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Extracts of *Bletilla Striata* and *Indigofera Stachyodes* alleviate the enhanced cxidative stress in SH-SY5Y cells exposed to fluoride by regulating the Nrf2/HO-1 pathway

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ABSTRACT

Purpose: Extracts of *Bletilla striata* and *Indigofera stachyodes* radix (traditional Chinese herbs) were selected to observe whether the phytomedicines could interfere with the enhanced oxidative stress in SH-SY5Y cells exposed to fluoride by the regulating the nuclear factor erythroid 2-related factor 2 (Nrf2)/ heme oxygenase-1 (HO-1) pathway.

Methods: SH-SY5Y cells were treated with fluoride and the extracts of *B. striata* and *I. stachyodes* radix. The cellular proliferation rates, the activity of superoxide dismutase (SOD), and the content of malondialdehyde (MDA) were measured by biochemical assays. The protein expressions of Nrf2, HO-1 and extracellular signal-regulated protein kinases 1 and 2 (Erk 1/2) were determined by Western blotting analysis.

Results: The results showed that decreased activity of SOD and increased content of MDA were found in SH-SY5Y cells exposed to fluoride. In addition, the protein expressions of Nrf2 and phospho-Erk1/2 were increased, but HO-1 declined, in the cultured cells exposed to fluoride. Interestingly, the extract of *B. striata* or *I. stachyodes* radix resulted in the raised activity of antioxidant enzyme SOD, and reduced content of MDA. Meanwhile, both of the phytomedicinal extracts resulted in enhanced expressions of Nrf2 and HO-1, but had no obvious influence on phospho-Erk1/2, in the cells exposed to fluoride.

Conclusions: These results indicate that extracts of *B. striata* or *I. stachyodes* can alleviate oxidative stress induced by fluorosis, which might involve, as the mechanism, the activation of the Nrf2/HO-1 pathway.

Key-words: *Fluorosis; Bletilla striata; Indigofera stachyodes; Oxidative stress; Nrf2/HO-1 pathway*

INTRODUCTION

The long-term exposure to excessive fluoride can result in injury of multiple systems or organs.[1] At present, the damage from fluoride exposure to the central nervous system (CNS) has attracted great attention and been extensively studied.[2] Epidemiological data revealed that the level of intelligence quotient of the children was lowered and the cognitive function of the adult patients with chronic fluorosis was reduced in areas of endemic fluorosis as compared to those in non-endemic areas.[3,4] Experimental studies showed a high dose of fluoride reduced the expression of neuronal nicotinic acetychole receptors, which are involved in the decline of the abilities of learning and memory of rats induced by chronic fluorosis.[5] Additionally, fluoride-exposed animals exhibit anxiety, depression, and memory deficits.[6, 7]

The mechanism of the neurotoxicity induced by fluoride is more complex. Importantly, fluoride can cross the blood-brain barrier, accumulate in neurons, and result in neurotoxicity.[8] On the basis of accumulating evidence, it has been indicated that enhanced oxidative stress might be an important factor in the CNS damage induced by fluoride exposure.[9] Increased levels of metabolites of lipid peroxidation such as malondialdehyde (MDA), carbonylated proteins, and 8-hydroxy-deoxyguanine in DNA oxidation and the reduced activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase have been determined. The effects of chronic fluorosis on the dynamics of mitochondria in neurons of the rat cerebral cortex were found, reflecting that abnormalities in mitochondrial dynamics may lead to high levels of oxidative stress.[10]

The Nrf2 signaling pathway plays a crucial role in cellular oxidative stress, regulating the expression levels of antioxidant enzyme-related genes to provide antioxidant effects and beneficial impact on multiple organs.[11] It has been demonstrated that fluoride activates the Nrf2 signaling pathway, which may enhance the levels of downstream antioxidant proteins, including heme oxygenase-1 (HO-1) and SOD.[12] Therefore, the activation of the Nrf2 signaling pathway may contribute to the protection against fluoride-induced neurotoxicity.

In recent years, the therapeutic effect of phytomedicine on fluorosis in nerve system has been widely recognized. Ginkgo biloba extract attenuated neurotoxicity in rats and SH-SY5Y cells exposed to fluoride.[13] Bacopa monniera protected the neuropathological alterations induced by fluorosis.[14] The methanol extract of Matricaria recutita was confirmed to have a potent neuroprotective activity against fluoride-induced oxidative stress in rats.[15]

Bletilla striata, a traditinal Chinese herb, is a perennial herbaceous plant known for its medicinal properties and has been extensively studied for its diverse bioactive compounds, particularly polysaccharides and neolignan glycosides.[16] These compounds exhibit a range of pharmacological activities, including neuroprotection, antioxidant properties, immunomodulation, and anti-inflammatory effects, which contribute to the plant's potential in treating various diseases and health conditions.[17] *Indigofera stachyodes* radix, a herb originating from the Miao people, one of China's ethnic minorities, has been used for various purposes, which exhibits a range of pharmacological activities, including anti-inflammatory effects, pain relief and antioxidant properties.[18,19]

In this investigation, we exposed SH-SY5Y neuroblastoma cells to fluorideto observe the effect of *B. striata* and *I. stachyodes* radix on attenuating oxidative stress damage induced by fluorosis. Our results presented a potential defensive mechanism against fluoride neurotoxicity via activating Nrf2/HO-1 signaling pathway and antioxidants.

MATERIAL AND METHODS

Materials

The extracts of *B. striata* (XG-12) and *I. stachyodes* radix (XRS-45-2) (obtained from Natural Products Research Center of Guizhou Province, China); sodium fluoride (NaF) (Sigma-Aldrich Inc., USA)； DMSO (Solarbio， China); Dulbecco's Modified Eagle Medium (DMEM/F12), Bicinchoninic acid kit, protease inhibitor (Thermo Scientific Inc., USA); Cell counting kit-8 (CCK-8) (APExBIO Inc., USA); antibodies against phospho-ERK1/2, rabbit IgG, mouse IgG (Cell Signalling Technology, USA); antibodies against α-Tubulin (Proteintech, China)； antibodies against HO-1 (Abcam, USA); SOD assay kit and MDA assay kit (Nanjing Jiancheng Inc., China); and all remaining chemicals (Sigma-Aldrich, USA) were obtained from the sources indicated.

Purification and isolation of the natural compounds

XG-12: The air-dried plant samples of B. striata (3.0 kg) were powdered and extracted with 95% aqueous ethanol (EtOH) (3 × 70 l, each 2 hr) under reflux. The crude EtOH extract was obtained by evaporation under reduced pressure, then the crude extract subjected to column chromatography and eluted by CH_2Cl_2: methanol (MeOH) (0:1→1:0) to yield five fraction (Fr. A-E). Compound XG-12, mainly containging bibenzyl compounds, was obtained from Fr. B by semi-preparative HPLC (MeOH/H_2O, 58:42, tR 22.0 min, 18 mg).

XRS-45-2: The air-dried plant samples of I. stachyodes radix (5.0 kg) were smashed and extracted with 95% aqueous EtOH (3 × 70 l, each 2 hr) under reflux. The crude EtOH extract was obtained by evaporation under reduced pressure, then the crude extract was subjected to macroporous adsorption resin column chromatography and gradient eluted by MeOH: H_2O (0:1→1:0) to afford seven fraction (Fr A-G). Fr. C was further separated by Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1) and semi-preparative HPLC (MeOH/H_2O, 55:45, tR 28.0 min, 24.8 mg) to yield XRS-45-2, mainly containging flavonoid compounds, .

Cell culture

SH-SY5Y cells were cultured in 75-cm² flasks until they reached 70–80% confluence in a mixture of DMEM/F12 (1:1) that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. The cells were incubated under 5% CO_2 at a temperature of 37°C. Following a 48-hour co-incubation period with XG-12 and XRS-45-2, respectively, and 160 ppm NaF, they were washed using ice-cold phosphate-buffered saline, harvested, and resuspended in RIPA cell lysis buffer with a protease inhibitor cocktail. The cell lysates were then sonicated on ice, and the protein concentration was measured using the Bradford assay.

Cell viability assay

Cell viability was assessed using the CCK-8 kit following the guidelines provided by the manufacturer. Approximately 1 × 10^5 cells were plated in 96-well plates. Following treatment, 10 µl of CCK-8 solution and 100 µl of medium were introduced to each well, which was then incubated for 2 hours at 37°C. Finally, the absorbance at 450 nm was recorded with the Multiskan FC Microplate Reader (Thermo Fisher Scientific).

SOD activity and MDA content detected by biochemical analysis

The cell proteins in supernatant were extracted and the protein concentrations measured with the BCA assay kit. The OD values of these supernatants were determined according to the instructions provided by the manufacturer (Nanjing Jiancheng Inc., China), and the SOD activity and MDA content were calculated based on the standard values, respectively, supplied with the instruction manual.

Protein expressions of Nrf2, HO-1 and Erk detected by Western blotting

The boiled samples were separated on sodium dodecyl sulfate (SDS) -polyacrylamide gel at a concentration of 10-12% (w/v). Following this, the proteins that had been separated were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). These membranes were subsequently blocked using a solution of 5% (w/v) nonfat milk for a duration of one hour. For the membrane incubation step, primary antibodies against Nrf2 (diluted 1:2000), HO-1 (1:1000), phospho-Erk1/2 (1:2000), and α-Tubulin (1:5000) were applied, respectively, overnight at 4℃ while gently shaking. The membranes were then washed three times with Tris-buffered saline containing Tween, after which they were incubated for one hour with a diluted secondary antibody. The bound immunoglobulins, detected using either anti-mouse or anti-rabbit secondary antibodies, were visualized with a ChemiDoc™ MP imaging system (Bio-Rad, USA). Band density analysis was carried out using ImageJ software. Relative quantification of the western blots was employed from a minimum of three independent experiments.

Statistical analysis

Analyses were conducted using SPSS software, version 26.0 (IBM, USA), along with GraphPad Prism version 9.5.1 (GraphPad, USA). Data are reported as the mean ± standard deviation (SD). To assess statistical significance, a one-way ANOVA was employed, followed by Tukey's multiple comparison test. A *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Concentration selections of fluoride, XG-12 and XRS-45-2 for the treatments of cells

To establish an *in vitro* model of fluoride toxicity, the CCK-8 assay was employed to

determine the optimal fluoride concentration exposed to SH-SY5Y cells. A concentration of 160 ppm fluoride for 48 hours of incubation induced a significant alteration in the viability of SH-SY5Y cells compared to the control group (Figure 1A). Based on these findings, 160 ppm fluoride was selected for the treatment. Additionally, 25 µM XG-12 (Figure 1B) and 50 µM XRS-45-2 (Figure 1C) were chosen for subsequent treatment of SH-SY5Y cells in this study since the concentrations of these two phytomedicinal extracts used here had no toxic effect on the cultured cells.

SOD activity and MDA content in SH-SY5Y cells* exposed to fluoride, *XG-12 and XRS-45-2

SOD activity was significantly decreased in the cells exposed to fluoride (Figure 2). The treatments with XG-12 (Figure 2A) or XRS-45-2 (Figure 2B) to the fluoride-exposed SH-SY5Y cells enhanced SOD activity. The MDA content was significantly elevated in the cultured cells exposed to fluoride compared to the control group (Figure 3). The treatments with XG-12 (Figure 3A) or XRS-45-2 (Figure 3B) to fluoride-exposed cells significantly suppressed the MDA levels.

Protein expressions of Nrf2, HO-1 and ERK1/2 in SH-SY5Y cells exposed to fluoride, XG-12 and XRS-45-2

In Figure 4A, significant increases in Nrf2 expression at protein level were detected in SH-SY5Y cells exposed to fluoride or XG-12 as compared to controls; while when co-treatments with fluoride and XG-12, a more obviously raised Nrf2 in the cells was found compared to fluoride or XG-12 exposure alone. In Figure 4B, exposure of fluoride to the cells significantly decreased the HO-1 expression as compared to controls; the treatment with XG-12 alone did not induce any change of HO-1 compared to the controls; the co-treatments with fluoride and XG-12 significantly attenuated the decreased level of HO-1 induced by fluoride alone. In Figure 4C, the exposure of fluoride to the cells significantly increased the p-ERK1/2 expression as compared to controls; the treatment with XG-12 alone did not induce any change in p-ERK1/2 expression compared to controls; the co-treatments with fluoride and XG-12 did not result in alteration compared to fluoride exposure alone.

In Figure 5A, a significant increase in Nrf2 expression at protein level was detected in SH-SY5Y cells exposed to fluoride as compared to controls, but no change was found by the treatment of XRS-45-2 alone; while when the co-treatments with fluoride and XRS-45-2,were used an obvious decline in Nrf2 in the cells was found as compared to the exposure of fluoride alone. In Figure 5B, the exposure of fluoride to the cells significantly decreased the HO-1 expression compared to controls; but the treatment of XRS-45-2 alone did not induce any change of HO-1 compared to controls; co-treatment with fluoride and XG-12 significantly attenuated the decreased level of HO-1 induced by fluoride alone. In Figure 5C, the exposure of fluoride to the cells significantly increased the p-ERK1/2 expression as compared to controls; the treatment with XRS-45-2 alone did not induce any changes of p-ERK1/2 compared to fluoride exposure alone; and the co-treatment with fluoride and XRS-45-2 also did not induce any change in p-ERK1/2 compared to fluoride exposure alone.

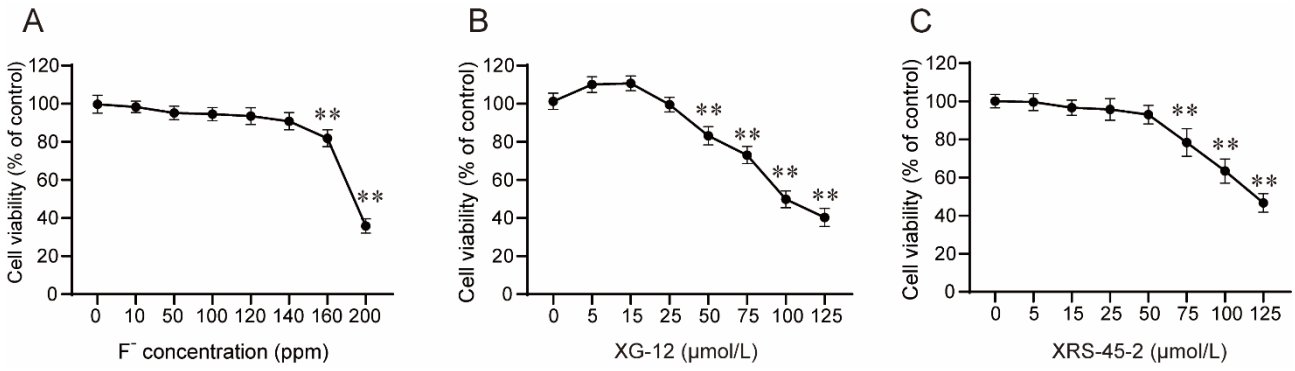

Figure 1. Cell viability in SH-SY5Y cells exposed to fluoride, and extracts of XG-12 and XRS-45-2. The CCK8 assay was used to detect the effect of different concentrations of NaF (A), XG-12 (B) or XRS-45-2 (C) on the viability of SH-SY5Y cells. F^-=fluoride. N= 4 batch experiments per group. The data are presented as the mean ± SD; **P<0.01 compared to the control group.

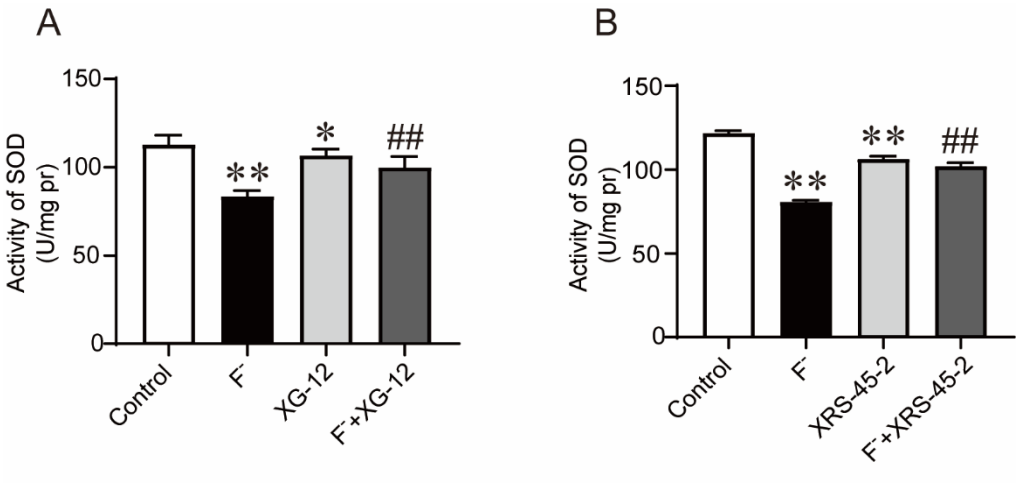

Figure 2. SOD activity in SH-SY5Y cells exposed to fluoride, and extracts of XG-12 and XRS-45-2. The SOD activity in the cultured cells was detected by biochemical assay. A. The cells exposed to fluoride and XG-12. B. The cells exposed to fluoride and XRS-45-2. F^-=fluoride. N=5 batch experiments per group. The data are presented as the mean ± SD; *P<0.05 or **P<0.01 compared to the control group; $^{\#\#}$P<0.01 compared to the fluoride group.

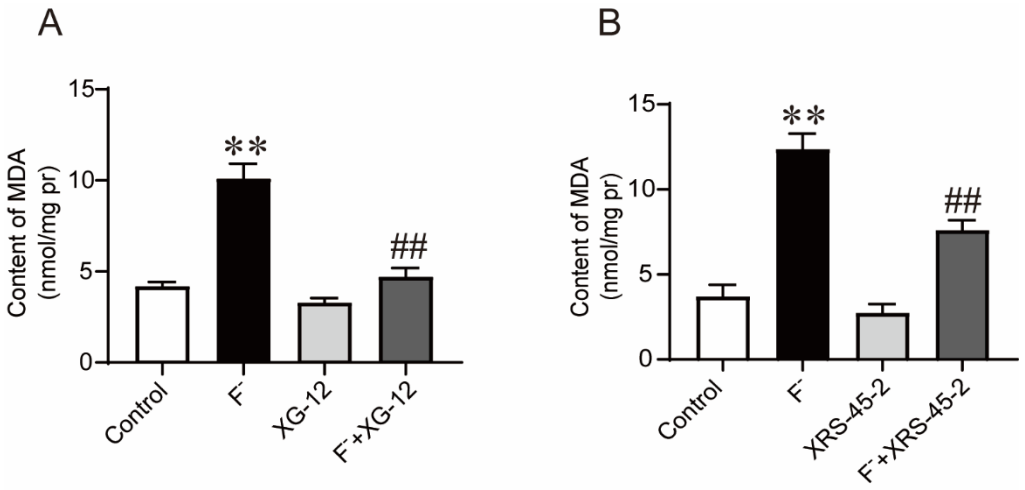

Figure 3. MDA content in SH-SY5Y cells exposed to fluoride, and extracts of XG-12 and XRS-45-2. The MDA content in the cultured cells was detected by biochemical assay. A. The cells exposed to fluoride and XG-12. B. F^-=fluoride. The cells exposed to fluoride and XRS-45-2. N=5 batch experiments per group. The data are presented as the mean ± SD; **P<0.01 compaired to the control group, and $^{\#\#}$P < 0.01 compared to the fluoride group.

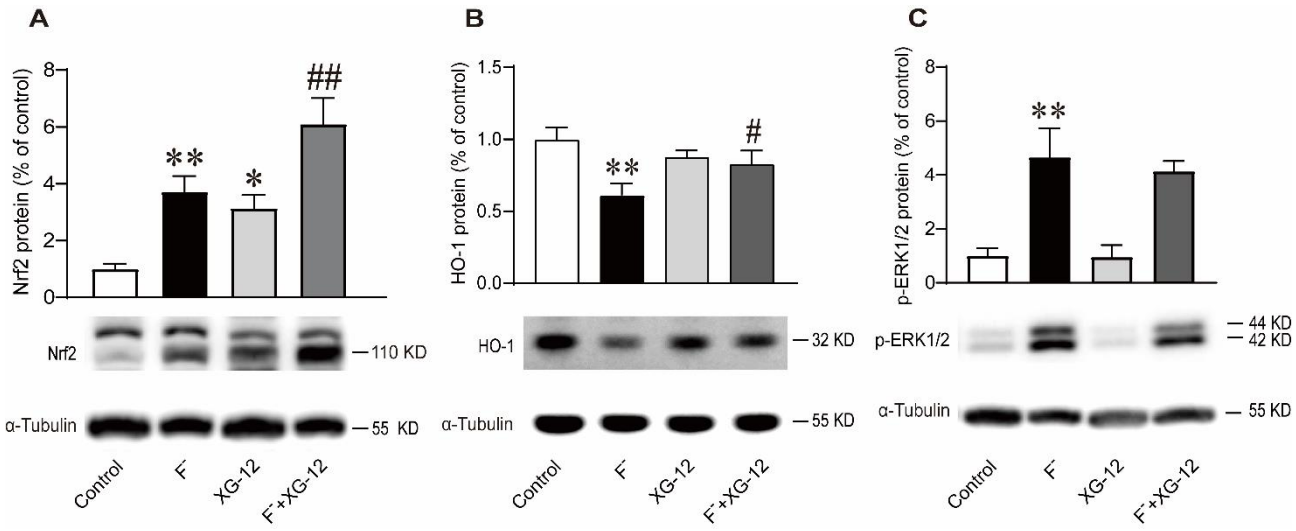

Figure 4. The protein expressions of Nrf2, HO-1 and ERK1/2 in SH-SY5Y cells exposed to fluoride and extract of XG-12. The protein expressions of Nrf2, HO-1 and ERK1/2 in cultured cells were detected by Western blotting. A. Nrf2, B. HO-1 and C. p-ERK1/2. NaF: dose used as F^- concentration. F^-=fluoride. N=3 batch experiments per group. The data are presented as the mean ± SD; *P<0.05 and **P<0.01 compared to the control group; $^{\#}$P<0.05 and $^{\#\#}$P < 0.01 compared to the fluoride group.

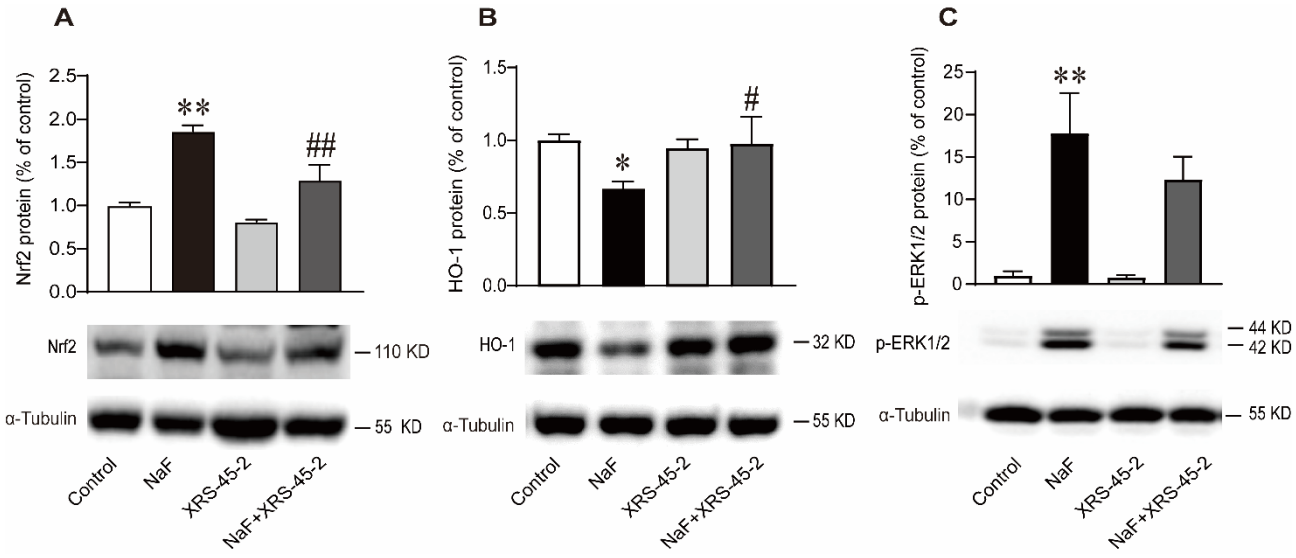

Figure 5. The protein expressions of Nrf2, HO-1 and ERK1/2 in SH-SY5Y cells exposed to fluoride and extract of XRS-45-2. The protein expressions of Nrf2, HO-1 and ERK1/2 in cultured cells were detected by Western blotting. A. Nrf2, B. HO-1 and C. p-ERK1/2. F^-=fluoride. N=3 batch experiments per group. The data are presented as the mean ± SD; *P<0.05 and **P<0.01 compaired to the control group; $^{\#}$P<0.05 and $^{\#\#}$P < 0.01 compared to the fluoride group.

DISCUSSION

Natural plants with antioxidant properties have been extensively studied for their ability to fight against diseases caused by free radicals.[20] Here, we assessed the oxidative stress levels in SH-SY5Y cells induced by fluorosis and also the protective ability of the extracts of *B. striata* and *I. stachyodes* radix against fluorosis. The main compounds in the extract of B. striata bibenzyls are bibenzyls and *I. stachyodes* radix are flavonoids. In the experiment, 160 ppm fluoride was used to expose the cultured SH-SY5Y cells and induced the decreased survival rates of the nerve cells, indicating a successful cellular model of fluorosis.

SOD is an important index to measure antioxidant level, and meanwhile MDA a marker of oxidative stress since that excessive

production of reactive oxygen species (ROS) leads to lipid peroxidation[4]. In the study, an inhibited activity of SOD and an increased content of MDA were observed, showing a high level of oxidative stress in the cultured cells induced by fluorosis, which is consistent with the findings in our or other groups.[4,5]

During the radical chain reaction of lipid peroxidation, various oxidized lipid products, including MDA, accumulate in cells, followed by organelle dysfunction and the unfolded protein response.[21] SOD can catalyze the decomposition of superoxide into oxygen and hydrogen peroxide; therefore, any substance that can enhance SOD activity, and inhibit ROS and MDA generation may alleviate the deterioration of oxidative stress.[22]

B. striata is a perennial herb with anti-inflammatory and antioxidant activities.[23] *I. stachyodes* radix is a traditional medicine frequently utilized by ethnic minorities in China and plays an antioxidant role through multicomponent, multitarget, and multi-pathway synergy.[24] In the study, the treatment with an extract of *B. striata* or *I. stachyodes* radix did not induce obvious changes in the SOD activity and MDA. However, both of the extracts attenuated the high oxidative level in SH-SY5Y cells induced with fluoride exposure, showing the increased SOD activity and decreased MDA content. This suggests that *B. striata* or *I. stachyodes* radix can play efficiently protect role when the level of oxidative stress in the internal environment increases.

The Nrf2/HO-1 signaling axis provides robust multi-organ protection against a spectrum of endogenous and exogenous insults, particularly oxidative stress.[25] In the study, fluoride exposure significantly activated the expression of Nrf2. The upregulation of Nrf2 after fluoride exposure can be regarded as a cellular self-defense.[26] In addition, we observed that fluoride exposure inhibited the expression of HO-1, indicating the damage aspect of fluorosis.

It has been indicated that the effective fraction of *B. striata* can upregulate the level of Nrf2/HO-1 pathway.[27] We determined that the extract of *B. striata* enhanced the expression of Nrf2 in the SH-SY5Y cells alone or co-treatment with fluoride, and attenuated the action of fluoride on HO-1. Furthermore, the extract of *I. stachyodes* radix did not stimulate the expression of Nrf2, however attenuated the action of fluoride on Nrf2/HO-1 pathway, playing a protective effect.[28] Importantly, the activation of the Nrf2 antioxidant pathway may lead to increased expression of SOD.[29]

Excessive phosphorylation of ERK 1/2 was observed in both *in vivo* and *in vitro* models induced by fluoride[30], which may be related to fluoride-induced neurotoxicity. In our previous investigation, the increases of phospho- and total-ERK1/2 at the protein levels were found in the brains of rats with fluorosis as compared to controls.[31] In the study, here, we found the significant increases of phospho-ERK1/2 in SH-SY5Y cells after fluoride exposure, consistent with previous research results.[30,31] Nevertheless, there was no obvious effect of *B. striata* or *I. stachyodes* radix on the expression of phospho-ERK 1/2 in the cultured cells exposed to fluoride.

CONCLUSIONS

Fluoride exposure induced the decreased activity of SOD and increased content of MDA in SH-SY5Y cells. For the protein expressions, fluoride exposure stimulated Nrf2 and phospho-ERK1/2, but HO-1 in the cultured cells. Interestingly, the extract of *B. striata* or *I. stachyodes* radix resulted in the raised activity of antioxidant enzyme SOD, and reduced content of MDA in SH-SY5Y cells exposed to fluoride. Meanwhile, the both of the phytomedicinal extracts resulted in enhanced expressions of Nrf2 and HO-1 but had no obvious influence on phospho-ERK1/2 in the cells exposed to fluoride. These results indicate that *B. striata* or *I. stachyodes* radix can alleviate oxidative stress induced by fluorosis, which might involve, as the mechanism, the activation of the Nrf2/HO-1 pathway.

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CONFLICT OF INTERESTS

None of the authors has any conflicts of interest.

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