ATTENUATION BY 7-NITROINDAZOLE OF FLUORIDE-INDUCED TOXICITY IN SH-SY5Y CELLS EXPOSED TO HIGH FLUORIDE: EFFECTS ON NITRIC OXIDE, NITRIC OXIDE SYNTHETASE ACTIVITY, nNOS, AND APOPTOSIS

Ming-Fen Deng,[a,b] Dan Zhu,[a] Yu-Ping Liu,[a] Wen-Wen He,[a] Chuan-Zhi Gui,[a,b,*] Zhi-Zhong Guan[a,c]

Guiyang, People's Republic of China

ABSTRACT: The purpose of the present research was to study the influence of a high level of fluoride on the expression of nitric oxide (NO) and the activity of nitric oxide synthase (nNOS) in a human neuroblastoma cell line (SH-SY5Y cells) and the attenuating effect of 7-nitroindazole (7-NI) on any fluoride-induyced toxicity. The cultured SH-SY5Y cells were exposed to different concentrations of fluoride [0.02–4.0 ppm of fluoride ion (F^-) prepared with NaF] and also treated with 7-NI (0.001 ppm) plus fluoride (2 ppm of F^-). The detection of the content of NO and the activity of nNOS, in the cultured cells and the medium, was by the photometric method; the detection of apoptosis of the cells was by flow cytometry; and the detection of the expression of nNOS at the protein level was by Western blotting. The results showed that after exposure to high fluoride the content of NO and the activity of NOS in both the SH-SY5Y cells and their culture medium, the protein expression of nNOS, and the apoptotic rate in the cells were all significantly increased in a dose-dependent manner. Interestingly, the increases in the content of NO, the activity of NOS, the protein level of nNOS, and apoptosis in the cells, resulting from the exposure of high fluoride, were all significantly attenuated by using 7-NI. The data indicate that 7-NI may be able to play an important role in ameliorating the toxic effects of fluoride on the NO system and apoptosis.

Key words: 7-Nitroindazole; Fluorosis; Nitric oxide; Nitric oxide synthase; SH-SY5Y cells.

Abbreviations: Analysis of variance (ANOVA); cell counting kit-8 (CCK-8); central nervous system (CNS); horseradish peroxidase (HRP); hydroxyl ($HO^{-}\cdot$); inducible NOS (iNOS); neuronal NOS (nNOS); nicotinamide-adenine-dinucleotide phosphate (NADPH); nitric oxide synthase (NOS); 7-Nitroindazole (7-NI); N-methyl-D-aspartic acid (NMDA); oxidant peroxynitrite ($ONOO^{-}\cdot$); poly-ADP-ribose polymerase (PARP); polyvinylidene difluoride (PVDF); phosphatidylserine (PS); propidium iodide (PI); room temperature (RT); superoxide anion ($O_2^{-}\cdot$).

INTRODUCTION

The excessive accumulation of fluoride over a long period can cause a vast array of symptoms and pathological changes in many tissues and organs, in addition to its well-known effects on the skeleton and teeth.[1-3] Fluoride can cross cell membranes and affect various soft tissues, leading to impairment of tissue functions. Especially, chronic fluorosis leads to dysfunction of the central nervous system (CNS), which results in lethargy, insomnia, and deterioration of learning and memory.[4-6] Chronic fluorosis may influence the course of intrauterine fetal life and induce certain harmful effects on the developing brain of the fetus.[7] It has been found that Intelligence Quotient was reduced in the children living in an endemic fluorosis area.[8-10] In a previous study, we found that coal burning fluorosis induced decreased learning and memory of rat offspring.[11] However, the molecular mechanisms of the toxic effects of fluoride on the CNS remain unclear.

[a]Department of Pathology in the Affiliated Hospital of Guizhou Medical University, Guiyang 550004, PR of China; [b]Department of the First People's Hospital of Guiyang, Guiyang 550001, PR of China; [c]Key Laboratory of Endemic and Ethnic Diseases of Ministry of Education of PR of China (Guizhou Medical University); *For correspondence: Chuan-Zhi Gui, Department of Pathology in the Affiliated Hospital of Guizhou Medical University, Guiyang 550004, PR of China; E-mail: guichuanzhi07@sina.cn.

Nitric oxide synthase (NOS) has three different isoforms, viz., neuronal NOS (nNOS, or NOS I), inducible NOS (iNOS, or NOS II), and endothelial NOS (eNOS, or NOS III).[12] All isoforms of NOS utilize L-arginine as the substrate, and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates.[13] nNOS, a cytosolic enzyme, is prevalent in the CNS. Because of the instability of NO, the expression of nNOS is closely related to the content of NO in the brain tissues. NO can be released by activating microglia, and has been described to be an important mechanism *in vitro* by which microglia cause neuronal death.[14-16] In addition, NO is a by product of metabolism and is harmful in a variety of ways.[17]

It has been found that excessive fluoride can promote oxidative stress.[18] Importantly, NO plays a physiological role as a neurotransmitter in the CNS. An important mode of the inactivated NO is its reaction with the superoxide anion ($O_2^{-}\cdot$) to form the potent oxidant peroxynitrite ($ONOO^{-}\cdot$), which can cause oxidative damage, nitration, and S-nitrosylation of biomolecules, including proteins, lipids, and DNA.[13,19,20] An excessive release of nNOS, which is dependent on NO, during stimulation at a high rate of N-methyl-D-aspartic acid (NMDA) receptors results in the production of the hydroxyl ($HO^{-}\cdot$) and $ONOO^{-}\cdot$ radicals, which are responsible for oxidative injury.[21] Nitrosative stress by peroxynitrite has been implicated in DNA single-strand breakage, followed by poly-ADP-ribose polymerase (PARP) activation.[13,22] In mammals, NO can be produced by nNOS.[12] There is evidence to show that a high level of fluoride can significantly increase the NOS activity and the NO content in BV-2 microglia cells in a dose-dependent manner.[23, 24]

Interestingly, 7-Nitroindazole (7-NI) is a selective inhibitor of nNOS by competing with both L-arginine and tetrahydrobiopterin.[25-27] There are a number of studies showing the beneficial effect of inhibiting nNOS activity as a means of reducing NO/ NMDA-induced neurotoxicity, of attenuating the development of tolerance to psychoactive agents, and of reducing withdrawal effects after the removal of psychoactive agents.[26,28,29] However, no results regarding the effect of 7-NI on chronic fluorosis have been reported.

In order to further understand the mechanism of the molecular effects of NO in the damage caused by chronic fluorosis, we exposed cells of a human neuroblastoma cell line (SH-SY5Y cells) to different amounts of fluoride and investigated the changes in NO content, NOS activity, and nNOS expression at the protein level to reveal whether 7-NI can have a neuroprotective effect on the toxicity induced by fluoride.

MATERIALS AND METHODS

Materials: Sodium fluoride (NaF, analytical reagent) and 7-NI (analytical reagent) (Sigma-Aldrich, USA); rabbit monoclonal antibody directed towards nNOS subunit (GeneTex Inc., USA); mouse monoclonal anti-β-actin antibody (Abcam, USA); anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich, USA); annexin V-FITC/ propidium iodide (PI) apoptosis detection kit (Becton-Dickinson Biosciences, USA); and all other general chemicals and reagents (Sigma- Aldrich, USA) were purchased from the sources indicated.

Exposure of SH-SY5Y cells to fluoride: The SH-SY5Y cells purchased from the German Collection of Microorganisms and Cell Cultures (Germany) were maintained in DMEM-high glucose solution supplemented with 10% fetal calf

serum, with replacement of this medium once every 3 days. These cells were exposed to various concentrations of fluoride (0.02–4.0 ppm F^- prepared with NaF) for 24 hr, 48 hr, or 72 hr. On the basis of their viability, as evaluated by cell counting kit-8 (CCK-8) reduction, 0.2 and 2.0 ppm F^- were selected as the low and high doses, respectively, for the exposure of 48 hr. In addition, the cultured cells were exposed to 0.001 ppm 7-NI together with 0.2 or 2.0 ppm F^- for 48 hr.

Assessment of viability in SH-SY5Y cells by cell counting kit-8: SH-SY5Y cells were exposed to various concentrations of fluoride for 24 hr, 48 hr, or 72 hr. By using CCK-8, 10 μL of CCK-8 solution was added to cultured cells at 37ºC for 2 hr and then the cell viability was quantified by a microplate reader (Bio-Tek Corp., USA) at 450 nm.

Examination of NO content and NOS activity: The contents of NO in the cultured cells and medium were detected by the nitrate reductive enzymatic method (Nanjing Jiancheng Biological Company, China).[23,30] The reaction was monitored by using a microplate spectrophotometer reader for 10 min with 550 nm at room temperature (RT), and then a formula used to calculate the results. NOS activity was detected by the colorimetry method (Nanjing Jiancheng Biological company, China) to detect the production of L-citrulline from L-arginine as described previously.[23,30] The reaction was monitored by using a microplate spectrophotometer reader with 530 nm at RT.

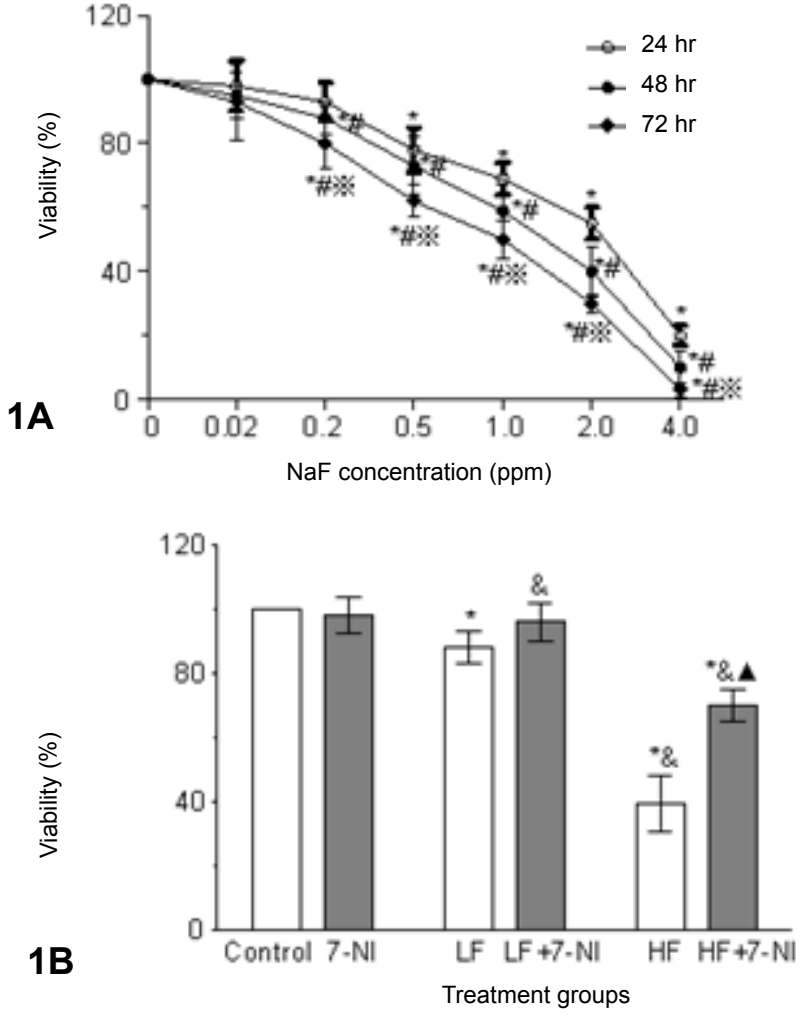
Assessment of apoptosis in SH-SY5Y cells measured by flow cytometry: The Annexin V/PI double staining assay recognizes the externalization of phosphatidylserine (PS) on the cell membrane, a hallmark of apoptotic cells. In brief, 5×10^5 SH-SY5Y cells were seeded on a 100-mm petri dish and treated with NaF or NaF plus 7-NI. Cells were suspended with trypsin, harvested, and stained with Annexin V/PI. Afterwards, the cells were analyzed by a flow cytometer (FACS Calibur; Becton-Dickinson, USA).[31]

Quantification of nNOS at protein level in SH-SY5Y cells by Western blotting: SH-SY5Y cells treated with NaF or NaF plus 7-NI were washed twice with PBS and then dissolved in 0.1 mL of lysis buffer.[20] After centrifugation at 12,000 × g for 20 min at 4ºC, the protein concentration of the supernatants thus obtained was determined using the BCA protein assay kit. The proteins were then separated by 8% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes employing a transfer unit (Bio-Rad Inc., USA). For quantification of nNOS and β-actin, these PVDF membranes were thereafter incubated with antibodies (diluted 1:1,000 and 1:10,000, respectively) at 4ºC overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (1:5 000) respectively, for 60 min. Finally, these membranes were incubated in ECL Plus reagent for 3 min and the signals thus produced visualized by exposure to Hyper Performance Chemiluminescence film.

Statistical analysis: The results were expressed as mean±SD. These means were examined for statistically significant differences by analysis of variance (ANOVA). Correlations between the level of nNOS protein and neurotoxic parameters (cell viability) or the level of nNOS and apoptotic rate in SH-SY5Y cells were analyzed employing the Pearson correlation test in the SPSS 22.0 software (SPSS Inc., USA).

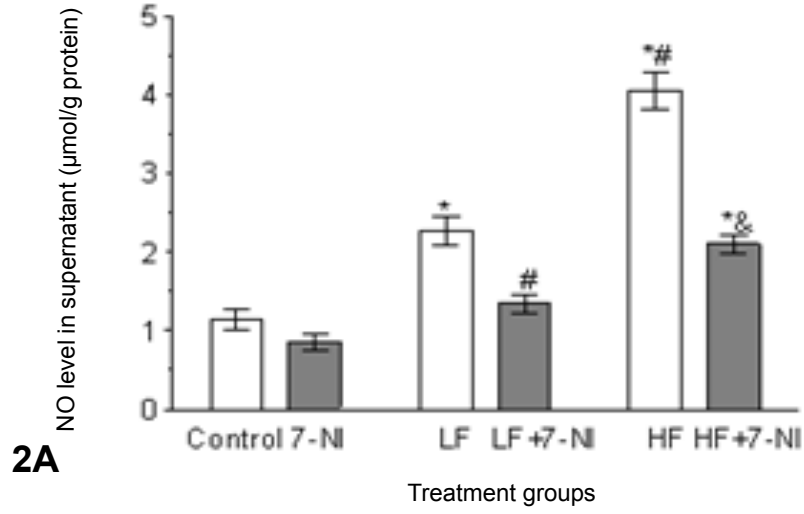
RESULTS

Cell viability in SH-SY5Y cells influenced by fluoride and/or 7-NI: As shown in Figure 1A, cell viabilities significantly declined in the SH-SY5Y cells exposed to 0.2 to 4.0 ppm F^-, and the changes were more obvious in the cells treated with high fluoride for 72 hr. The viability of the cells treated with 7-NI did not differ significantly from those of the control group (Figure 1B). When the cells were treated with 0.001 ppm 7-NI together with 0.2 or 2.0 ppm F^-, the cellular viabilities were distinctly and significantly attenuated (Figure 1B).

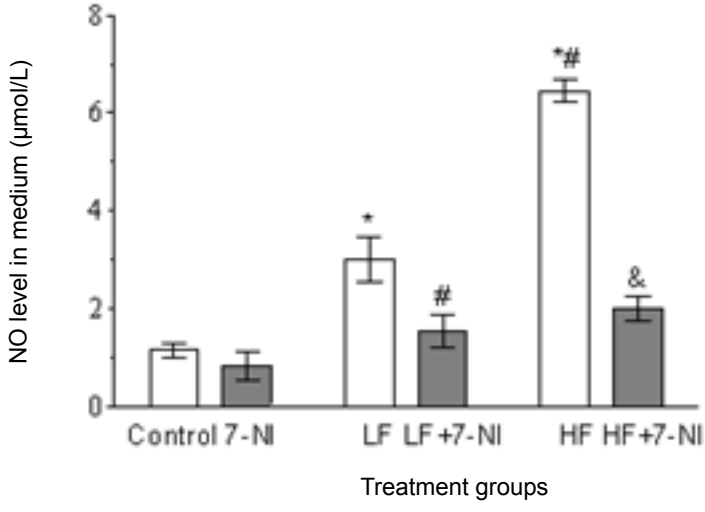


Figures 1A and 1B. Cell viability in SH-SY5Y cells treated by fluoride or fluoride plus 7-NI. **1A:** The cells were exposed with various concentrations of NaF for 24 hr, 48 hr, and 72 hr; **1B:** The cells were exposed, for 48 hr, to (from left to right): (i) 0 ppm F^- and 0 ppm 7-NI (control), (ii) 0 ppm F^- and 0.001 ppm 7-NI, (iii) 0.2 ppm F^- (low concentration of fluoride, LF), (iv) 0.2 ppm F^- and 0.001 ppm 7-NI, (v) 2.0 ppm F^- (high fluoride, HF), and (vi) 2.0 ppm F^- and 0.001 ppm 7-NI. The cell viabilities were measured by CCK-8 assay, and the data are presented as a percentage of the controls. The values presented are mean±SD of the CCK-8 reduction in 6 independent experiments. *$p<0.05$ compared to the control group; $^{\#}p<0.05$ compared to 24 hr; ※ $p<0.05$ compared to 24 hr and 48 hr; $^{\&}p<0.05$ compared to the LF group; and ▲ $p<0.05$ compared to the HF group.

NO content and NOS activity in SH-SY5Y cells exposed to fluoride or fluoride plus 7-NI: In the cells exposed to low or high fluoride, both the NO content and the NOS activity were significantly increased with a dose-dependent manner in the cells or in the culture medium (Figures 2A-2D). Interestingly, the content of NO and the activity of NOS were significantly decreased in the cells exposed to the combination of NaF plus 7-NI as compared to the treatment with only NaF (Figures 2A-2D).

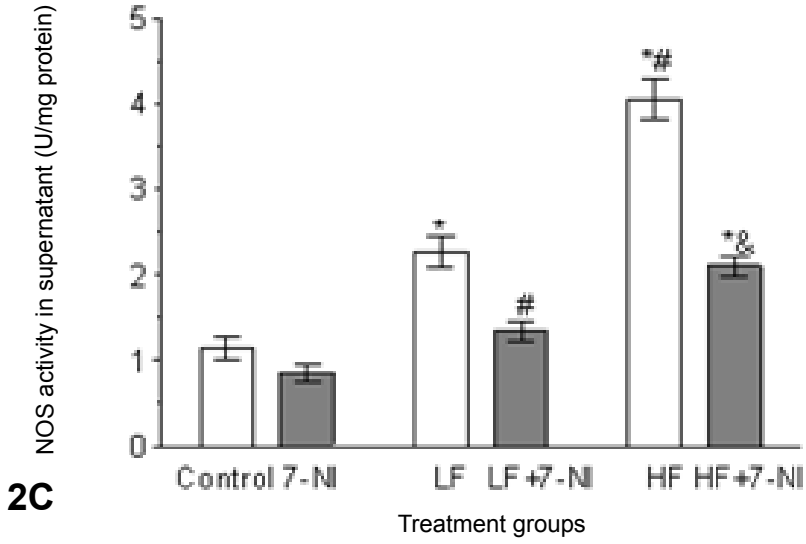


2A



2B

Figures 2A and 2B. 2A: The levels of NO production in the supernatant of SH-SY5Y cells treated by fluoride or fluoride combined with 7-NI. **2B:** The levels of NO production in the medium of SH-SY5Y cells treated by fluoride or fluoride combined with 7-NI. The cells were exposed, for 48 hr, to (from left to right): (i) 0 ppm F^- and 0 ppm 7-NI (control), (ii) 0 ppm F^- and 0.001 ppm 7-NI, (iii) 0.2 ppm F^- (low concentration of fluoride, LF), (iv) 0.2 ppm F^- and 0.001 ppm 7-NI, (v) 2.0 ppm F^- (high fluoride, HF), and (vi) 2.0 ppm F^- and 0.001 ppm 7-NI. The NO content in the supernatant and the medium were measured with commercial kits. The values presented are mean±SD of the optical density (OD) values in 6 independent experiments. *$p<0.05$ compared to the control group; $^{\#}p<0.05$ compared to the LF group; and $^{\&}p<0.05$ compared to the HF group.



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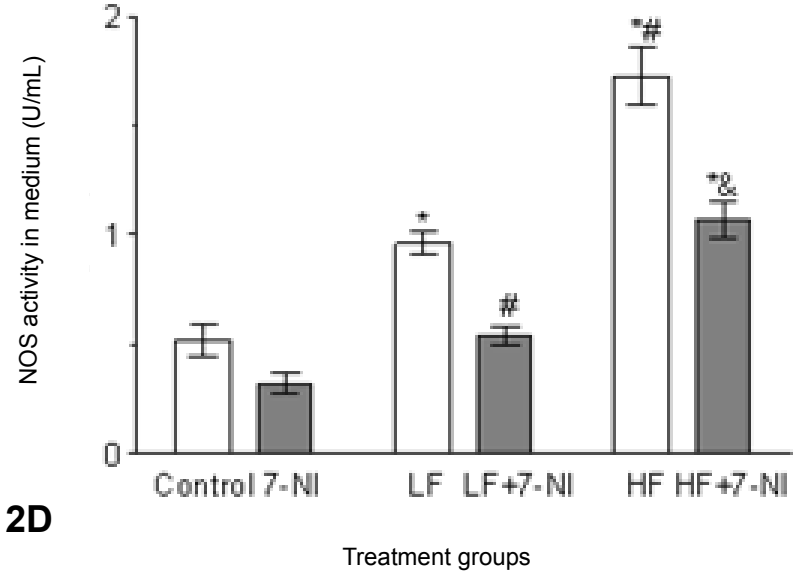
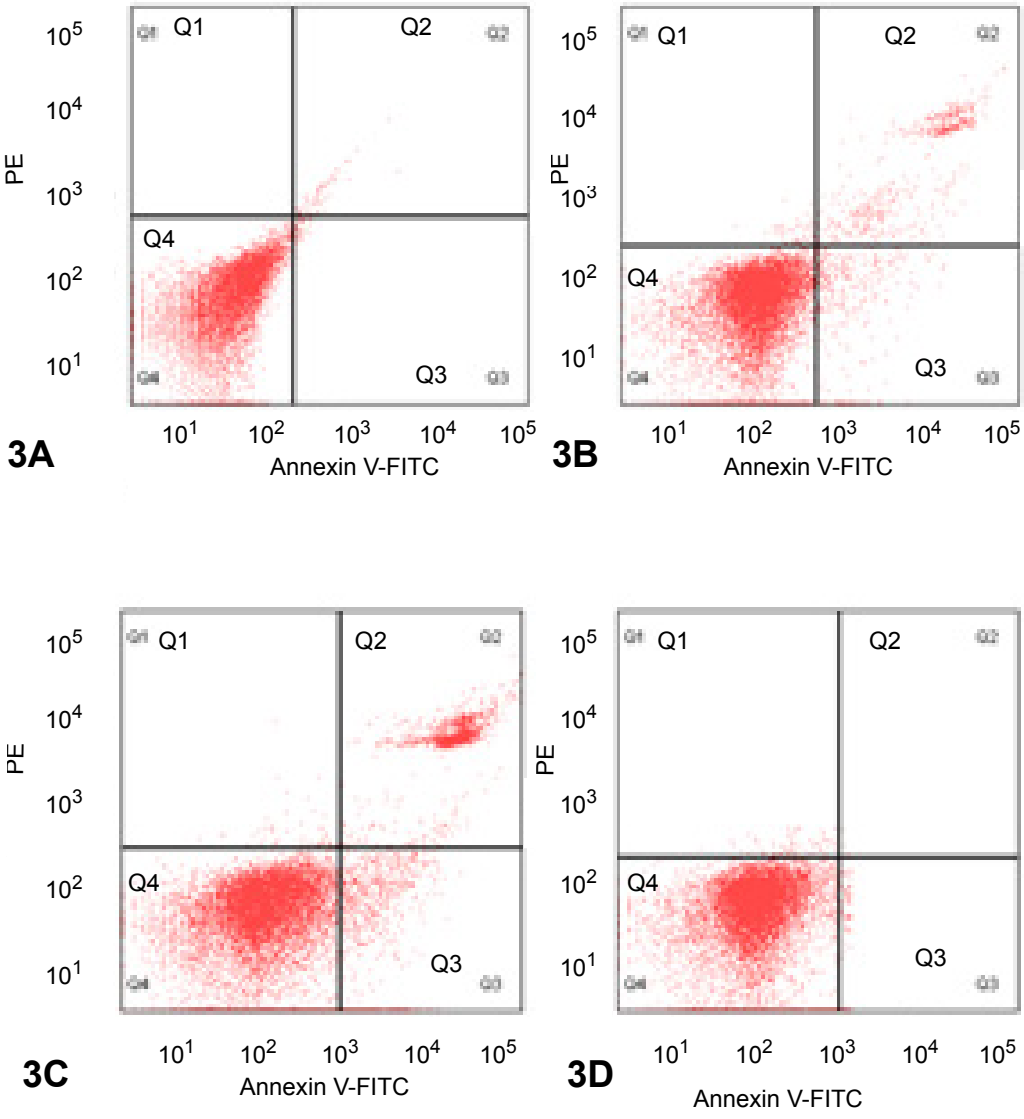


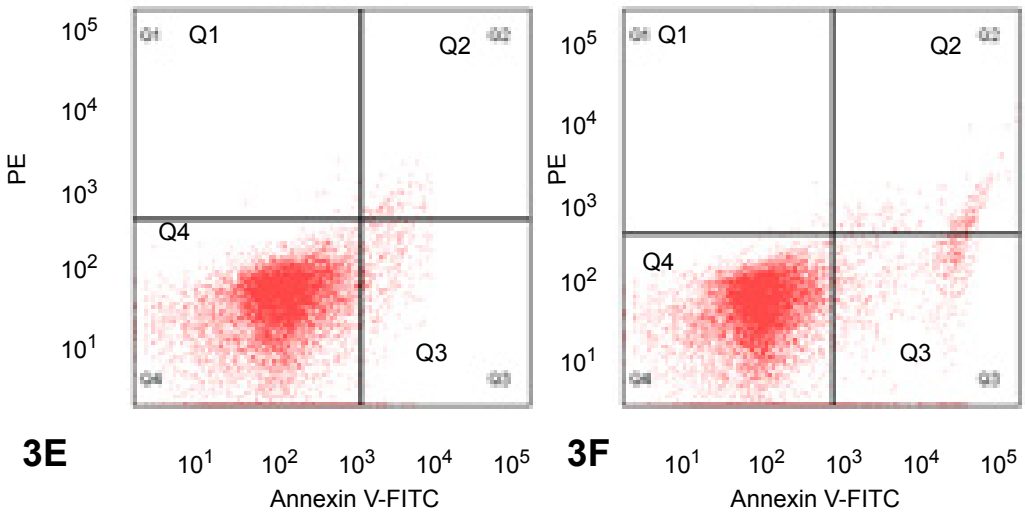
2D

Figures 2C and 2D. 2C: The levels of NOS activity in the supernatant of SH-SY5Y cells treated by fluoride or fluoride combined with 7-NI. **2D:** The levels of NOS activity in the medium of SH-SY5Y cells treated by fluoride or fluoride combined with 7-NI. The cells were exposed, for 48 hr, to (from left to right): (i) 0 ppm F^- and 0 ppm 7-NI (control), (ii) 0 ppm F^- and 0.001 ppm 7-NI, (iii) 0.2 ppm F^- (low concentration of fluoride, LF), (iv) 0.2 ppm F^- and 0.001 ppm 7-NI, (v) 2.0 ppm F^- (high fluoride, HF), and (vi) 2.0 ppm F^- and 0.001 ppm 7-NI. The NOS activity in the supernatant and the medium were measured with commercial kits. The values presented are mean±SD of the optical density (OD) values in 6 independent experiments. *$p<0.05$ compared to the control group; $^{\#}p<0.05$ compared to the LF group; and $^{\&}p<0.05$ compared to the HF group.

Apoptotic rate of SH-SY5Y cells exposed to fluoride or fluoride plus 7-NI: As shown in Figures 3A-3F, an increased apoptotic rate, shown by staining by Annexin V-FITC/PI double staining, was detected in SH-SY5Y cells exposed to a high level of fluoride. In addition, when the SH-SY5Y cells were exposed to fluoride in combination with 7-NI the raised level of apoptosis was significantly attenuated (Figure 3G).



Figures 3A, 3B, 3C, and 3D. Apoptotic rate of SH-SY5Y cells not exposed to fluoride or 7-NI, of cells exposed to fluoride at low and high concentrations (0.2 ppm and 2.0 ppm, respectively), and of cells exposed to 7-NI (0.001 ppm). Photographs of apoptosis in different groups detected by Flow Cytometry. **3A:** Control group (0 ppm F^- and 0 ppm 7-NI); **3B:** Low fluoride (LF) group (0.2 ppm F^-); **3C:** High fluoride (HF) group (2.0 ppm F^-); and **3D:** 7-NI alone group (0.001 ppm 7-NI).



Figures 3E and 3F. Apoptotic rate of SH-SY5Y cells exposed to fluoride at low and high concentrations (0.2 ppm and 2.0 ppm, respectively) and 7-NI (0.001 ppm). Photographs of apoptosis in different groups detected by Flow Cytometry. **3E:** Low fluoride (0.2 ppm F^-) and 7-NI (0.001 ppm); and **3 F:** High fluoride (2.0 ppm F^-) and 7-NI (0.001 ppm).

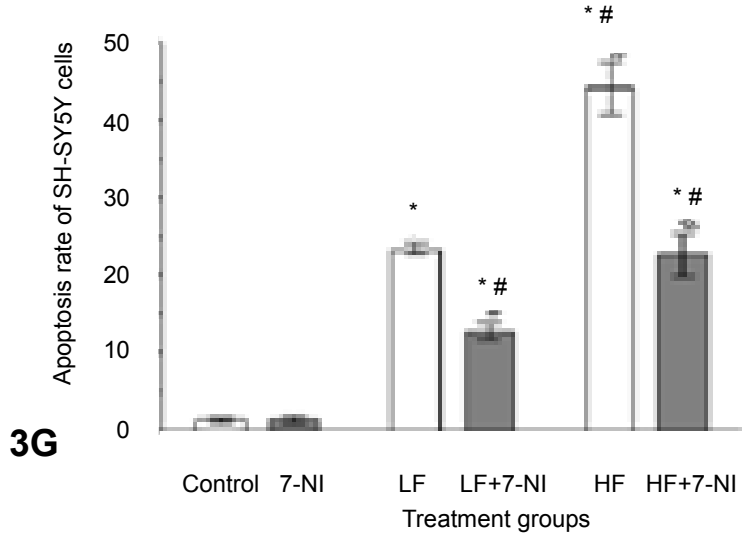


Figure 3G. Apoptotic rate of SH-SY5Y cells exposed to fluoride at low and high concentrations (0.2 ppm and 2.0 ppm, respectively) and 7-NI (0.001 ppm) and LF plus 7-HI or HF plus 7-NI. The cells were exposed, for 48 hr, to (from left to right): (i) 0 ppm F^- and 0 ppm 7-NI (control), (ii) 0 ppm F^- and 0.001 ppm 7-NI, (iii) 0.2 ppm F^- (low concentration of fluoride, LF), (iv) 0.2 ppm F^- and 0.001 ppm 7-NI, (v) 2.0 ppm F^- (high fluoride, HF), and (vi) 2.0 ppm F^- and 0.001 ppm 7-NI. The values presented are mean±SD of the apoptosis rates in 6 independent experiments. *$p<0.05$ compared to the control group; $^{\#}p<0.05$ compared to the LF group; and $^{\&}p<0.05$ compared to the HF group.

Activation of nNOS at the protein level in SH-SY5Y cells: As shown in Figure 4, the protein level of nNOS in the cells treated with fluoride was significantly increased. Furthermore, the rise in the protein level of nNOS induced by fluoride was significantly attenuated by treatment combining the fluoride with 7-NI.

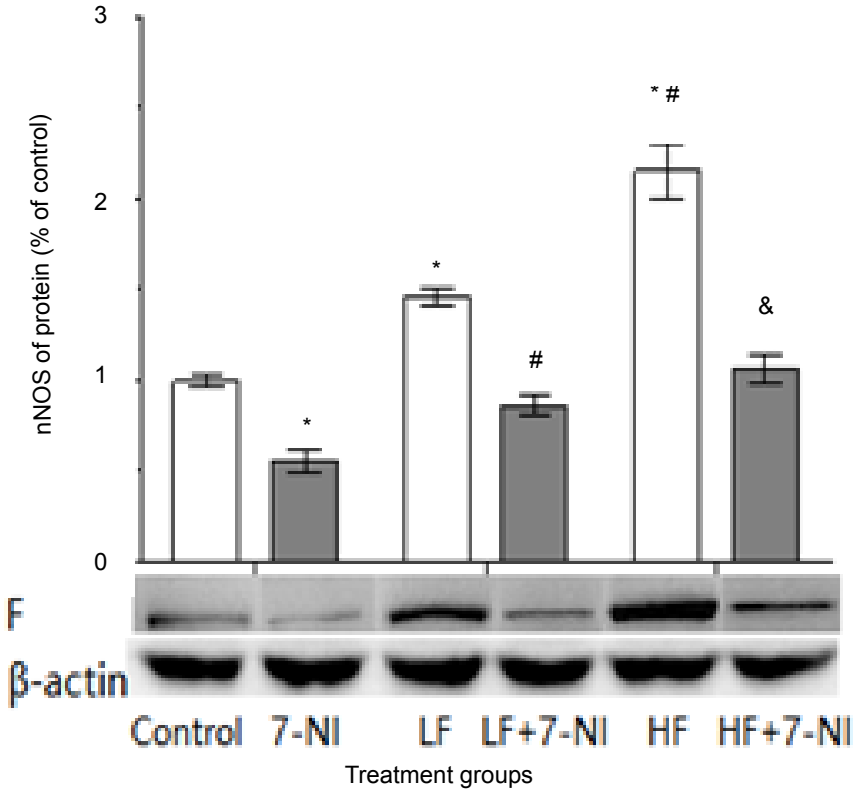


Figure 4 The protein expression of nNOS in the cultured SH-SY5Y cells exposed to 7-NI or 0.2 ppm F^- (LF) or 2.0 ppm F^- (HL) alone; or LF or HF plus 7-NI, was determined by Western Blotting. The order of the groups, from left to right, is: (i) 0 ppm F- and 0 ppm 7-NI (control), (ii) 0 ppm F- and 0.001 ppm 7-NI, (iii) 0.2 ppm F- (low concentration of fluoride, LF), (iv) 0.2 ppm F- and 0.001 ppm 7-NI, (v) 2.0 ppm F^- (high fluoride, HF), and (vi) 2.0 ppm F^- and 0.001 ppm 7-NI. The values presented are mean±SD of gray values with 6 independent experiments. *$p<0.05$ compared to the control group, $^{\#}p<0.05$ compared to the LF group, and $^{\&}p<0.05$ compared to the HF group.

Correlations between the levels of nNOS protein and toxicity in SH-SY5Y cells exposed to a high level of fluoride: Correlation analysis (Table 1) revealed that the elevated level of nNOS protein in the SH-SY5Y cells exposed to high fluoride was positively correlated with the reduction in the cell viabilities and the increase in the apoptotic rates.

Table 1. Correlations between the levels of nNOS protein and toxicity in SH-SY5Y cells exposed to a high level of fluoride (r value).

Protein	Viability	Apoptosis
nNOS	r=0.999 p<0.05	r=1.000 p<0.01

DISCUSSION

Abnormal NO signaling is involved to a variety of neurodegenerative pathologies, such as excitotoxicity following a stroke, multiple sclerosis, Alzheimer's disease, and Parkinson's disease.[13,32] A high level of NO can also produce energy depletion due to the inhibition of mitochondrial respiration and the inhibition of glycolysis.[33]

In the present study, we observed that cell proliferation was inhibited, and the levels of NO and NOS were increased in SH-SY5Y cells exposed to fluoride. Thus, it can be hypothesized that the increase of NO in neurons could be one of the causative factors for fluoride-induced neurotoxicity. When excessive NO is released it can react with the superoxide anion ($O_2^{-}\cdot$) and produce the $ONOO^{-}\cdot$ radical that is responsible for oxidative injury.[21] Here, we found that high fluoride induced an increased activity of NOS in SH-SY5Y cells, which may result in oxidative injury and thereby inhibit cell proliferation.

NO could activate microglia and contribute to the oxidative stress accompanying the inflammatory process,[34] which may be an important mechanism in neuronal death.[14-16] In our study, we found that the protein level of nNOS in the SH-SY5Y cells exposed to excessive fluoride was significantly higher than that in the controls. In addition, we found that a significantly increased apoptotic rate was induced in the SH-SY5Y cells by exposure to a high fluoride level, which may be the result of the high fluoride level causing an increase in the level of NO.

7-NI is considered to be a selective inhibitor of nNOS.[35-38] Several studies have reported that 7-NI plays an important role in neuroprotection.[39-41] In the present study, we examined the possible neuroprotective effect of 7-NI in culture cells exposed to a high amount of fluoride. The results indicated that 7-NI induced the increased proliferation of cells and attenuated the increased apoptosis in SH-SY5Y cells exposed to fluoride. It may be speculated that 7-NI can inhibit the fluoride-induced over-expression of nNOS protein and overproduction of NO, and that the neuroprotective effect of 7-NI may be attributed to its interaction with the nitric oxide pathway.

CONCLUSION

In conclusion, after exposing SH-SY5Y cells to high fluoride, there was a significant increase, in a dose-dependent manner: (i) in the content of NO and the activity of NOS, in both the cells and their culture medium, (ii) in the protein expression of nNOS, and (iii) in the apoptotic rate in the cells. Interestingly, the increases in the NOS activity, the NO content, the nNOS protein, and the apoptosis rate in the cells resulting from the exposure to high fluoride were all significantly attenuated by using 7-NI in the cultured cells. The data indicate that 7-NI may be able play an important role in ameliorating the toxic effect of fluoride on the NO system and apoptosis.

ACKNOWLEDGMENTS

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