have also been known to cause osteoporosis and osteosclerosis [6]. The highest concentration of fluoride has been observed in the cementum of roots in animal [7] and human [8] studies. That concentration has also been found to increase with age [9-13] and is dependent upon the level of fluoride in the water consumed [10, 12].

The biochemical effects of fluoride include a decrease in antioxidant enzymatic activity that results in the accumulation of reactive oxygen species. This further alters the intra-cellular metabolism and causes activation of the general stress response protein SOD1. Research studies have shown that high concentrations of fluoride can stimulate cell apoptosis through an oxidative stress dependent pathway, which can cause increased lipid peroxidation in cells, leading to the dysfunction of mitochondria and the activation of downstream pathways [14, 15].

Autophagy is a quintessential component of a multitude of cellular and tissue metabolic events [16] and it has also been considered as a key component of tissue protective mechanisms against the detrimental effects of extrinsic factors [17]. Autophagy-related 5 (ATG5), Beclin1, LC3 and p62 play pivotal roles in the autophagy pathway. ATG5 is essential for the generation of autophagosomes by forming a complex with ATG12. This is, in turn, a necessary step for the stimulation of LC3-I and the formation of LC3-II and subsequently the formation of autophagosomes [18]. Beclin1 is important for early stages of autophagy, where it helps in recruiting other ATG proteins for the activation of downstream genes [19]. Similarly, the levels of LC3 can indicate the number of autophagosomes and their successful formation [20]. LC3 is activated by binding its substrate p62 to promote the degradation of autophagosomes [21]. Therefore, ATG5, Beclin1, LC3 and p62 are considered indicators of autophagic influences in this study.

In recent years, the mutually stimulatory crosstalk between autophagy and inflammation has also been characterized. The inflammatory marker NF-κB has been found to negatively regulate autophagy in *in vitro* experiments [22], highlighting a confluence of these signaling pathways. It has also been elucidated that fluoride activates autophagy in many ways in different cell lines [23], which either protect or impair the cellular metabolism of an organism [24].

There is a close correlation between oxidative stress and autophagy [25]. Studies have revealed that autophagy, apoptosis, and necrosis are associated with the development of diseases at the cellular level [26]. Several studies have demonstrated that the suppression of autophagy pathways can result in excessive apoptosis, as seen in several neoplasms and other neurological disorders [27]. Fluoride has also been reported to impair the process of autophagic degradation in the testes of rats [24], and autophagy may play a crucial role in protective responses towards fluoride-induced oxidative damage [23, 28]. Previously, our research group reported that the cell motility of gingival cells can be induced by low-level fluoride [29]. In recent years, it has been observed that autophagy in osteoblasts can be modulated by fluoride. It is also quite possible that fluoride may influence autophagy in stem cells from apical papillas and their differentiation [30].

The effect of fluoride on autophagy in several tissues leads to tissue damage. However, the effects of high-level NaF exposure in periodontal tissues and its mechanism are still unclear. Similar ambiguity

exists regarding the relationship of autophagy and apoptosis in cases of NaF toxicity. Therefore, this study aimed to explore the roles of oxidative stress, apoptosis, and autophagy in NaF toxicity, and to particularly focus on the regulatory role of apoptosis and inflammation in autophagy in periodontal structures exposed to high levels of NaF. This study used an *in vitro* model of 5 mM NaF-treated cementoblast cells and an *in vivo* mouse model of 5 mM NaF exposure from 12 to 58 weeks. We observed an increase in apoptosis associated with activation of the HIF-1α signaling pathway due to the suppression of autophagy mediated by the toxicity of 5 mM NaF. This finding was further supported by suppression of the levels of markers of autophagy after an increase in apoptosis was induced by high-level NaF exposure.

Methods

Cell culture

Human cementoblast cells (HCEM2) were a gift of Hiroshima University, Japan. HCEM2 cells were cultured in Dulbecoo's Modified Eagle Medium (DMEM) (Wako, Tokyo, Japan) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin under 5% CO_2 at 37°C in a humidified environment. The cells were sub-cultured at 70-80% confluency. About 1.2 x 10^5 HCEM2 cells were seeded in 6-well plates with 2 ml normal growth medium and 24 h later were treated with 5 mM NaF for 5 min.

Animals

C57BL/6 mice (10-week-old, n=12, male, body weight 29.4 ± 0.8 g) were obtained from Japan SLC (Shizuoka, Japan) and were individually housed under a 12:12 h light-dark cycle in pathogen-free conditions. The mice were divided into 2 groups: a control group (n=6) and a 5 mM NaF treated group (n=6). The mice were provided water with or without 5 mM NaF at the age of 12 weeks and stopped at 58 weeks. Mice were sacrificed by cervical dislocation under deep anesthesia with a mixture of medetomidine hydrochloride at a dose of 0.3 mg/kg, midazolam at a dose of 4 mg/kg and butorphanol tartrate at a dose of 5 mg/kg (0.1 ml/10g body weight). All animal experiments were performed after approval by the Institute's Ethics Committee at Nihon University School of Dentistry at Matsudo (AP17MD015).

Measurement of alveolar bone resorption and micro-computed tomography

The distance between the cemento-enamel junction and the alveolar bone crest at seven buccal sites per mouse was measured, standardized to provide measurements in millimeters. Three-dimensional (3D) changes for each mandibular bone were captured using micro-computed tomography (micro-CT) (R_mCT2, Rigaku Corp., Tokyo, Japan) under the following exposure conditions: tube voltage, 90 kV; tube current, 200 μA; voxel size, 20 × 20× 20 μm.

MTS assay

The MTS assay was performed as previously described [29]. Briefly, 3 x 10^3 HCEM2 cells per well were seeded and cultured in 96-well plates for 24 h. Serum-free medium was changed after 24 h and then replaced with 5 mM NaF for 5 min. Cell viability was assessed by the MTS assay after NaF supplementation according to the manufacturer's instructions. Cell Titer 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) was used to detect cell viability. The results were quantitated by absorbance at 490 nm.

Flow cytometry

Cell apoptosis was detected using a BD Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, CA, USA) performed as per the manufacturer's instructions. HCEM2 cells were trypsinized and collected after treatment with 5 mM NaF for 5 min. One x 10^6 cells/mL were resuspended in fresh culture medium and stained with or without Annexin V-FITC and Propidium Iodide for 15 min. The cells were then washed with binding buffer and immediately analyzed using flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA, USA).

Western blot

HCEM2 cells were solubilized by RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Twenty μg protein were applied on each polyacrylamide-SDS gel (Wako, Osaka, Japan) and then transferred to PVDF membranes (Millipore, St. Louis, MO, USA). After blocking with 5% skim milk, the membranes were incubated with anti-ATG5 (1:500, ab108327, Abcam, Tokyo, Japan), anti-Beclin1 (1:500, ab62557, Abcam, Tokyo, Japan) anti-LC3-I/II (1:1000, #12741, Cell Signaling Technology, Danvers, MA, USA), anti-p62 (1:1000, ab56416, Abcam, Tokyo, Japan), anti-Bax (1:1000, ab32503, Abcam, Tokyo, Japan) anti-p-NF-κB (1:800, bs-3543R, Bioss, Woburn, MA, USA), anti-NF-κB (1:800, #4746, Cell Signaling Technology, Danvers, MA, USA), anti-SOD1 (1:500, ab13498, Abcam, Tokyo, Japan), anti-HIF1-α (1:500, ab2185, Abcam, Tokyo, Japan), anti-Cleaved-caspase3 (1:500, ab49822, Abcam, Tokyo, Japan) or anti-GAPDH (1:1000, #2118, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. An ECL Plus Western Blotting Detection System (GE Healthcare, Tokyo, Japan) was used to visualize the images of western blots.

RT-PCR

RNA was extracted from HCEM2 cells using a RNeasy Mini Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's protocol. Briefly, cDNAs were transcribed from 1 μg of each RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed to detect expression of the following target genes using TaqMan gene Expression Assays: ATG5 (Assay ID Hs00169468_m1), Beclin1 (Assay ID Hs01007018_m1), LC3-I (Assay ID Hs01076567_g1), LC3-II (Assay ID Hs00797944_s1) and ACTB (Assay ID Hs01060665_g1). Each detection was performed in triplicate.

Immunohistochemical staining

Tissues of the upper jaw were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and decalcified with 10% EDTA (pH: 8.0). The specimens were subjected to antigen retrieval (pH: 6.0) and peroxidase blocking and were then incubated with anti-ATG5 (1:75, ab108327, Abcam, Tokyo, Japan), anti-Beclin1 (1:100, ab62557, Abcam, Tokyo, Japan), anti-Cathepsin K (1:50, LS-B2512, Abcam, Tokyo, Japan), anti-RANKL (1:50, NB100-80849Novus, Centennial, CO, USA), anti-Bax (1:100, ab32503Abcam, Tokyo, Japan), anti-p-NFkB (1:100, bs-3543R, Bioss, Woburn, MA, USA), anti-HIF1-a (1:100, ab2185, Abcam, Tokyo, Japan) or anti-8OHdG (1:20, MOG-020P, JaICA, Shizuoka, Japan) overnight at 4°C. Further, the slides were then incubated with secondary antibodies (Rat MAX-PO, Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 min. Images were captured using a microscope (OLYMPUS, Tokyo, Japan).

Immunofluorescent staining

As described above, the slides were incubated with anti-Runx2 (1:50, 5356-1, Epitomics, Tokyo, Japan), anti-Osterix (1:75, ab94744, Abcam, Tokyo, Japan), anti-ATG5 (1:75, ab108327, Abcam, Tokyo, Japan) or anti-Beclin1 (1:100, ab62557, Abcam, Tokyo, Japan) overnight after antigen retrieval and peroxidase blocking. The slides were incubated with anti-rat IgG-Alexa Fluor 488 secondary antibody at room temperature for a duration of 60 min in a dark chamber. A fluorescence microscope was used to capture images (OLYMPUS, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 and the results were assessed by an independent two-tailed Student's t-test or analysis of variance (ANOVA). *p value* less than 0.05 showed significant difference.

Results

High level NaF induces HCEM2 cell apoptosis via the downregulation of autophagy

To understand the effects of high level NaF on HCEM2 cells, we treated the cells with 5 mM NaF for 5 min. MTS assays showed that 5 mM NaF significantly reduced HCEM2 cell viability (Fig. 1A, $p<0.05$). Flow cytometry confirmed that treatment with 5 mM NaF significantly induced HCEM2 cell apoptosis compared with the control group (Fig. 1B, C, $p<0.01$). Consistently, the expression of Bax and Cleaved-caspase3 was also induced in the 5 mM NaF treated group (Fig. 1E). Autophagy is one of the key regulators of cell death, and WB and RT-PCR were performed to investigate the expression of autophagy markers in HCEM2 cells after 5 mM NaF treatment. The expression of ATG5, Beclin1, LC3-I and LC3-II were all significantly down-regulated by treatment with 5 mM NaF, as shown by RT-PCR and WB (Fig. 1D, E, $p<0.05$) while the expression of p62 protein was upregulated (Fig. 1E). Further, the autophagy related reactive oxidative stress markers SOD1, p-NFkB and HIF1-a were upregulated with the suppressed autophagy (Fig. 1E). These results revealed that treatment with 5 mM NaF may induce the apoptosis of HCEM2 cells via the suppression of autophagy.

High level NaF reduces the expression of autophagy in cementoblasts and periodontal ligament cells

We provided mice with water enriched with 5 mM NaF from 12-weeks old and continued that until they were sacrificed at 58-weeks old (Fig. 2A). Mice treated with 5 mM NaF supply had reduced expression of Osterix and Runx2 (Fig. 2B). The expression levels of ATG5 and Beclin1 were both suppressed by 5 mM NaF in cementoblasts and in periodontal ligament cells (Fig. 3). IHC staining showed that the expression of ATG5 and Beclin1 was decreased as shown above, whereas Bax, p-NFkB and HIF1-a were inductively expressed (Fig. 4). These results confirmed that 5 mM NaF induced a high expression of the HIF1-a/p-NFkB axis, which suppressed autophagy and promoted apoptosis.

High level NaF enhances alveolar bone resorption via the downregulation of autophagy

To determine the influence of 5 mM NaF treatment on alveolar bone, we performed micro CT on the upper and lower jaws of the mice. Treatment with 5 mM NaF enhanced the alveolar bone resorption in both the upper jaw and the lower jaw compared to the control group (Fig. 5A, B, $p<0.05$). IHC results also revealed that 5 mM NaF induced the expression of Cathepsin K and RANKL in periodontal tissues (Fig. 5C). Furthermore, we investigated the expression level of autophagy markers in periodontal tissues and observed that ATG5 and Beclin1 were also decreased after treatment with 5 mM NaF. This was consistent with the data shown above where 8-OHdG, p-NFkB and HIF1-a were upregulated in periodontal tissues, which may enhance the differentiation and ability of osteoclasts, resulting in alveolar bone loss (Fig. 6). Treatment with 5 mM NaF also upregulated the expression of autophagy related proteins (ROS, p-NFkB, HIF1-a), suppressed the expression of cementoblast markers and induced apoptosis via the downregulation of ATG5 and Beclin1 expression. The reduced autophagy in the periodontal ligament and alveolar bone may indirectly promote the differentiation of osteoclasts and augment their function (Fig. 7).

Discussion

In this study, we demonstrated that in HCEM2 cells *in vitro* 5 mM NaF induces HIF-1α gene expression, NF-κB phosphorylation and caspase 3 activity. We also observed that 5 mM NaF reduces autophagy in cementoblasts and increases the expression of HIF-1α. The oxidative stress activation by 5 mM NaF was also observed with the suppression of autophagy through 5 mM NaF-mediated apoptosis. *In vivo*, the results demonstrated that mice treated with high levels of NaF have increased expression of HIF-1α, Bax and NF-κB phosphorylation in their cementoblasts and periodontal tissues. Those mice also had decreased levels of autophagy-related proteins in cementoblasts and the periodontal ligament after 5 mM NaF ingestion.

Relatively few studies have highlighted the histopathological changes of periodontal tissues exposed to high level NaF. The results of the present study and those of previous *in vitro* studies provide evidence that 5 mM NaF has severe effects on the cementum, the periodontal ligament and alveolar bone by inducing inflammation and decreasing mineralization and autophagy. It is known that the best method for the systemic absorption of NaF is via the gastrointestinal tract. Hence, deionized water mixed with

NaF to a concentration of 100 ppm [7, 11, 12] was used to feed the mice. This meant that they were exposed to 5 mM NaF every day from week 12 to week 58, which covered their adult and aging periods. Hence, such a design helped us to assess alveolar bone damage due to NaF, as manifested by micro-CT and histopathological changes. These findings were consistent with evidence reported in the literature [29, 31, 32]. This also suggested that our mouse model to evaluate the toxicity of exposure to 5 mM NaF was successfully constructed. To highlight the underlying mechanisms of the toxicity of NaF *in vitro* and *in vivo*, this animal model and NaF treated HCEM2 cementoblasts were used, respectively.

The protective effect of NaF on cementum is dependent on its concentration in systemic or local mode of exposures [33]. High concentrations of NaF (1 mM or more) have been known to result in the arrest of cell growth and cell death either by causing cellular necrosis or apoptosis [15]. It has also been observed that exposure of adult rats to 60 or 120 mg/L NaF for a period of 10 weeks resulted in exacerbated apoptosis in the hippocampus and cortex of the brain [34]. Since there was a paucity of scientific literature on the cytotoxicity of high concentrations of NaF on cementoblasts, this study was planned and conducted. We now report the inhibition of cell proliferation and increased apoptotic rates after treatment with 5 mM NaF, which suggests that excessive NaF may be cytotoxic. We also provide significant evidence that exposure of mice to 5 mM NaF accelerates apoptosis, as demonstrated by established markers such as Bax and caspase-3 activation in HCEM-2 cells (Fig. 1). Immunofluorescence microscopy showed that 5 mM NaF-treated cellular cementum had dispersed resorption concavities along with periodontal ligament cells in the vicinity (Fig. 2). These results are consistent with a previous study in terms of alterations of oxidative stress and apoptosis, indicating their impediment following exposure to high concentrations of NaF. Suzuki et al. reported that ROS generation is induced by fluoride, which results in oxidative damage and apoptosis [23]. Treatment with 5 mM NaF alleviated oxidative stress in cementoblasts in this study, which indicates that oxidative stress and apoptosis signaling related to it are involved in the periodontal cell death and toxicity associated with NaF.

Oxidative stress plays a vital role in fluoride-induced toxicities [35, 36], and the involvement of this stress response in fluoride-induced autophagy remains speculative. In the present study, levels of ATG5 and Beclin1, which reflect the autophagy status [37], were obviously decreased in cementoblasts and periodontal tissues following NaF exposure. Furthermore, the fluorescence microscopy results clearly showed that 5 mM NaF caused autophagy impairment, which suggested that 5 mM NaF exposure might lead to oxidative stress. As expected, our results provided *in vivo* and *in vitro* evidence that 5 mM NaF exposure induces oxidative stress that is followed by the activation of inflammation. The transcription factor NF-κB plays a key role in the regulation of inflammatory responses, thereby suggesting the importance of autophagy and NF-κB activation in inflammatory environments [38]. Fluoride is an oxidative stress inducer [39], which suggests a potential role for defective autophagy in the toxicity following exposure to a high concentration of NaF.

The results of our study confirmed that 5 mM NaF-treated autophagy was not sufficient to counteract the NaF-induced cellular damages in HCEM2 cells. That can be explained by the hypothesis that 5 mM NaF increased the expression of HIF-1α and oxidative stress in cementoblasts and periodontal ligament cells

and the associated activation of the pathways of mitochondrial apoptosis. A better understanding and clinical correlation to disease progression in the periodontium and discerning its molecular and therapeutic interventions are exciting prospects for future research.

Our findings highlighted that the reduction of oxidative stress by 5 mM NaF also blocked autophagy, which resulted in the decreased expression of markers such as Beclin-1, p62 and LC3-II in cementoblasts and periodontal ligament cells treated with NaF. Immunofluorescence staining also verified these findings to be secondary to excessive oxidative stress. Autophagy is known to be governed by multiple upstream signaling molecules. Among them, HIF-1α is a marker that acts as a culminating node to many stimuli. The above results indicated that exposure to NaF causes activation of the HIF-1α-ROS signaling pathway, promotes apoptosis and suppresses autophagy. In a clinical scenario, this can increase the damage of periodontal tissues (Fig. 7). Since oxidative stress induces the expression of HIF-1α and apoptosis, this can be extrapolated to use NaF in adequate concentrations to alleviate oxidative stress responses in periodontal tissues.

Autophagy is regarded as a cellular process required for the maintenance of cellular homeostasis and the elimination of damaged proteins and cellular organelles [40]. ATG5 is critical for autophagosome formation [41], and deletion of ATG5 in cells causes defects in autophagy and even cell death [42]. Knockdown of ATG5 has also been used to block autophagy pathways [43] and over-expression ATG5 is known to be inhibited by the accumulation of NF-κB p65 in the nucleus [44]. We observed that mRNA and protein expression levels of factors related to ATG5 were considerably down-regulated in 5 mM NaF-treated cells and tissues. This further indicates that periodontal cells are less sensitive to autophagy. Lei et al. demonstrated that high fluoride could cause autophagy of HAT-7 cells, especially related to the expression of Beclin1 [30]. It has also been postulated that the regulation of p62 is responsible for controlling the formation of intracellular inclusion bodies in autophagy, and Beclin1 is involved in the initial steps of the formation of autophagosomes. Recently, it was demonstrated that fluoride also induces autophagy due to an increased expression of Beclin1 and LC3 and a decreased expression of p62 in osteoblasts [17, 45]. Similarly, a dose-response pattern in LC3-II protein expression in ameloblast-derived cells has been observed with fluoride treatment [30], which decreases after increasing the concentration of fluoride. The present study demonstrated that NaF treated groups had increased mRNA and protein expression levels of p62 and suppressed levels of Beclin1 (Fig. 1). Beclin1 is regarded as the molecular basis of the inter-relationship of the apoptosis and autophagy pathways. In this study, we reported an increase in the levels of cleaved caspase-3 in the 5 mM NaF group, suggesting that autophagy may not be able to protect the cells from 5 mM NaF-induced cell-death. Alteration of the expression of LC3 has been found to be involved in the fluoride-induced autophagy in Leydig cells of mice or through JNK signaling mediated by ROS [23]. Simultaneously, we observed a significant decrease in LC3 mRNA and protein expression levels, and an alteration in the ratio of LC3-II/LC3-I (Fig. 1) following treatment with 5 mM NaF. This suggests that the downregulation of autophagy is a dynamic process associated with a high dose of NaF.

We postulated that 5 mM NaF inhibits autophagy which in turn suppresses apoptosis [46]. Additionally, the present study provides evidence regarding the role of fluoride toxicity in terms of its role in autophagy and apoptosis. Overall, these results suggest that exposure to 5 mM NaF caused an induction of the HIF-1α-ROS signaling pathway and the related apoptosis and suppressed autophagy that can eventually impair cementoblasts, the periodontal ligament and functions of the periodontium. The regulation of oxidative stress and the proposed signaling pathway based on our results are summarized schematically in Fig. 7. These findings suggest an essential role of autophagy in modulating the cellular mechanisms and hence can have direct implications in resorption and possibly offer potential therapeutic strategies in the future.

Abbreviations

NaF: Sodium fluoride; micro-CT: micro-computed tomography; SOD1: Superoxide dismutase 1; ATG5: Autophagy-related 5; LC3I/II: Microtubule-associated proteins I/II; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; HIF-1α: Hypoxia inducible factor 1 Subunit Alpha; HCEM2: Human cementoblast cell line; 8-OHdG: 8-Oxo-2'-deoxyguanosine; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; ROS: Reactive oxygen species; p: *p* value; SD: Standard deviation.

Declarations

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Authors' contributions

SO, XL, FZ and UKB performed the experiments, XL and UKB wrote the manuscript. SO, XL, FZ and UKB conceived the study and analyzed the data. FZ, CT, KA and UKB designed the animal experiments. NT, ISK, LZ and YL supervised interpretation of the data and critical review of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author (bhawal.ujjal.kumar@nihon-u.ac.jp) on reasonable request.

Ethics approval

All animal experiments were performed after approval by the Institute's Ethics Committee at Nihon University School of Dentistry at Matsudo (AP17MD015).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

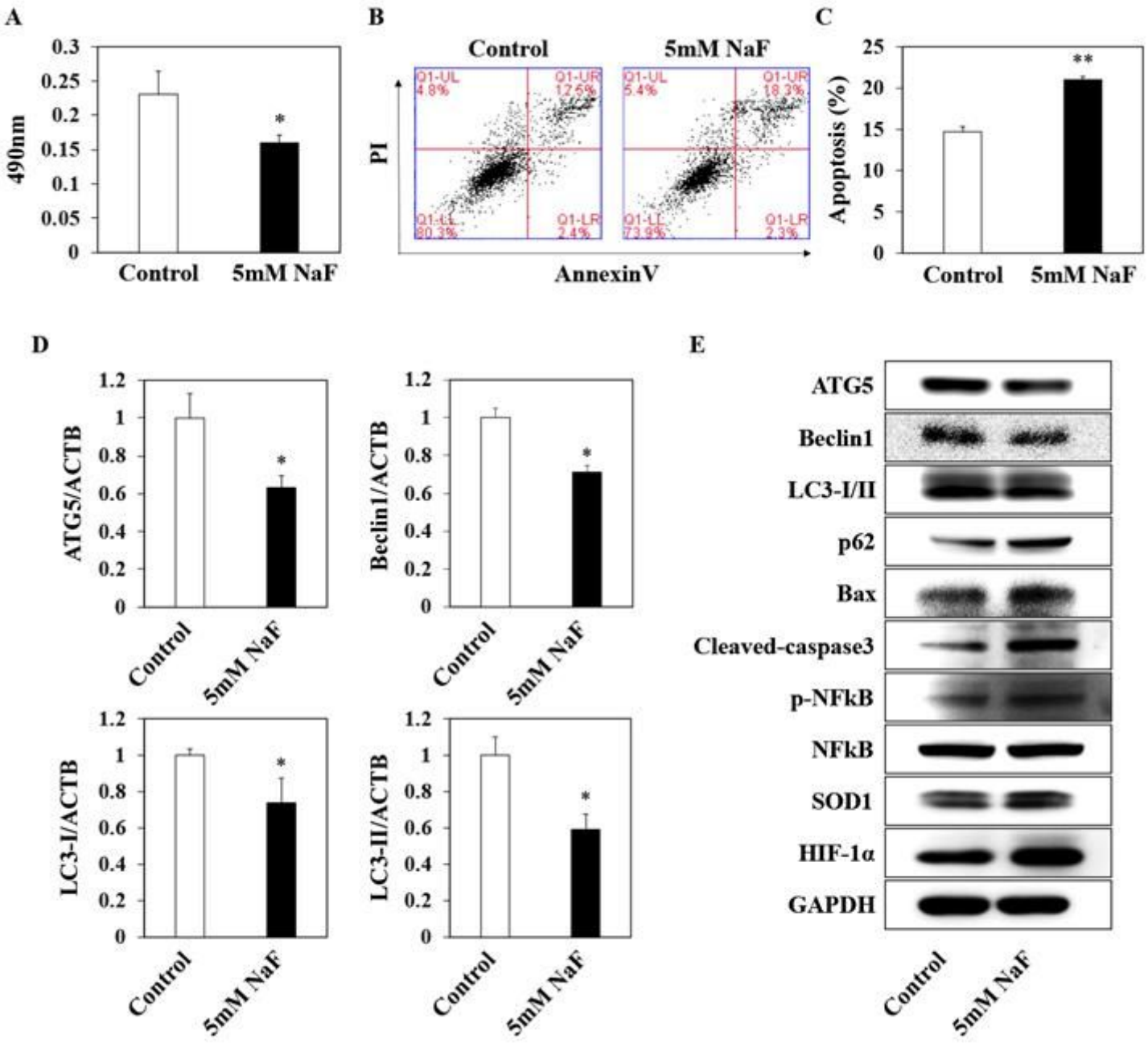

Figure 1

High level NaF induces HCEM2 cell apoptosis via the downregulation of autophagy. A MTS assays showing that 5 mM NaF reduces cell viability; * indicates $p<0.05$. B, C Flow cytometry results showing that 5 mM NaF treatment significantly induces HCEM2 cell apoptosis compared with the control group (** indicates $p<0.01$). D The expression of ATG5, Beclin1, LC3-I and LC3-II are all significantly down-regulated by 5 mM NaF treatment; results are expressed relative to β-actin; * indicates $p<0.05$. E Western blot confirming the expression of markers of oxidative stress, apoptosis, autophagy, and related proteins in HCEM2 cells.

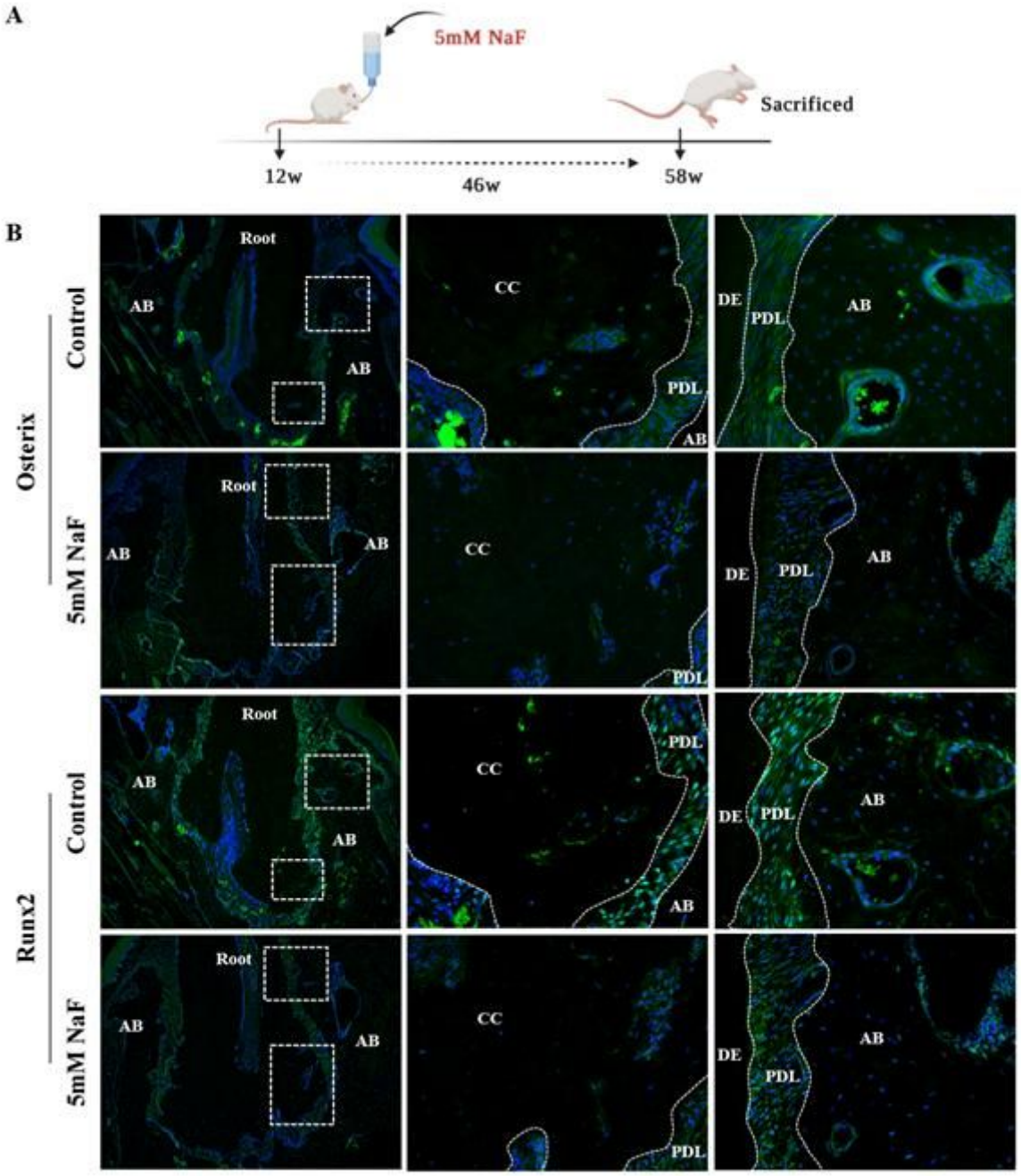

Figure 2

High level NaF reduces the expression of cementoblast markers. A Mice were provided with water enriched with or without 5 mM NaF from 12-weeks old and were sacrificed when they became 58-weeks old. B 5 mM NaF reduces the expression of Osterix and Runx2. Fields in white dashed boxes in the left column are shown at higher magnification in the middle and right columns.

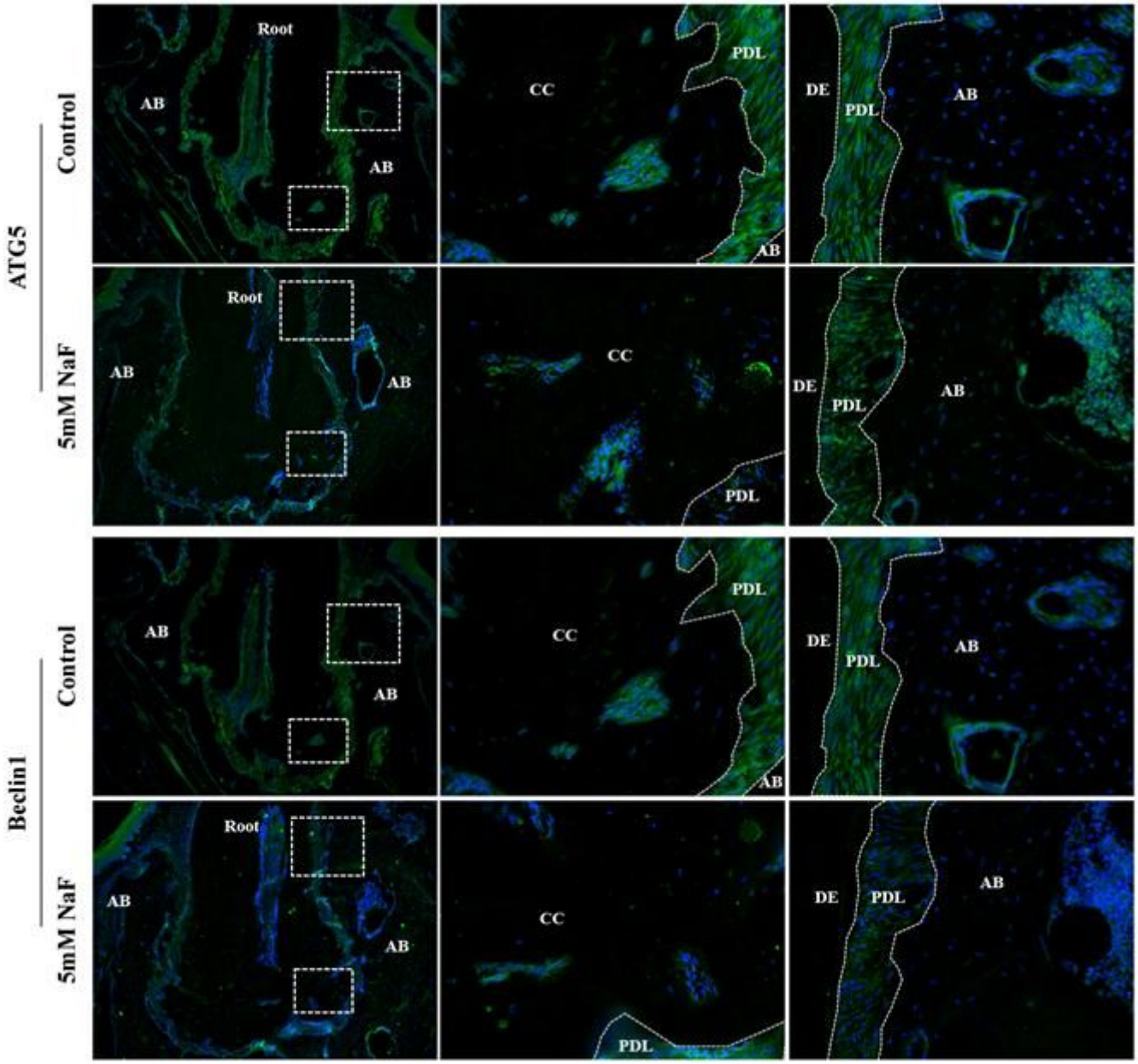

Figure 3

High level NaF reduces the expression of autophagy markers in cementoblasts and periodontal ligament cells. The expression level of ATG5 and Beclin1 were suppressed by 5 mM NaF both in cementoblasts and in periodontal ligament cells. Fields in white dashed boxes in the left column are shown at higher magnification in the middle and right columns.

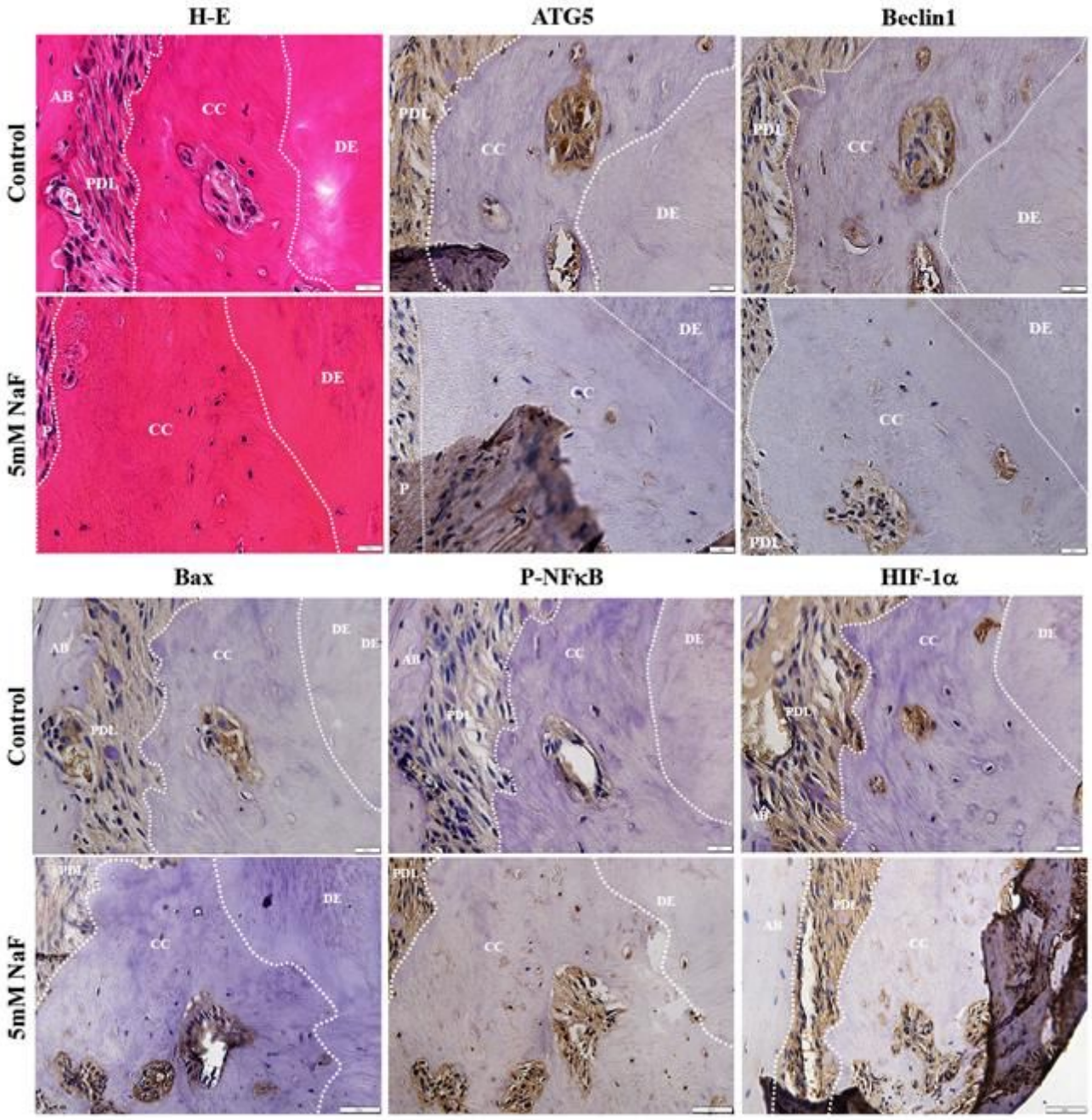

Figure 4

High level NaF suppresses autophagy via the induction of autophagy upstream proteins in the cementum. The expression of ATG5 and Beclin1 were decreased by 5 mM NaF, whereas 8-OHdG, p-NFκB and HIF1-α were inductively expressed by 5 mM NaF.

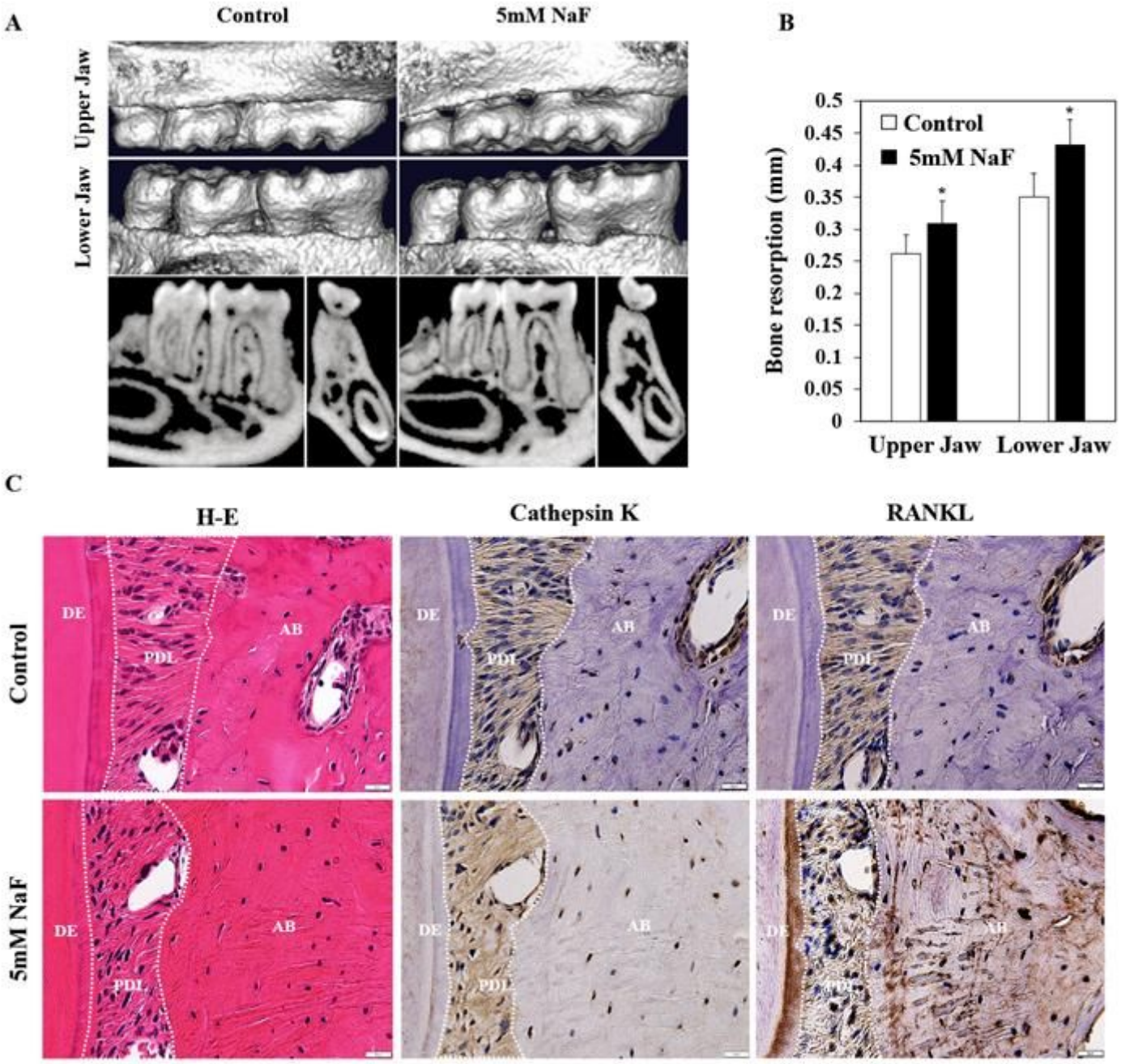

Figure 5

High level NaF treatment enhances alveolar bone resorption via down-regulated autophagy. A, B microCT showing that 5 mM NaF significantly enhanced alveolar bone resorption both in the upper jaw and the lower jaw compared to the control; * indicates $p<0.05$. C Treatment with 5 mM NaF induces the expression of Cathepsin K and RANKL in periodontal tissues.

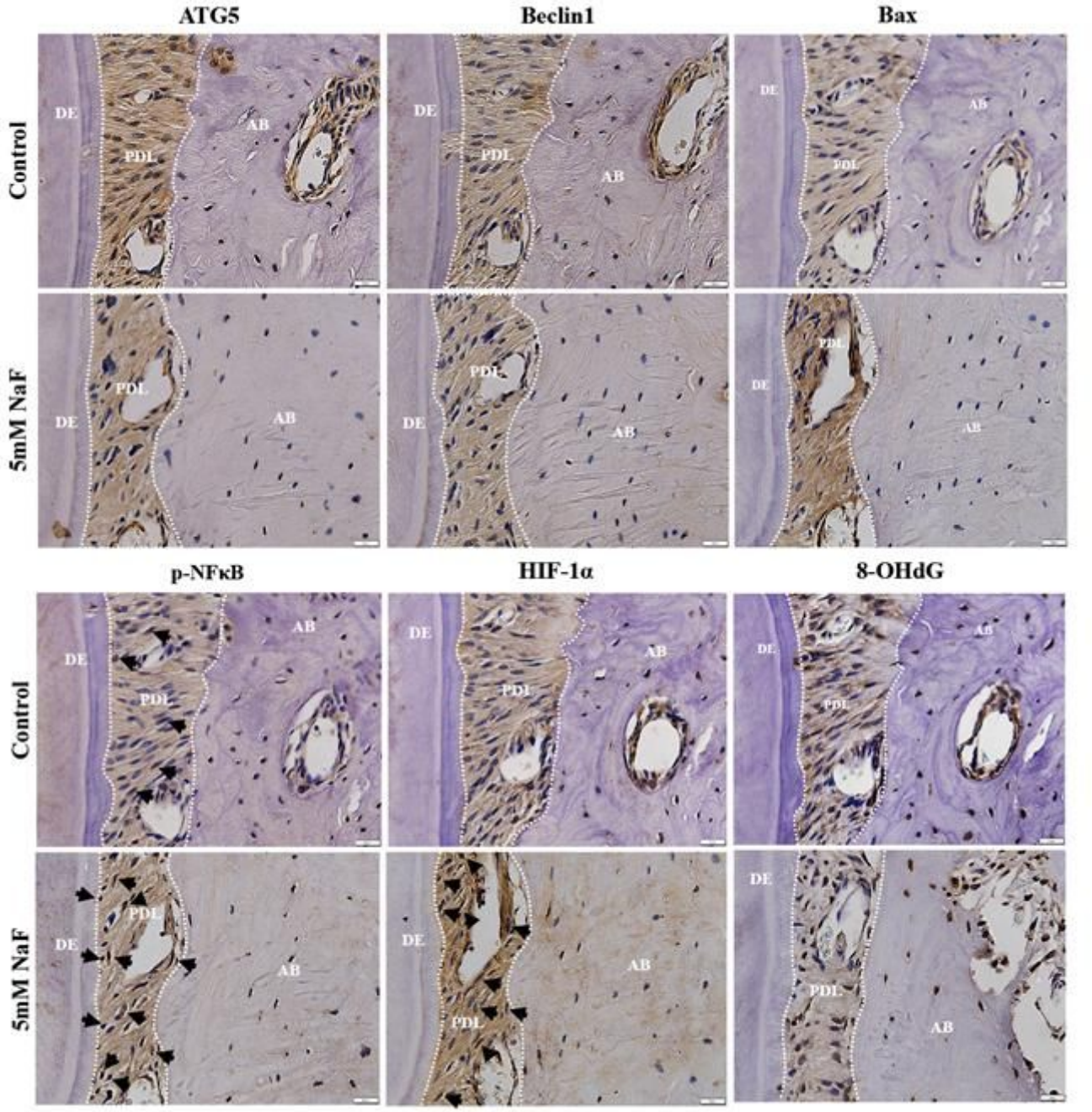

Figure 6

Treatment with 5 mM NaF suppresses autophagy via the induction of autophagy upstream protein expression in periodontal tissues. ATG5 and Beclin1 were decreased after treatment with 5 mM NaF, while 8-OHdG, p-NFκB and HIF1-α were upregulated in periodontal tissues.

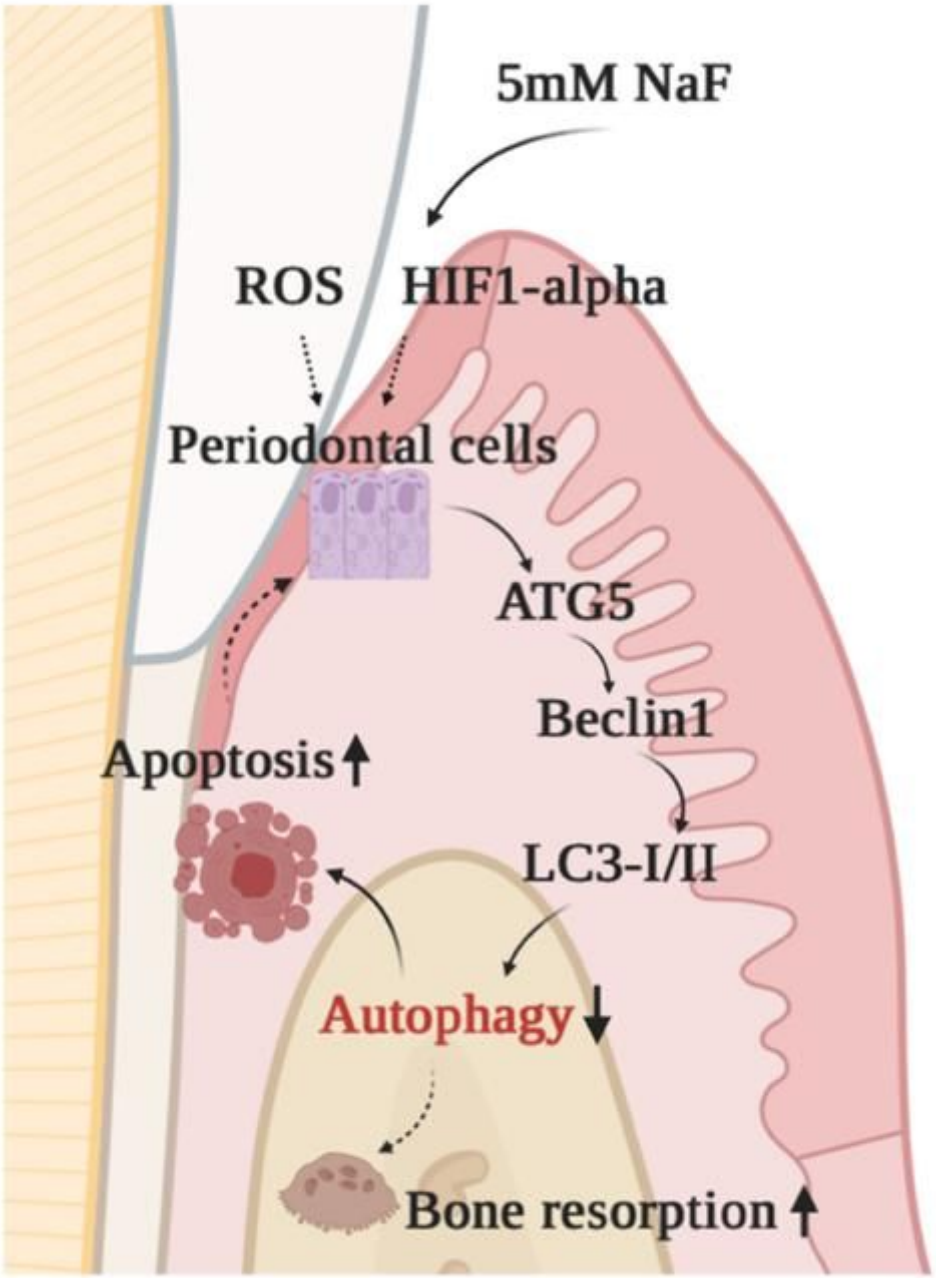

Figure 7

Graphic summary. Treatment with 5 mM NaF upregulates the expression of autophagy related genes, ROS, and HIF1-α, and suppressed the expression of cementoblast markers and induced apoptosis by downregulating ATG5 and Beclin1 expression. The reduced autophagy in the periodontal ligament and alveolar bone may indirectly promote the differentiation of osteoclasts and augment their functions.

Supplementary Files

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