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Sodium Fluoride and Sulfur Dioxide Derivatives Induce TGF-β1-Mediated NBCe1 Downregulation Causing Acid–Base Disorder of LS8 Cells

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Abstract

The aim of the present work was to assess whether the combination of sodium fluoride (NaF) and sulfur dioxide derivatives (SO_2 derivatives) affects the expression of the electrogenic sodium bicarbonate cotransporter NBCe1 (SLC4A4), triggering an acid–base imbalance during enamel development, leading to enamel damage. LS8 cells was taken as the research objects and fluorescent probes, quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and factorial analysis were used to clarify the nature of the fluoro-sulfur interaction and the potential signaling pathway involved in the regulation of NBCe1. The results showed that exposure to fluoride or SO_2 derivatives resulted in an acid–base imbalance, and these changes were accompanied by inhibited expression of NBCe1 and TGF-β1; these effects were more significant after fluoride exposure as compared to exposure to SO_2 derivatives. Interestingly, in most cases, the toxic effects during combined exposure were significantly reduced compared to the effects observed with fluoride or sulfur dioxide derivatives alone. The results also indicated that activation of TGF-β1 signaling significantly upregulated the expression of NBCe1, and this effect was suppressed after the Smad, ERK, and JNK signals were blocked. Furthermore, fluoride and SO_2 derivative-dependent NBCe1 regulation was found to require TGF-β1. In conclusion, this study indicates that the combined effect of fluorine and sulfur on LS8 cells is mainly antagonistic. TGF-β1 may regulate NBCe1 and may participate in the occurrence of dental fluorosis through the classic TGF-β1/Smad pathway and the unconventional ERK and JNK pathways.

Keywords Dental fluorosis · Fluoride · Sulfur dioxide · NBCe1 · TGF-β1

Introduction

Fluoride and sulfur are widely distributed in various forms in nature, especially in southwest China, where there is a high incidence area of coal-burning fluorosis. Importantly, the distribution of fluorine pollution and sulfur pollution in coal overlaps [1]. When coal is burned, the majority of the fluorine and sulfur are released into the air in the form of hydrogen fluoride (HF) and sulfur dioxide (SO_2). In recent years, the control of fluoride and SO_2 emissions has resulted in a significant reduction in exposure to pollution sources, although serious pollution still exists in some remote areas [2]. Chronic exposure to high fluoride levels results in dental fluorosis [3]. Dental fluorosis is clinically characterized by chalky, opaque plaques or enamel defects and can seriously affect the appearance and function of the teeth [4]. Dental fluorosis has become an increasingly serious worldwide problem; however, the precise mechanisms underlying the development of dental fluorosis are unclear. After SO_2 enters the body, it is transformed into its metabolic derivatives, sulfites, and bisulfites, in the blood. These are then distributed to all organs of the body in a dynamic equilibrium with a molar concentration of 3:1. Therefore, the damage caused by SO_2 in an organism is actually caused by its derivatives [5]. Studies demonstrated that both individual and combined exposures to fluoride and SO_2 resulted in pathological changes and DNA damage in brain, the liver morphology,

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and DNA integrity of rats were adversely affected by fluoride and/or SO_2 exposure [6, 7]. Research also showed that a marked decrease in sperm quality and altered morphology and ultrastructure of blood testis barrier in the testis of mice exposed to fluoride or/and SO_2 [8]. However, most studies of the mechanisms of dental fluorosis have only focused on the effects of fluoride. The effect of co-exposure to fluoride and SO_2 on enamel remains unclear.

Enamel formation is a unique cell-regulated biological mineralization process, which mainly occurs in two stages: in the secretion stage, characterized by the initiation and slow lengthening of enamel crystals; in the maturation stage, the crystal expands rapidly in width and thickness. The development of enamel requires strict pH control at all stages of formation. The formation of hydroxyapatite crystals produces a large number of protons, which need to be buffered to maintain mineralization [9, 10]. Most studies support the concept that ameloblasts secrete bicarbonate to buffer protons released by mineral formation. Ameloblasts express many transmembrane proteins, which are typical proteins for pH regulation and ion transport in epithelial cells [11]. The Na^+/HCO_3^- cotransporter (NBCe1) encoded by SLC4A4 is a member of SLC4 (solute carrier bicarbonate transport) family is one of the important transmembrane proteins of cells [12]. NBCe1 has three main variants, NBCe1-A, NBCe1-B, and NBCe1-C. Human NBCe1 mutations cause kidney development defects, leading to acidosis (proximal tubular acidosis) and changes in the eyes and teeth [13]. NBCe1-/- mice had a severe and fatal phenotype, died before weaning, and had chalky white enamel that was easily broken [14]. NBCe1 is expressed in ameloblasts in a polarized manner and plays a key role in enamel mineralization by transporting HCO_3^- and Na^+ to regulate the pH of ameloblasts. Humans with NBCe1 mutations have been found to have enamel abnormalities [15]. Similar findings have been observed in mice lacking NBCe1 [14].

The development of enamel is regulated by a variety of signal molecules, including transforming growth factor-beta (TGF-β). The TGF-β/Smad signaling pathway is the classical TGF-β pathway. TGF-β phosphorylates Smad2 and Smad3 by binding to their receptors and forms a complex with Smad4, which is then transferred into the nucleus to further regulate the expression of the TGF-β target gene and other DNA-binding factors [16]. Yet, TGF-β may also signal through other pathways, such as the mitogen-activated protein kinase (MAPK) pathway and related molecules including JNK, p38 MAPK, and p-p44/42 MPAK (ERK), which may act independently or in concert with the classical pathways [17]. It was found that overexpression of TGF-β1 resulted in cyst-like enamel in mice with low mineral content, no columnar enamel structure in developing enamel, and enamel mineralization defects in Smad3 knockout mice [18, 19]. Studies have demonstrated that the expression of TGF-β1 in rat incisor ameloblasts is inhibited in chronic fluorosis, with the degree of inhibition positively correlated with the fluoride concentration [20]. Moreover, fluoride can mediate tooth formation through the MAPK signaling pathway [21]. Research has also indicated that SO_2 reduces DNA synthesis by inhibiting the ERK/MAPK pathway and prevents vascular smooth muscle cells (VSMCs) from entering the S phase from the G1 phase, thus negatively regulating proliferation [22]. It can also reduce collagen remodeling by inhibiting the TGF-β/Smad pathway of VSMCs [23]. These studies indicate that both fluoride and sulfur play corresponding regulatory roles through the TGF-β1/Smad and MAPK signaling pathways. Under pathophysiological conditions, the K1 channel blockers 4AP and TGF-β1 in the extracellular space are activated, and by binding to the TGF-β receptor and activating the Smad, ERK, and JNK signaling pathways to autocrine and/or paracrine, it acts on astrocytes and regulates the functional expression of NBCe1, thereby participating in the pH inside and outside the cell [24, 25]. At present, no studies have shown the regulatory relationship between TGF-β1 and NBCe1 in ameloblasts. In this case, further studies are needed to clarify the regulatory mode of NBCe1 in ameloblasts and its possible mechanism in the combination of fluoride and sulfur in tooth enamel damage.

Thus, it was hypothesized that the TGF-β1/Smad and MAPK signaling pathways may be involved in enamel injury induced by fluoride and sulfur through the regulation of NBCe1. The results of this study indicated that combined exposure to fluoride and SO_2 derivatives had an antagonistic effect on LS8 cells. Additionally, the TGF-β1 pathway was found to be involved in the regulation of LS8 cell acid–base disorder via inhibition of the downstream TGF-β1/Smad, ERK, and JNK signaling pathways, which, in turn, downregulated the expression of NBCe1, resulting in dental fluorosis. These findings provide an experimental basis for the analysis of the potential molecular mechanism of dental fluorosis.

Materials and methods

Cell culture

The LS8 mouse ameloblast-derived cell line (provided by Special Key Laboratory Of Oral Disease Research, Higher Education Institution in Guizhou Province) and the SV40-immortalized mouse ameloblast-like cell line were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (10%), penicillin (50 units/ml), and streptomycin (50 μg/mL) (Solarbio, Beijing, PR China) at 37 °C in a 5% CO_2-humidified atmosphere.

Cell viability assays

A Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to determine cell viability. Approximately 6000 cells per well were transferred to 96-well plates with 100 μL of DMEM medium containing 10% FBS. Cells were cultured in a humidified incubator at 37 °C. At a cell confluence of around 80%, the culture medium was changed and the cells were incubated for 24, 48, or 72 h in 100 μL of medium containing varying doses of sodium fluoride (NaF; Sigma-Aldrich, St. Louis, MO, USA) (0, 0.25, 0.5, 1.0, 2, 4, 8, 16 mM) or sulfur dioxide (SO_2) derivatives (Sigma-Aldrich, St. Louis, MO, USA) (0, 1, 2, 3, 4, 5, 6, 7, 8 mM). CCK8 reagents were added to each well and the cells were incubated for 1.5 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the cell survival rate was calculated according to the following formula: (absorbance of the experimental group − absorbance of the blank group)/(absorbance of the untreated control group − absorbance of the blank group) × 100%. The experiments were performed in triplicate.

Cell treatment

2×10^5 cell suspensions/well (2 mL) was seeded into 6-well plates; the final concentrations of fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for the combination treatment were selected based on the cell viability assays. According to the 2 × 3 factorial design, the experiment was divided into 9 groups: (1) no treatment control group; (2) NaF (0.2 mM, $F_{0.2}$) treatment group; (3) NaF (2 mM, F_2) treatment group; (4) SO_2 derivatives (0.3 mM, $S_{0.3}$) treatment group; (5) SO_2 derivatives (3 mM, S_3) treatment group; (6) $F_{0.2} + S_{0.3}$ combined treatment group; (7) $F_{0.2} + S_3$ combined treatment group; (8) $F_2 + S_{0.3}$ combined treatment group; (9) $F_2 + S_3$ combined treatment group. Cells were treated with NaF and SO_2 derivatives for 24 h. For the intervention trials, the serum was removed from the medium 12 h before treatment, and the cells were incubated with recombinant human TGF-β1 (PeproTech; Rocky Hill, NJ, USA); doses of 0, 2.5, 5, and 10 ng/mL were used to treat LS8 cells for 1 h and 10 ng/mL. TGF-β1 was used to treat LS8 cells for different time periods (0, 15, 30, 45, 60 min). Then, cells submitted to the 1 h TGF-β1 treatment were submitted to one of the following combination treatments: F_2S_3, Smad3 inhibitor SIS3 (5 μM; Selleckchem, Houston, TX, USA), ERK inhibitor PD98059 (10 μM; Selleckchem), or JNK inhibitor SP600125 (10 μM; Selleckchem). After 1 h of treatment with TGF-β1, the $F_2 + S_3$ was added and the cells were cultured for 24 h, or the cells were incubated with SIS3, ERK, or PD98059 for 2 h before the addition of TGF-β1 or $F_2 + S_3$.

Measurement of intracellular pH

To detect the intracellular pH level of LS8 cells, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Beyotime Biotechnology, Shanghai, PR China) was used. After treatment, the cells were washed twice with 10 mmol/L HEPES buffer, and 5 umol/L BCECF-AM fluorescent probe was added to each well (from a total of 500 μL); the cells were then incubated in an incubator at 37 °C for 30 min in the dark. Next, the dye solution was absorbed, and the fluorescent probes that did not enter the cells were flushed twice with a HEPES buffer. Finally, HEPES buffer was added to test fluorescence intensity of LS8 cells. Image J software was used to calculate the average fluorescence intensity.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

LS8 cells were cultured and treated as described above. The total RNA was isolated from the LS8 cells using a Trizol reagent (Invitrogen, Carlsbad, California, USA). RNA (1 μg) was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Shiga, Japan), according to the manual. qRT-PCR was performed for 40 cycles at 95 °C for 30 min, 54 °C for 60 s, and 72 °C for 30 s using a CFX Connect Thermocycler (Bio-Rad, Hercules, Calif, USA) with SYBR Green dye (Takara, Shiga, Japan), according to the manufacturer's protocol. A complete list of all primers used in this study can be found in Table 1; all primers were synthesized by Gene Pharma (Sangon Biotech, Shanghai, PR China). The relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Western blot

The cells were lysed with a Radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Beyotime Biotechnology, Shanghai, PR China). The protein content was measured using a BCA protein assay (Beyotime Biotechnology,

Table 1 Primer sequences

Gene	Forward	Reverser
GAPDH	5′-GGTTGTCTCCTGCGACTTCA-3′	5′-CGGTTCATGTCATGGATGGTG-3′
TGF-β1	5′-TACGGCAGTGGCTGAACCA-3′	5′-CGGTTCATGTCATGGATGGT-3′
Smad4	5′-CCTGTTGTGACTGTGGATGG-3′	5′-CCAAACGTCACCTTCACCTT-3′
NBCe1	5′-GGCAGGCTGGGTTTGACTAG-3′	5′-TGGGTGACTTTAGCCCTGAGT-3′

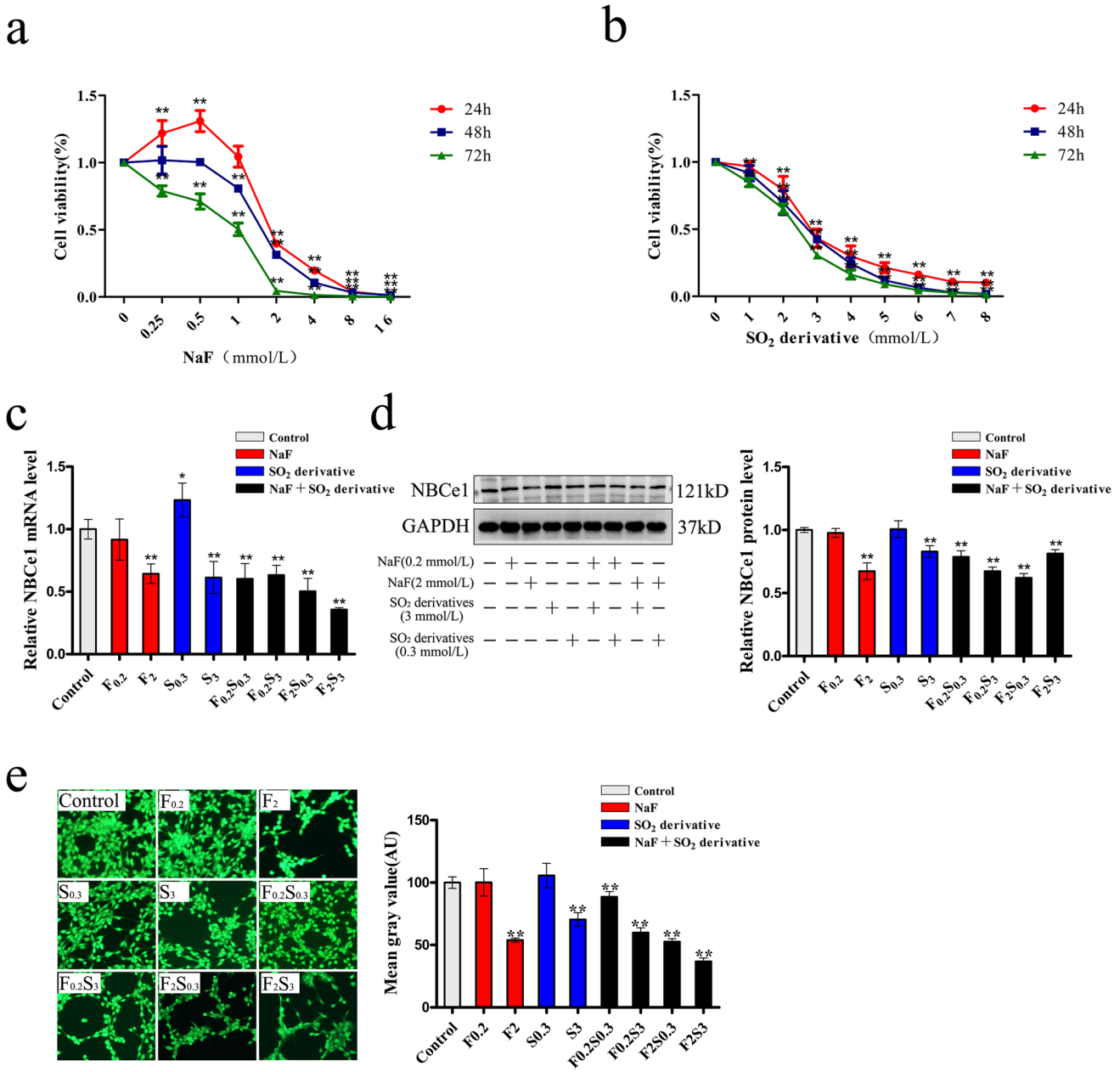


Fig. 1 NBCe1 response to fluoride and SO_2 derivatives in LS8 cells. **a**, **b** CCK8 assays were carried out to determine the viability of LS8 cells treated with fluoride (0, 0.25, 0.5, 1.0, 2, 4, 8, 16 mM) or SO_2 derivatives (0, 1, 2, 3, 4, 5, 6, 7, 8 mM) for 0, 24, 48, and 72 h. $n=6$. **c** Effects of individual and combined exposure to fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for 24 h on the intracellular pH of LS8 cells; cells were incubated with 5 μm BCECF-AM in HEPES solution at 37 °C for 30 min; the images were taken under a fluorescence microscope with a 20 lens. **d**, **e** Effects of individual and combined exposure to fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for 24 h on mRNA (qRT-PCR analysis of cDNA from LS8 cells) and protein (20 mg of protein was loaded per lane) expression of NBCe1. Statistical data were analyzed by LSD-t. Data are expressed as the mean ± SD, $n=3$, $^{*}P<0.05$, $^{**}P<0.01$, compared with control group

Shanghai, PR China). A total of 20–25 μg of protein lysate per sample was loaded for 6 ~ 10% polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Biotechnology, Shanghai, PR China) and was transferred from the gel to 0.45 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk for 2 h at room temperature and incubated with antibodies against TGF-β1 (ab260408, 1:1000, abcam, Cambridge, MA, USA), Smad4 (ab40759, 1:1000, abcam), NBCe1 (ab187511, 1:1000, abcam), Phospho-Smad2 (Ser245/250/255) (#3104, 1:1000, Cell

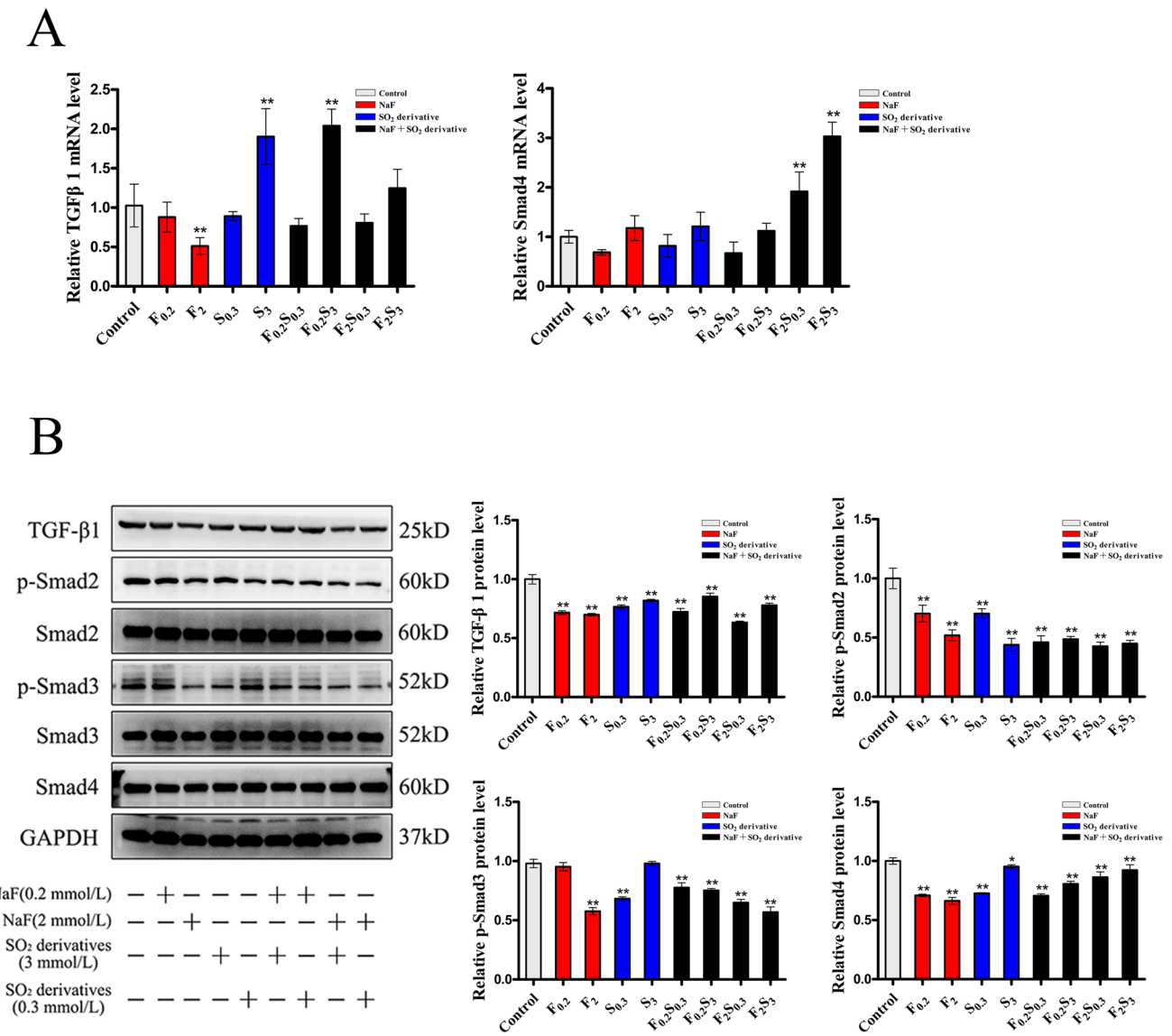


Fig. 2 Effects of individual and combined exposure to fluoride and SO_2 derivatives on the TGF-β1/Smad signaling pathways. **a**, **b** qRT-PCR analysis of TGF-β1 and Smad4 mRNA levels in LS8 cells treated with fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for 24 h. The relative gene expression was normalized to the expression of the housekeeping gene, GAPDH. **c** LS8 cells were treated with fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for 24 h and TGF-β1, p-Smad2, p-Smad3, and Smad4 protein expression were quantified by western blot analysis; 25 mg of protein was loaded per lane; GAPDH served as the loading control protein. Statistical data were analyzed by LSD-t. Data are expressed as the mean ± SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, compared with control group

Signaling Technology, Danvers, MA, USA), Smad2 (#5339,1:1000, CST), Phospho-Smad3 (Ser423/425) (#9520, 1:1000, CST), Smad3 (#9523,1:1000, CST), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370, 1:1000, CST), p44/42 MAPK (Erk1/2) (#9102, 1:1000, CST), Phospho-p38 MAPK (Thr180/Tyr182) (#4511, 1:1000, CST), p38 MAPK (#8690, 1:1000, CST), Phospho-SAPK/JNK (#4668, 1:1000, CST), SAPK/JNK (#9252, 1:1000, CST), and GAPDH (ab8245, 1:5000, abcam) at 4 °C overnight. The membranes were then washed with a Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with secondary antibodies for horseradish peroxidase (HRP)-conjugated Affnipure Goat Anti-Mouse lgG (H + L) (SA00001-1, 1:8000, Proteintech Group, Inc. Wuhan, PR China) and HRP-conjugated Affnipure Goat Anti-Rabbit (SA00001-2, 1:8000, Proteintech Group, Inc. Wuhan, PR China) for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (ECL) detection reagents (Millipore, Billerica, MA, USA). Images of western blots were acquired on a ChemiDoc MP system (Bio-Rad

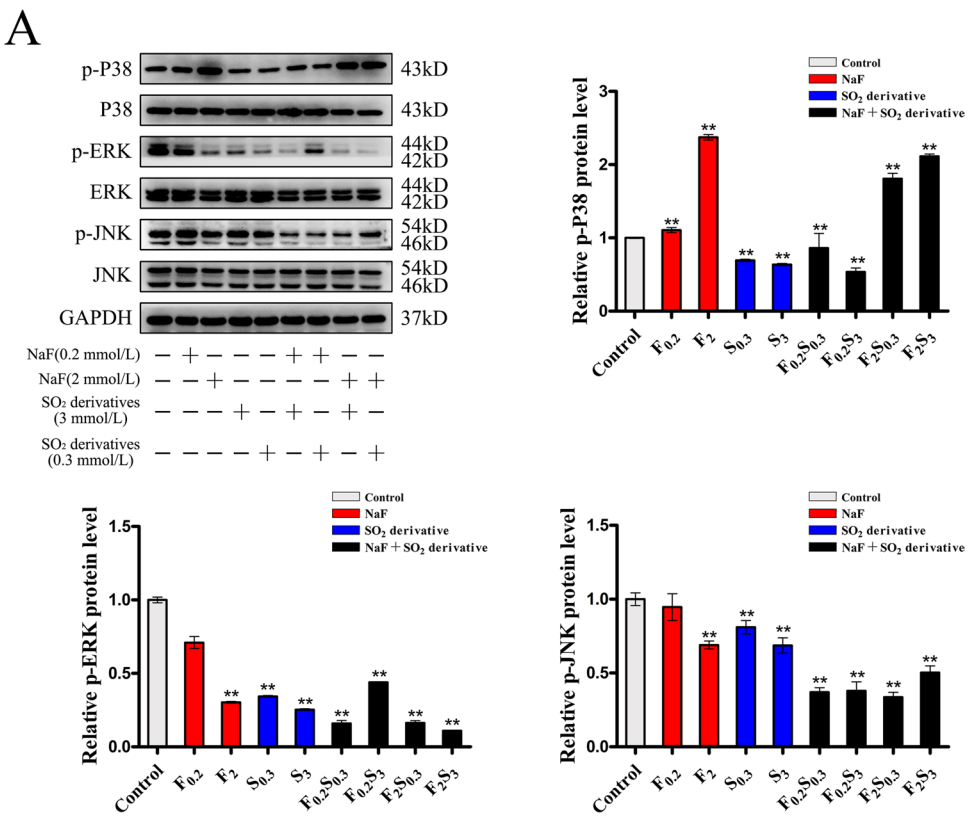


Fig. 3 Effects of individual and combined exposure to fluoride and SO_2 derivatives on the MAPK signaling pathway. **a** Western blot analysis of p-P38, p-ERK, and p-JNK in LS8 cells treated with fluoride (0, 0.2, 2 mM) and SO_2 derivatives (0, 0.3, 3 mM) for 24 h; 25 mg of protein was loaded per lane; GAPDH served as the loading control protein. Statistical data were analyzed by LSD-t. Data are expressed as the mean ± SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, compared with control group

Laboratories), and quantification of blots was performed using ImageJ.

Statistical analysis

Data are expressed as the median (range) or mean ± standard deviation (SD). SPSS v23.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Where appropriate, statistical analysis was undertaken using one-way analysis of variance (ANOVA) and the least significant difference test (LSD-t-t) for multiple comparisons. Pearson's correlation analysis was used in the correlation analysis; $r > 1$ shows that the two variables are positively correlated; $r < 1$ shows that the two variables are negatively correlated; $r = 1$ shows that there is no correlation. Statistical significance was defined as $P < 0.05$; $P < 0.01$ was considered highly significant. All experiments were repeated in triplicate.

Results

Viability of fluoride- and SO_2 derivative-treated LS8 cells

To examine the cytotoxic effects of fluoride and SO_2 derivatives on LS8 cells, the cells were exposed to fluoride and SO_2 derivatives for 24, 48, and 72 h, respectively. The results indicated that cell viability increased at first and then decreased with increasing fluoride dose after treatment for 24 h, as compared to the control group. After 48 h of fluoride treatment, the cell viability result revealed no significant change when the fluoride treatment concentration was less than 1 mM, but when the exposure concentration was greater than 1 mM, there was a gradual decline in cell viability. After treatment with fluoride for 72 h, the viability of LS8 cells decreased significantly (Fig. 1a). In addition, SO_2 derivative-induced inhibition of cell viability exhibited dose- and time-dependent effects. The results

Table 2 Correlation analysis of NBCe1 protein expression with TGF-β1/Smad pathway and MAPK pathway related protein expression

		TGF-β1	p-Smad2	p-Smad3	Smad4	p-P38	p-JNK	p-ERK
NBCe1	*r*	0.435	0.708	0.518	0.116	-0.41	0.798	0.577
	P	0.023	0.000	0.006	0.563	0.034	0.000	0.000

indicated that LS8 cells were sensitive to both fluoride and SO_2 derivatives, and there was a good dose–response relationship after a short period of action (Fig. 1b). The cell viability trends were similar after treatment for different periods of time. Consequently, 24 h was selected as the treatment time for subsequent experiments. After 24 h of treatment with fluoride or SO_2 derivatives, the cell viability decreased to 45.0% (± 0.02) with 2 mM fluoride and 47.3% (± 0.06) with 3 mM SO_2 derivatives, as compared with the control. Thus, in the subsequent experiments, 2 mM fluoride and 3 mM SO_2 derivatives were used as the highest concentrations, and one tenth of their concentrations were selected as the lowest concentrations, respectively.

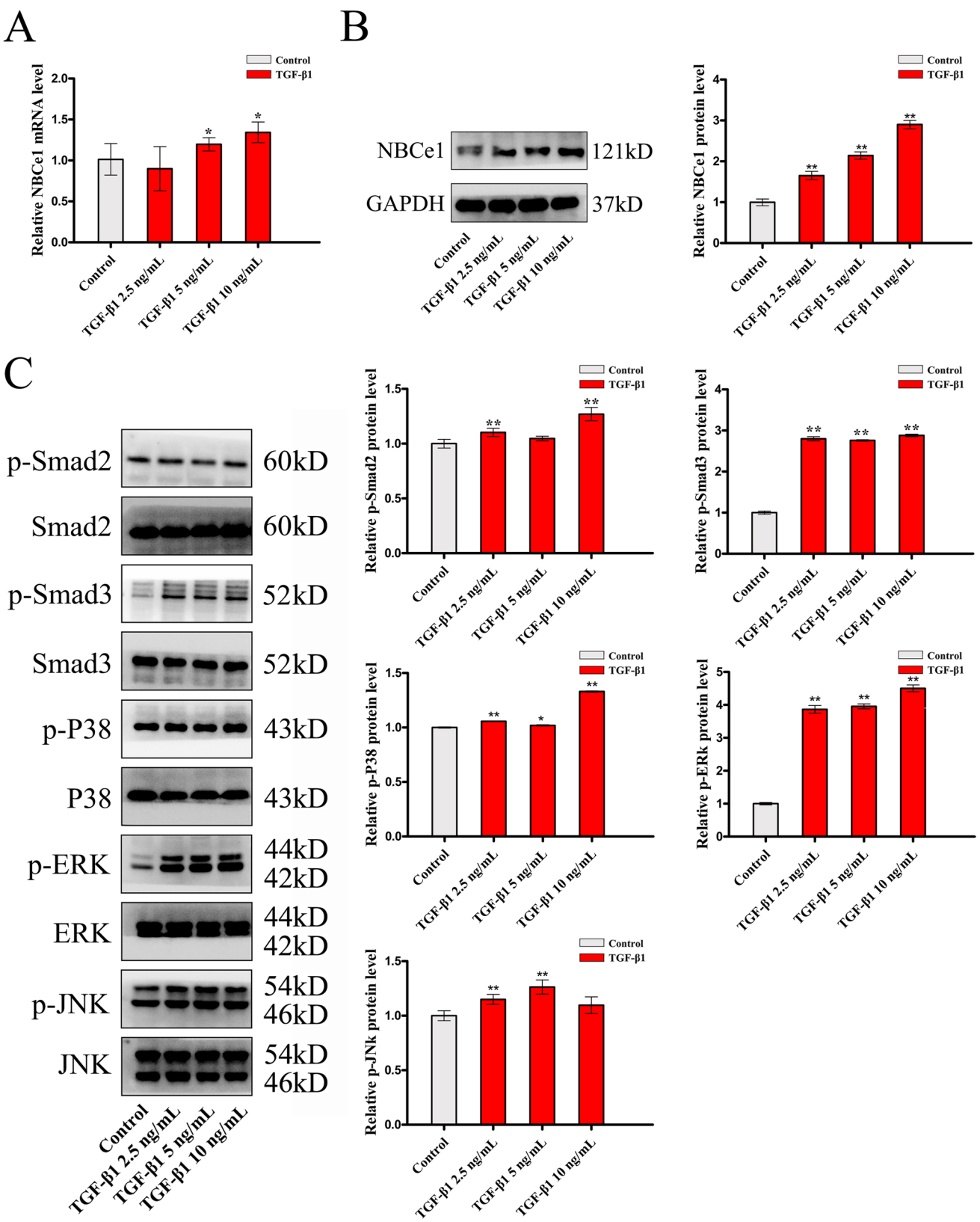
Response of NBCe1 to individual and combined exposure to fluoride and SO_2 derivatives in LS8 cells

Previous data have shown that NBCe1 is a potential pH regulator in enamel organ cells during enamel development in mice [22]. Thus, we first investigated whether NBCe1 was downregulated in LS8 cells after exposure to fluoride and SO_2 derivatives, thereby influencing cell pH regulation, in order to determine whether NBCe1 is a potential research target. To this end, LS8 cells were treated with various concentrations of fluoride and SO_2 derivatives and subsequently, NBCe1 mRNA and protein abundance, and intracellular pH was determined by qRT-PCR, western blot, and BCECF-AM analysis, respectively. The results indicated that exposure to F_2 and S_3 alone produced appreciable decreases in intracellular pH while there were no changes in pH with $F_{0.2}$ and $S_{0.3}$. Interestingly, there was a more pronounced decrease in intracellular pH with co-exposure to fluoride and SO_2 derivatives, particularly with combined exposure to F_2 and S_3 (Fig. 1e).

The results also indicated that $F_{0.2}$ and $S_{0.3}$ alone produced no appreciable changes in NBCe1 mRNA and protein levels. Conversely, there were marked increases with F_2 and S_3 exposure individually. With co-exposure to $F_{0.2}$ and $S_{0.3}$ or S_3, depletion of the NBCe1 protein was more pronounced compared to exposure to each individually. Compared with the F_2 group, there were no appreciable changes in the NBCe1 mRNA and protein expression levels in the $F_2S_{0.3}$ group. Moreover, the NBCe1 protein level in the F_2S_3 group was lower than F_2 group. Compared to individual exposure to SO_2 derivatives, the protein expression level of NBCe1 in F_2S_3 group had no significant change, and the mRNA and protein expression levels of NBce1 in other combined groups were significantly lower than those in $S_{0.3}$ group or S_3 group (Fig. 1c and d). The interaction analysis showed that the combined effects of fluoride and sulfur on the mRNA and protein expression of NBCe1 in LS8 cells showed interaction ($F = 32.650, 18.846$, $p < 0.05$). Moreover, the mean square of NBCe1 mRNA expression in the combined groups (0.097) was less than the sum of the mean square of NBCe1 mRNA expression exposed to fluoride (0.251) and SO_2 derivatives (0.451) alone. Similarly, the mean square of NBCe1 protein expression in the combined groups (0.051) was less than the sum of the mean square of NBCe1 protein expression exposed to fluoride (0.134) and SO_2 derivatives (0.029) alone. According to the criterion of interaction analysis, if the individual effect difference between different levels of one factor is greatly reduced due to the influence of another factor level, and the difference is statistically significant, it can be considered that the two factors have antagonistic interaction. Thus, our results suggest that the effects of fluoride and SO_2 derivatives on NBCe1 are interactive with primarily antagonistic effects.

Fluoride and SO_2 derivatives inhibit the TGF-β1/Smad and MAPK signaling pathways in LS8 cells

To examine the relevant mechanism, the TGF-β1 and Smad4 mRNA expression levels of LS8 cells were examined by qRT-PCR. TGF-β1 mRNA expression was decreased by fluoride but increased by SO_2 derivatives. Conversely, TGF-β1 and Smad4 mRNA expression were significantly higher in the group exposed to the combination of SO_2 derivatives and fluoride than in the single exposure group (Fig. 2a). The results of the interaction analysis showed that fluoride and SO_2 derivatives had synergistic effects on TGF-β1 and Smad4 mRNA expression. The influence of the TGF-β1/Smad (TGF-β1, p-Smad2, p-Smad3, and Smad4) signaling pathway (Fig. 2b) and several MAPK family members (ERK, JNK, and p38) was evaluated by western blot (Fig. 3a). The data indicated that exposure to fluoride significantly reduced the expressions of TGF-β1, p-Smad2, p-Smad3, Smad4, p-ERK, and p-JNK while p-P38 was significantly increased with increasing fluoride exposure dose. When exposed to SO_2 derivatives alone, the protein abundance of TGF-β1, p-Smad3, and Smad4 decreased compared with the control group, but the decrease degree was greater in $S_{0.3}$ group compared with S_3 group, and p-Smad2, p-ERK, p-JNK, and p-P38 showed a gradual decreasing trend in a dose-dependent manner. Compared with $F_{0.2}$ group, TGF-β1 and Smad4 protein expression levels in $F_{0.2}S_{0.3}$ group did not change, but increased in $F_{0.2}S_3$ group; however, the expression of p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38 decreased in $F_{0.2}S_{0.3}$ group and $F_{0.2}S_3$ group. Compared with F_2 group, with the increase of SO_2 derivatives dose, the protein abundance of TGF-β1 decreased first and then increased, p-Smad2, p-ERK, p-JNK, and p-P38 all decreased, and, on the contrary, Smad4 increased. The protein expression level of p-Smad3 increased in $F_2S_{0.3}$ group; there was no significant change in $F_2S_{0.3}$ group. Compared with the $S_{0.3}$ group,


A
Relative NBCe1 mRNA level
Control
TGF-β1
Control
TGF-β1 2.5 ng/mL
TGF-β1 5 ng/mL
TGF-β1 10 ng/mL
B
NBCe1
121kD
GAPDH
37kD
Relative NBCe1 protein level
C
p-Smad2
60kD
Smad2
60kD
p-Smad3
52kD
Smad3
52kD
p-P38
43kD
P38
43kD
p-ERK
44kD
42kD
ERK
44kD
42kD
p-JNK
54kD
46kD
JNK
54kD
46kD
Relative p-Smad2 protein level
Relative p-Smad3 protein level
Relative p-P38 protein level
Relative p-ERk protein level
Relative p-JNk protein level

◂**Fig. 4** Activation of the TGF-β1 signaling pathway might modulate NBCe1 expression in a dose-dependent manner through the classical pathway and MAPK signaling pathway in LS8 cells. **a** Dose dependency of NBCe1 mRNA regulation in LS8 cells treated with TGF-β1 (0, 2.5, 5, 10 ng/mL) for 1 h. qRT-PCR analysis of cDNA from LS8 cells. **b** Western blot analysis of NBCe1; 25 mg of protein was loaded per lane. **c** The p-Smad2, p-Smad3, p-P38, p-ERK, and p-JNK protein expression in LS8 cells after treatment with TGF-β1 (0, 2.5, 5, 10 ng/mL) for 1 h. GAPDH served as a loading control protein. Statistical data were analyzed by LSD-t. Data are expressed as means ± SD, $n=3$, $^{*}P<0.05$, $^{**}P<0.01$, compared with control group

the expression of TGF-β1, p-Smad2, p-ERK, and p-JNK protein in the $F_{0.2}S_{0.3}$ group and the $F_2S_{0.3}$ group all decreased; however, the p-P38 protein expression increased. Compared with S_3 group, TGF-β1 and p-Smad2 protein expression did not change significantly in $F_{0.2}S_3$ group and F_2S_3 group, p-Smad3, Smad4, and p-JNK decreased, but p-P38 expression significantly increased; p-ERK showed an ascending and then descending trend. The results also indicated that fluoride and SO_2 derivatives had an interactive effect on the expression of TGF-β1/Smad and MAPK signal pathway-related molecules, primarily showing an antagonistic effect at low concentrations and a synergistic effect at high concentrations. Correlation analysis showed that the NBCe1 protein expression of LS8 cells was positively correlated with TGF-β1, p-Smad2, p-Smad3, p-JNK, and p-ERK protein expressions ($r=0.435$, 0.708, 0.518, 0.798, and 0.577, all $P<0.05$); there was no significant correlation between NBCe1 and Smad4 protein expression ($r=0.116$, $P>0.05$); however, NBCe1 protein expression was negatively correlated with p-P38 protein expression ($r=-0.41$, $P>0.05$). The correlation analysis results of this study can be found in Table 2. In a word, these data suggest that in vitro fluoride and SO_2 derivative models are appropriate tools for studying the regulation of NBCe1. In addition, the results indicate that NBCe1 is located downstream of TGF-β1 in fluoride- and SO_2 derivative-dependent signaling.

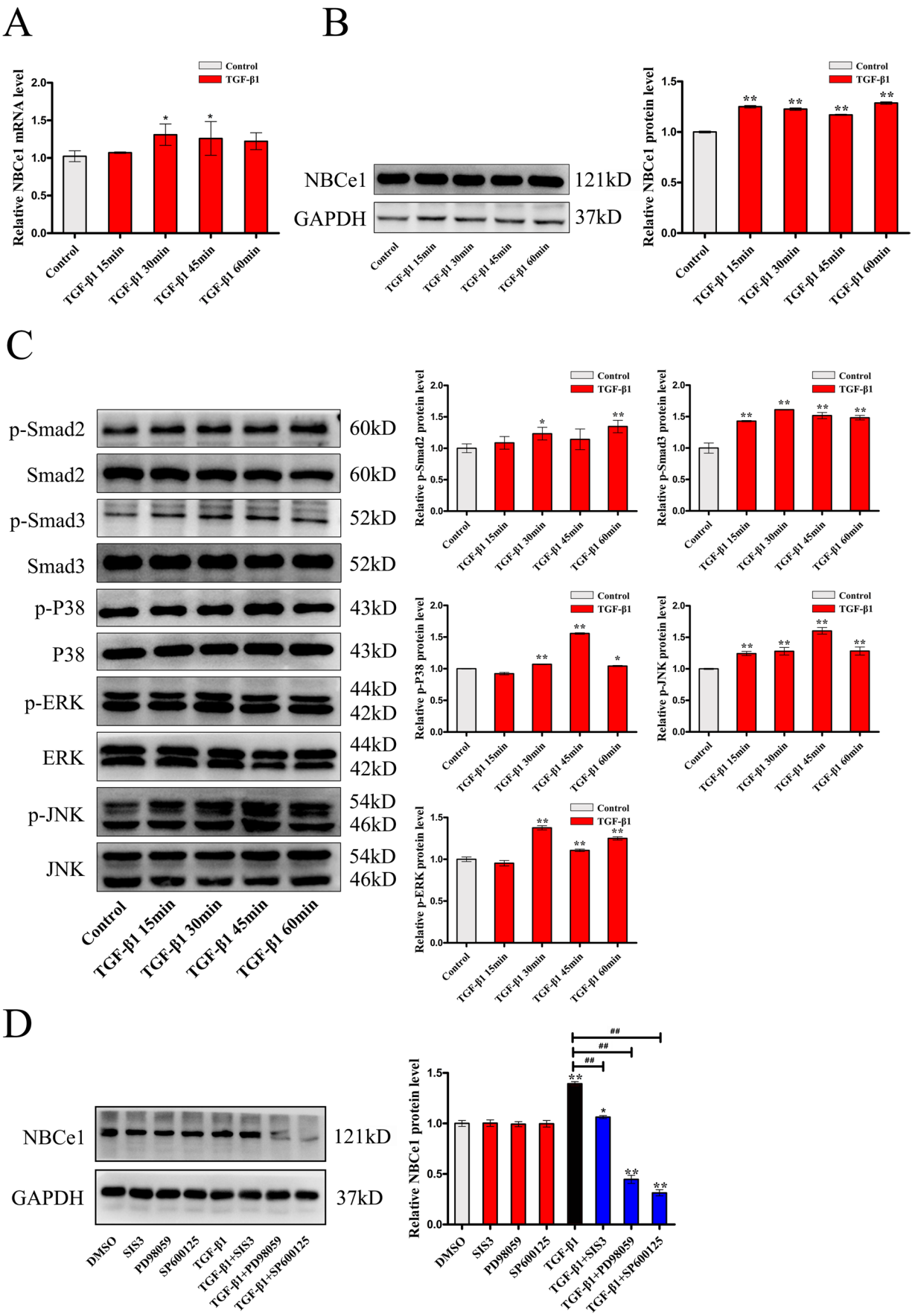
TGF-β1 regulates NBCe1 in LS8 cells through the TGF-β1/Smad and MAPK signaling pathways

To further explore the regulation mechanism of TGF-β1, we examined whether activation of the TGF-β1 signaling pathway might modulate NBCe1 expression in LS8 cells. Human recombinant TGF-β1 (0, 2.5, 5, 10 ng/mL) was used to treat LS8 cells for 1 h and 10 ng/mL TGF-β1 was used to treat LS8 cells for different time periods (0, 15, 30, 45, 60 min). Subsequently, the transcription and protein expression levels of the NBCe1 gene were determined by qRT-PCR and western blot, respectively. Compared with no TGF-β1 treatment, there was no significant difference in the NBCe1 mRNA expression level in the 2.5 ng/mL TGF-β1 treatment group, while there was significantly increased expression in the 5 and 10 ng/mL TGF-β1 treatment groups (Fig. 4 a). The protein expression level increased gradually in a dose-dependent fashion (Fig. 4b). Under the 10 ng/mL TGF-β1 treatment condition, the cells exposed to the treatment for 15 min exhibited no appreciable change in the mRNA expression levels of NBCe1, while the NBCe1 mRNA and protein expression levels of the other groups were significantly increased (Fig. 5a and b). The expression levels of p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38 protein were also detected, and the results showed that regardless of the dose or TGF-β1 treatment time, TGF-β1 significantly upregulated the protein expression levels of p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38 in LS8 cells (Figs. 4c and 5c). Combined with the results of the previous factorial experiment, these results indicate that co-exposure to fluoride and SO_2 derivatives significantly inhibits the expression of TGF-β1, but increases p-P38. However, the level of p-P38 increased significantly during exposure to exogenous TGF-β1, indicating that TGF-β1 cannot regulate NBCe1 activity through P38 in LS8 cells.

In order to understand how TGF-β1 regulates NBCe1 in LS8 cells, cells were treated with the Smad3 inhibitor SIS3 (5 μM), ERK inhibitor PD98059 (10 μM), and JNK inhibitor SP600125 (10 μM) for 2 h before treatment with TGF-β1 (10 ng/mL for 1 h). The results indicated that inhibition treatment alone had no significant effect on NBCe1 protein abundance. On the contrary, when cells were treated with both inhibitors and TGF-β1, the upregulation of NBCe1 by TGF-β1 was markedly blocked by SIS3, PD98059, and SP600125, and the activity of NBCe1 was decreased significantly (Fig. 5d). Taken together, these results indicate that TGF-β1 may regulate NBCe1 through the classical TGF-β1/Smad or the unconventional p-ERK and p-JNK pathways.

The downregulation of NBCe1 as a result of co-exposure to fluoride and SO_2 derivatives is dependent on TGF-β1

To investigate further whether the downregulation of NBCe1 due to fluoride and SO_2 derivatives co-exposure depends on TGF-β1, LS8 cells were treated with fluoride and SO_2 derivatives in the presence and absence of human recombinant TGF-β1 (10 ng/mL). Then, NBCe1 protein expression was determined by western blot. Based on the experimental results from the factorial analysis, it was obtained that the interaction would be most obvious with a fluoride concentration of 2 mM and SO_2 derivatives concentration of 3 mM (F_2S_3). Thus, this combined group was selected for the following experiments. The results showed that NBCe1 protein expression was significantly upregulated after TGF-β1 treatment compared with the control group. In contrast, in the F_2S_3 group, NBCe1 protein abundance was significantly


A
B
C
D
Control
TGF-β1
Relative NBCe1 mRNA level
Relative NBCe1 protein level
NBCe1
GAPDH
121kD
37kD
Control
TGF-β1 15min
TGF-β1 30min
TGF-β1 45min
TGF-β1 60min
p-Smad2
Smad2
p-Smad3
Smad3
p-P38
P38
p-ERK
ERK
p-JNK
JNK
60kD
52kD
43kD
44kD
42kD
54kD
46kD
Relative p-Smad2 protein level
Relative p-Smad3 protein level
Relative p-P38 protein level
Relative p-JNK protein level
Relative p-ERK protein level
DMSO
SIS3
PD98059
SP600125
TGF-β1
TGF-β1+SIS3
TGF-β1+PD98059
TGF-β1+SP600125

◂**Fig. 5** Activation of the TGF-β1 signaling pathway might modulate NBCe1 expression in a time-dependent manner through the classical pathway and MAPK signaling pathway in LS8 cells. Time dependency of NBCe1 regulation in LS8 cells treated with 10 ng/mL TGF-β1 (0, 15 min, 30 min, 45 min, 60 min). **a** NBCe1 mRNA was measured by qRT-PCR. **b** Western blot was used to measure the protein abundance of NBCe1. **c** The p-Smad2, p-Smad3, p-P38, p-ERK, and p-JNK protein expression in LS8 cells after treatment with 10 ng/mL TGF-β1 (0, 15 min, 30 min, 45 min, 60 min). **d** LS8 cells were treated with the Smad3 inhibitor SIS3 (5 μM), ERK inhibitor PD98059 (10 μM), and JNK inhibitor SP600125 (10 μM) for 2 h before treatment with TGF-β1 (10 ng/mL for 1 h); NBCe1 protein expression was measured by western blot; 25 mg of protein was loaded per lane; GAPDH served as a loading control protein. Statistical data were analyzed by LSD-t. Data are expressed as the mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with TGF-β1 group

downregulated. Surprisingly, simultaneous treatment with TGF-β1 and F_2S_3 increased the level of NBCe1 in LS8 cells, as compared with F_2S_3 treatment alone (Fig. 6a). The protein expression levels of p-Smad2, p-Smad3, p-ERK, and p-JNK were detected after the treatment described above and, consistent with the above results, it was found that compared to the F_2S_3 group, concomitant treatment with F_2S_3 in the presence of human recombinant TGF-β1 produced a marginal elevation (non-significant) in p-Smad2 and p-ERK protein abundance, while there were more pronounced increases in p-Smad3 and p-JNK protein expression (Fig. 6b).

Taken together, these data demonstrate that fluoride and SO_2 derivatives affect the transcriptional and functional expression of NBCe1 in LS8 cells via the TGF-mediated TGF-β1/Smad, ERK, and JNK signaling pathway.

Discussion

The pathogenic mechanisms underlying the effects of fluoride and sulfur in combination on dental fluorosis are largely unknown. In addition, NBCe1 is thought to play a key role in enamel development. The process of enamel mineralization is strictly pH-dependent, where NBCe1 is responsible for the reabsorption of HCO_3^- and plays a crucial role in maintaining crystal growth [26, 27]. Mutations in NBCe1 induce defects in human and mouse enamel development [14]. However, it is not clear whether the combination of fluoride and sulfur can damage tooth enamel via NBCe1. Here, it was demonstrated that both individual and co-exposure to fluoride and SO_2 derivatives inhibited the expression of NBCe1 and resulted in an acid–base imbalance in cells. In some cases, fewer toxic effects were observed during combined exposure compared to fluoride or SO_2 derivatives alone; mainly antagonistic effects were found after co-exposure to fluoride and SO_2 derivatives. Furthermore, stimulation of LS8 cells by human recombinant TGF-β1 resulted in partial reversal of the downregulation of NBCe1 induced by fluoride and SO_2 derivatives, while Smad3, ERK, and JNK inhibitors reduced the activation effect of TGF-β1 on NBCe1. These findings confirm that the TGF-β1 signaling pathway directly regulates NBCe1 expression through Smad, ERK, and JNK.

We found that low dose of fluoride can improve LS8 cell activity and high dose of fluoride can inhibit LS8 cell activity; this is consistent with previous studies showing that fluoride at different concentrations has both a stimulatory and an inhibitory effect on cell activity [28]. John R. Farley and colleagues found that fluoride treatment increased bone cell proliferation and alkaline phosphatase activity in vitro and increased bone formation in embryonic skulls in vivo [29]. Other reports indicate that fluoride enhances protein tyrosine phosphorylation in osteoblast-like cells by enhancing tyrosine kinase activity, thereby promoting the proliferation of osteoblast-like cells [30]. Therefore, the reason why low-dose fluoride promoted LS8 cell proliferation might be that fluoride increased alkaline phosphatase or tyrosine kinase activity and promoted LS8 cell division.

Ameloblast is one of the target cells highly sensitive to fluoride. Studies showed that after fluoride treatment for a period of time, the expression of Fas receptor and caspase-3 activity on ameloblast surface-mediated apoptosis were enhanced, and there was a dose–effect relationship. There was also a certain correlation between apoptosis rate and fluoride exposure dose [31, 32]. High doses of fluoride easily targeted the transitional/early maturation ameloblasts and caused apoptosis or cell death. Bcl-2 might be involved in this process [33]. The results showed that the activity of LS8 cells decreased with the increase of SO_2 derivatives does and the prolongation of time; these results are consistent with previous studies that SO_2 derivatives can decrease the viability of guard cells (0.4–4.0 mM) and Swan.71 cells (0.1–10.0 mM) and increase their mortality in a dose-dependent manner [34, 35].

CCK8 data showed that when exposed to 2 mM of NaF or 3 mM of SO_2 derivatives for 24 h, the cell survival rate decreased to about half (45.28 ± 3.14% and 47.35 ± 2.28%). Therefore, the concentration of NaF (2 mM) and SO_2 derivatives (3 mM) was selected as the highest dose in the experiment. Some scholars treated SH-SY5Y cells with 0.2 and 2 mM of NaF, respectively, to explore the mechanism of neural damage induced by different concentrations of fluoride [36, 37]. Based on the above reasons, 0.2 and 2 mM of NaF and 0.3 and 3 mM of SO_2 derivatives were selected to explore the possible mechanism of enamel damage induced by fluoride and sulfur.

Enamel biomineralization results in the release of protons into the matrix and acidification of the microenvironment. Ameloblasts require NBCe1 to transport bicarbonate to maintain mineral deposition [38, 39]. The current results demonstrated that the pH of LS8 cells was reduced under

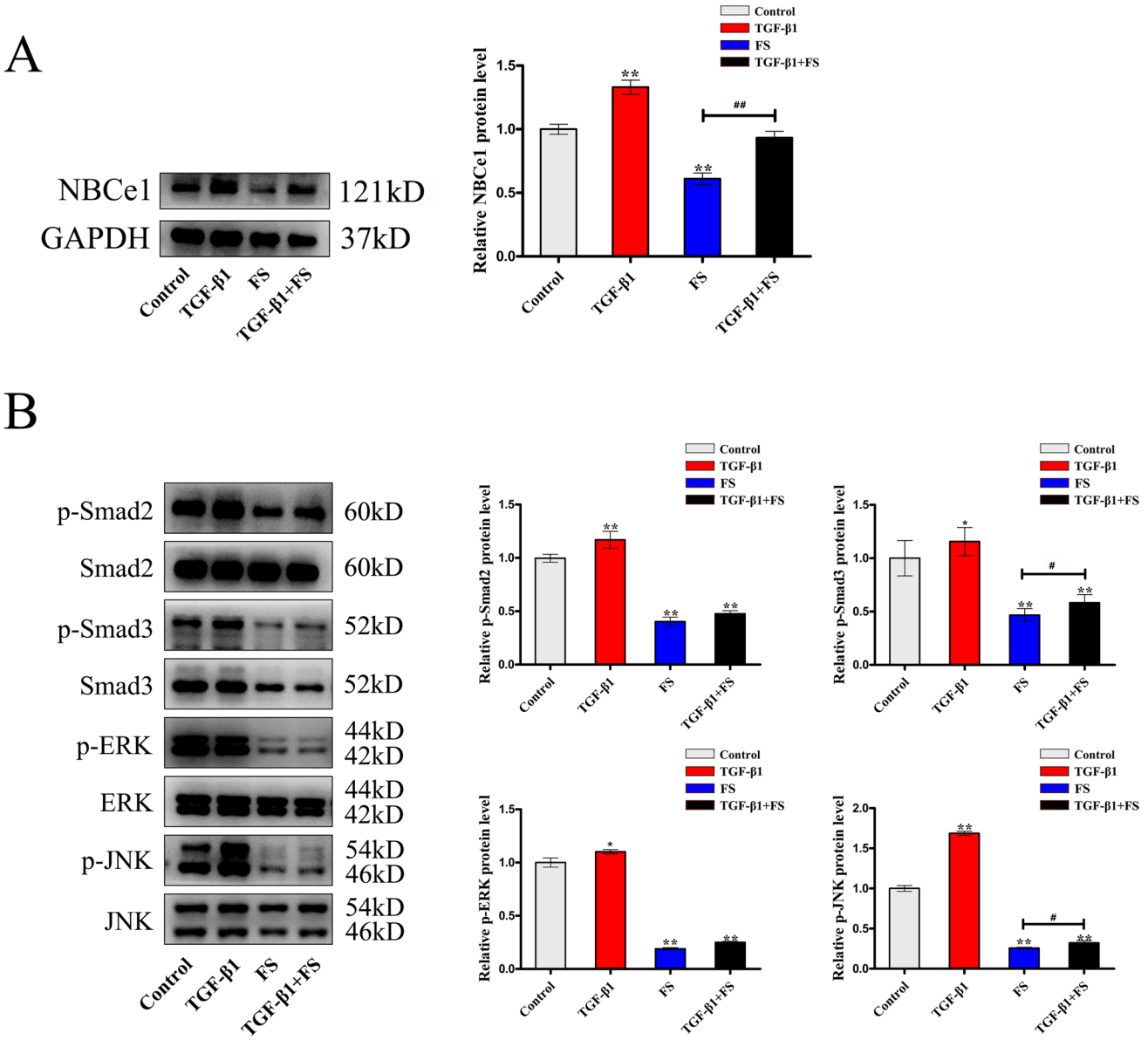


Fig. 6 Downregulation of NBCe1 by fluoride and SO_2 derivatives co-exposure depends on TGF-β1. **a** Protein abundance of NBCe1 by western blot in LS8 cells in the presence or absence of TGF-β1 (10 μM) and after the treatment with F_2S_3. **b** The p-Smad2, p-Smad3, p-ERK, and p-JNK protein expression in LS8 cells after treatment with TGF-β1 (10 ng/mL) in the presence or absence of F_2S_3. A total of 20 mg of protein was loaded per lane; GAPDH served as a loading control protein. Statistical data were analyzed by LSD-t. Data are expressed as the mean ± SD, $n=3$, $^{*}P<0.05$, $^{**}P<0.01$, compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with FS group

exposure to fluoride and SO_2 derivatives alone or in combination. The changes in the expression of NBCe1 were consistent with the changes in pH, suggesting that fluoride and SO_2 derivatives alone and in combination may lead to cell acid–base disorder by inhibiting NBCe1 expression. These findings are in contrast to the findings of Zheng and coworkers [40], who found that NBCe1 expression was not affected by fluoride. The possible reason for the inconsistent results may be that the current study used a higher concentration of fluoride than the study (50 ppm) by Zheng et al., and this could have a direct effect on NBCe1 expression. At high dose of fluoride, pH regulation becomes maladjusted, which leads to ameloblast dysfunction and abnormal enamel mineralization [27]. Combined with our data, it is suggested that inhibition of NBCe1 expression may contribute to this process.

TGF-β is a growth factor with many biological functions, which is widely involved in various physiological and pathological processes of mammals, as well as in the development of tooth germ and the formation of tooth tissue

[41]. Previous studies have demonstrated that fluoride significantly reduces TGF-β1 transcript levels in fluorosed enamel [42]. SO_2 derivatives markedly inhibit the TGF-β1-induced phosphorylation of Smad2 and Smad3 in VSMCs [23]. The current results indicated that both fluoride and sulfur markedly inhibited the expression of TGF-β1, p-Smad2, p-Smad3, and Smad4, and the trend was basically consistent with NBCe1. Research has revealed that TGF-β1 can directly regulate the transcription and functional expression of NBCe1 in mouse astrocytes [24], and, thus, these results suggest that fluoride and sulfur may inhibit the phosphorylation of the receptor substrate protein signaling molecules Smad2 and Smad3 and the expression of the Smad4 protein by downregulating TGF-β1 expression, thereby downregulating the expression of NBCe1. The results show that excessive fluoride can inhibit TGF-β1 expression in ameloblasts, and the downregulation of TGF-β1 expression by fluoride may contribute to the decrease of KLK4 protein level in enamel; it also explains the possible reason of abnormal protein content in dental fluorosis enamel [42]. Therefore, we speculated that fluoride and sulfur may downregulate NBCe1 expression by inhibiting TGF-β1 activation, so that the acidified enamel microenvironment cannot be fully neutralized by bicarbonate, thus interfering with subsequent matrix synthesis, secretion, and mineralization, which may be one of the cellular mechanisms of enamel damage.

The MAPK signaling pathway, as a non-classical pathway of TGF-β, can be activated by cytokines such as TGF-β1. The MAPK signal pathway is not only involved in cell proliferation, differentiation, and apoptosis, but is also involved in tooth germ development. One study found that a lack of the P38 gene in mice resulted in dysplasia of the tooth ectoderm phenotype due to a deficiency in ameloblast differentiation and activity, and ERK affected tooth development through the FGF10 signaling pathway [43]. Fluoride can activate MAPK to participate in the apoptosis of odontoblastin-like cells [44]. The results of the current study demonstrated that both fluoride and sulfur exposure alone, and in combination, can inhibit the phosphorylation of ERK and JNK, with a greater degree of inhibition during combined exposure. Sulfur alone inhibited P38 phosphorylation; however, in contrast, fluorine activated it. The combined exposure to fluorine and sulfur primarily produced an effect similar to fluoride, with the activation of P38. Consistent with the results of the current study, a previous study found that fluoride inhibited the phosphorylation of ERK and JNK but activated P38 [45]. The results also indicated that the inhibition of ERK and JNK phosphorylation leads to a decrease in caspase-3 expression, which is closely related to the induction of apoptosis; this may be one of the mechanisms of dental fluorosis. P38 and JNK, which are members of the MAPK family, have been shown to activate and promote apoptosis [46, 47]. In the current study, high concentrations of fluoride and sulfur induced apoptosis in a large number of LS8 cells. As to the specific mechanism, further studies are needed to evaluate the correlation between the upregulation of P38 expression and LS8 cell apoptosis after fluoride and sulfur treatment. In the current study, the expression of NBCe1 was consistent with the trend in TGF-β1, p-Smad2, p-Smad3, p-ERK, and p-JNK and was contrary to that of p-P38; thus, we speculate that NBCe1 is regulated by the TGF-β1/Smad, ERK, and JNK signaling pathways, mediated by TGF-β1.

According to the above assumption, our study demonstrated that activation of the TGF-β1 signaling pathway by human recombinant TGF-β1 upregulated the mRNA and protein expression of NBCe1. TGF-β1 also activated p-Smad2, p-Smad3, P-ERK, p-JNK, and p-P38. On the contrary, with combined exposure to fluoride and SO_2 derivatives, there was increased p-P38 levels but decreased TGF-β1 and NBCe1. These results further suggest that TGF-β1 may regulate NBCe1 via the classical SMAD pathway and unconventional ERK and JNK pathways, but not through p38. SIS3, PD98059, and SP600125 are effective inhibitors of Smad3, ERK, and JNK, respectively. The current study demonstrated that these inhibitors effectively blocked the upregulation of NBCe1 by TGF-β1. Thus, these results indicate that the regulation of NBCe1 by TGF-β1 depends on the activities of p-Smad3, p-ERK, and p-JNK.

In this study, TGF-β1 partially reversed the inhibition effect of fluoride and SO_2 derivatives on NBCe1 expression, and the results also showed that SIS3, PD98059, and SP600125 inhibitors had no effect on NBCe1 but could block the upregulation effect of TGF-β1 on NBCe1. This suggests that p-Smad3, p-ERK, and p-JNK are specific to NBCe1 in LS8 cells, and they are dependent on TGF-β1 regulation, rather than acting alone. A similar study found that SIS3, PD98059, and SP600125 inhibitors significantly reduced the dependent upregulation of TGF-β1 signaling on NBCe1 transport activity. However, the inhibitor alone had no effect on NBCe1 expression in astrocytes, suggesting that this effect was specific to TGF-β1, and that the results of this study were indeed derived from inhibition of TGF-β1 signaling [24].

TGF-β1 mainly transmits signals through the Smads family. Besides the SMAD signal, TGF-β1 also activates the MAPK signal transduction pathway, including JNK, ERK, and p38. TGF-β1 regulates the phosphorylation of Smad2/3 and the formation of the Smad2/3/4 complex via the JNK and p38 signaling pathways in HepG2 cells, thereby influencing the expression of plasminogen activator inhibitor 1 (PAI-1). MAP kinase signaling through ERK and p38 and via phosphorylation of the linker region of Smad2 mediates the effects of TGF-β1 on biglycan synthesis in VSMCs [48]. Therefore, we believe that in the process of TGF-β1-mediated fluorine-sulfur regulation of NBCe1, JNK and ERK may play a role by regulating the

phosphorylation of Smad2/3. On this basis, in our next study, we will continue to explore the interaction between JNK, ERK, and SMAD signaling molecules in mediating TGF-β1 regulation of NBCe1 in LS8 cells.

In conclusion, this study demonstrated that combined exposure to fluoride and sulfur can cause acid–base disorder in LS8 cells and has an antagonistic effect. Fluoride and sulfur can reduce the phosphorylation levels of Smad2, Smad3, ERK, and JNK by downregulating the expression of TGF, thereby inhibiting the expression of NBCe1. This may be one of the possible mechanisms underlying the enamel damage caused by fluoride and the sulfur-induced acid–base imbalance in LS8 cells. The present study provides new insights for further research on the pathogenesis of dental fluorosis. We will further clarify the interaction between Smad and both ERK and JNK in our next study and will further explore the specific mechanism of TGF-β1 regulation of NBCe1.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12011-022-03169-8.

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Data Availability "The datasets generated during and/or analyzed during the current study are not publicly available due to (reason(s) why data are not public) but are available from the corresponding author on reasonable request.."

Declarations

Conflict of Interest The authors declare no competing interests.

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