ALTERATIONS IN OXIDATIVE STRESS AND APOPTOSIS IN CULTURED PC12 CELLS EXPOSED TO FLUORIDE

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SUMMARY: This study was conducted to investigate the mechanism of nerve damage caused by fluoride ion (F) exposure. PC12 cells, from rat adrenal medulla pheochromocytoma, were treated with different F concentrations (0.005, 0.05, 0.5, 2.5, and 5 mM). Intracellular reactive oxygen species (ROS) levels were detected with a ROS Assay Kit after 2hr of F exposure. Cell viability was detected using the Cell Counting Kit-8 (CCK-8) at different time points (2, 4, 6, 8, 12, 24, and 48 hr). Morphological changes were observed with an inverted microscope after 24 hr of F exposure. Cell apoptosis was detected using Hoechst 33342 dye after 8 hr of F exposure. The results showed that cell viability was negatively correlated with F exposure duration. After 12 and 24 hr of F exposure, cell viability showed a dose-response relationship with the F content. For a certain duration of F exposure, the levels of intracellular ROS, cell damage, and cell apoptosis showed a approximately linear relationship with the level of F exposure. These findings suggest that intracellular oxidative stress, induced by F exposure, might be one of the reasons for the cell apoptosis induced by F and support the theory that F toxicity may result from fluoride-induced free radical damage. In addition, the results suggest that cell apoptosis might be a key step in the pathophysiology of F-induced nerve damage.

Keywords: Apoptosis; Fluorosis; Nerve damage; PC12 cells; Reactive oxygen species.

INTRODUCTION

Exposure to excessive amounts of the fluoride ion (F) causes fluorosis in susceptible individuals. The injury might be associated with oxidative stress induced by F, and cell apoptosis might be one of the key links. However, the exact mechanism of fluorosis remains unknown. The theory of free radical damage caused by F has been recognised by many scholars,[1] and a new hot topic is whether or not F toxicity results from F-induced endoplasmic reticulum stress leading to cell apoptosis. However, these studies have mostly focused on bone injury with there being few studies on the effects of fluorosis on nerve cells. In addition, the commonly used traditional indicators of toxicity, such as malondialdehyde and superoxide dismutase, have low specificity and sensitivity, so that the results from different studies have varied from one another.[2,3]

PC12 cells, derived from a rat adrenal medulla pheochromocytoma, can be induced by nerve growth factor (NGF) to proliferate into cells with the characteristics of sympathetic neurons. Changes in the intracellular redox state can promote the generation of reactive oxygen species (ROS) and the activation of apoptosis-inducing factors, leading to cell apoptosis and aggravation of the redox state alterations.[4] This study used cultured PC12 cells to investigate acute toxicity in nerve cells based on the previous research of our laboratory.[5-9] We explored the mechanisms underlying the nerve cell damage caused by F exposure by

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determining the alterations in intracellular ROS concentration with different concentrations of direct F exposure, by observing the morphology, survival rate, and cell apoptosis of the PC12 cells with different F concentrations, and by evaluating the relationship between the F dose and the duration of the F exposure.

MATERIALS AND METHODS

Materials: Dulbecco's modified Eagle medium (DMEM)-high glucose, horse serum (Gibco), foetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Material Company, China), trypsin, polylysine, L-glutamine, penicillin-streptomycin, Cell Counting Kit-8, Hoechst 33342, active oxygen detection kit (Beyotime Institute of Biotechnology, China), and other reagents of analytical grade (e.g., NaF, NaCl, $NaHCO_3$, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4 and EDTA) were purchased from Jinhua Reagent Company, China.

Subjects: The PC12 cells were purchased from the Institute of Neuroscience, School of Medicine, Zhejiang University.

Process method: The PC12 cells were divided into control and F groups. In the control group, the samples were cultured in DMEM-high glucose supplemented with 5% FBS, 10% horse serum, and 1% penicillin and streptomycin. In the F groups, the samples were respectively added to 0.005, 0.05, 0.5, 2.5, and 5 mM sodium fluoride (NaF) solution.

Intracellular ROS: The concentrations of intracellular ROS were detected by using ROS detection kits. Loading probe: Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was diluted in serum-free medium at a ratio of 1:1,000 to a final concentration of 10 μmol/L. The medium was removed. The cells were added to an appropriate amount of diluted DCFH-DA, and incubated for 20 min at 37ºC. The cells were washed three times with serum-free medium to remove the unbound DCFH-DA. The NaF-containing medium was then added and the cells were cultured for 2 hr as the ROS were generated in early apoptosis at the time point of 2 hr. The control group was then established. Finally photos were taken, and the cells were observed directly by laser scanning confocal microscopy. The following parameters were established: excitation wavelength of 488 nm and emission wavelength of 525nm.

Cell viability: CCK-8 was used to assay cell viability. Cells were seeded in 96-well plates, and the inocula density was 5,000 cells/100 μL. After the cells stabilised (approximately 12 hr later), the blank medium was replaced with the NaF-containing medium and cultured for 24 hr. Each concentration was assessed 12 times. The control group and the blank medium control group were established. After the F exposure, the cells were washed twice with phosphate buffered-saline (PBS) at pH 7.4. Then, the cells were added to plates containing 10% CCK-8, and incubated for 30 min. Lastly, the absorbance was determined at 450 nm.

Nerve cell morphology: The nerve cell morphology of each group was observed by fluorescence microscopy.

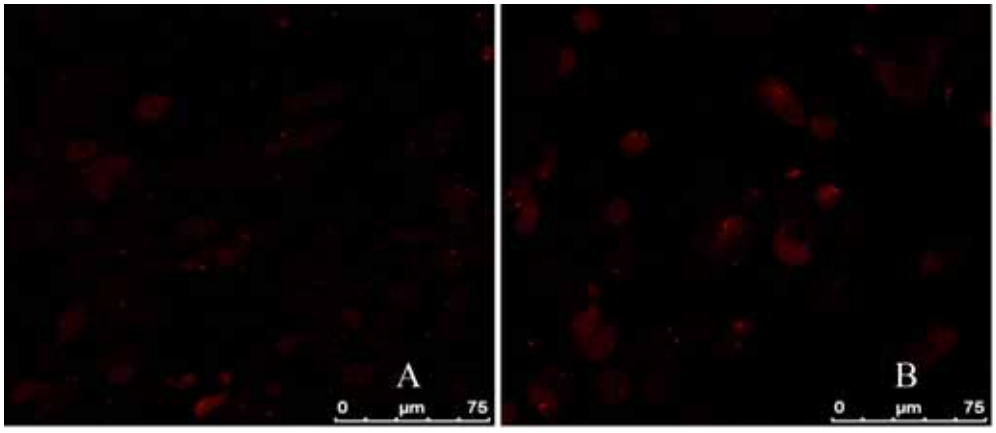
Cell apoptosis: After treating cells with NaF, they were washed with PBS to remove the residual medium. An appropriate amount of Hoechst 33342 dye was then added into the plates. The cells were incubated for 20 min to 30 min at 37ºC. The dye was then removed, and the treated cells were washed with PBS two or three times. The cells were observed by laser scanning confocal microscopy, and the following parameters were set: excitation wavelength of 346 nm and emission wavelength of 460nm. The fluorescence intensity of the confocal images was obtained by MetaMorph software (Molecular Devices, Inc; USA).

Statistical analysis: SPSS 18.0 was used for the statistical analysis. All the values were expressed as mean±SD. Data were analyzed by homogeneity of variance test and one-way ANOVA. The least significant difference test was used for further multiple comparisons. A value of $p<0.05$ was considered as a significant difference.

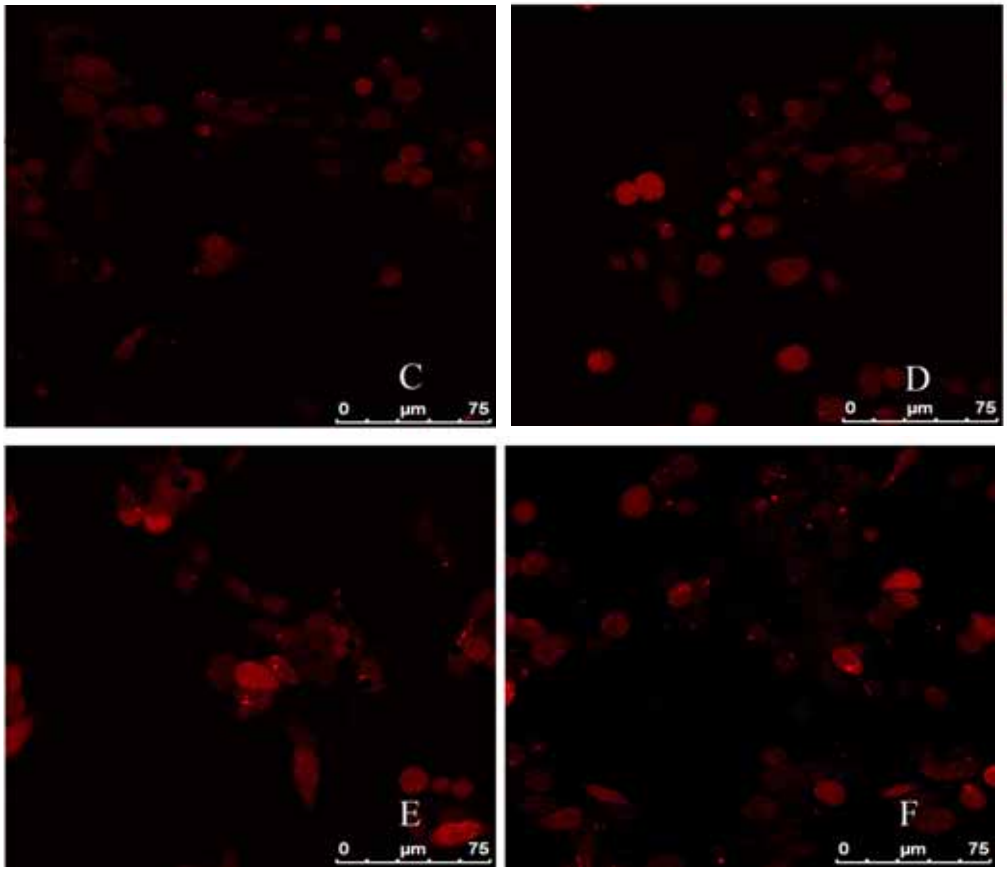
RESULTS

Preliminary experiments confirmed that the ROS level was so vulnerable that it needed to be determined early in the cell culture stage at the time point of 2 hr. A semi-lethal effect occurred in the PC12 cells after exposure to F for 24 hr and cell apoptosis was mostly obviously observed after F-exposure for 8 hr. In consequence, we determined the levels of intracellular ROS at the time point of 2 hr, observed the cell morphology at the time point of 24 hr, and detected the apoptosis at the time point of 8 hr.

Levels of intracellular ROS: The intracellular ROS concentration was detected using DCFH-DA, and the fluorescence intensity represented the levels of intracellular ROS (Figure 1 and Table 1). The fluorescence intensity of the cells labelled with DCF, after the cells were exposed to NaF for 2 hr, gradually rose with the greater concentrations of F suggesting that the concentration of the intracellular ROS also gradually increased. Compared with the control group, the ROS concentrations of all F groups were significantly increased ($p<0.01$).



Figures 1A and B. Detection of the intracellular ROS at the time point of 2hr. A: control group; B: 0.005 mM group.



Figures 1C-F. Detection of the intracellular ROS at the time point of 2hr. C: 0.05 mM group; D: 0.5 mM group; E: 2.5 mM group; F: 5.0 mM group.

Table 1. Detection of the levels of intracellular ROS at the time point of 2 hr

Group (F concentration in mM)	Fluorescence intensity
0 (control)	37.54±2.36
0.005	41.60±3.70**
0.05	46.93±2.45**
0.5	47.96±2.64**
2.5	52.37±3.03**
5.0	60.45±1.62**

The values were expressed as means±SD, n=20. Compared with the control group: **p<0.01.

Effects of NaF treatment on cell viability: After the PC12 cells were treated with different concentrations of NaF (between 0 and 5.0 mM) for 2 hr to 48 hr, cell proliferation was assessed by the CCK-8 assay (Figure 2). Compared with the control groups, the cell viability of the 0.5, 2.5, and 5 mM groups showed a tendency to reduced cell survival with increasing duration of exposure. After 8 and 48 hr of F treatment, the cell proliferation was significantly decreased ($p<0.05$ or $p<0.01$) in each F group, except for the 0.005 mM group for 8 hr exposure. After 12 and 24 hr of F treatment, the low-dose exposure group cells (0.005 mM) appeared to recover and proliferated at a slightly higher rate than that of the other treatment groups ($p<0.01$). Subsequently, at 48 hr, the cell survival rates of the 0.005 mM group was significantly decreased ($p<0.01$) compared to the control group.

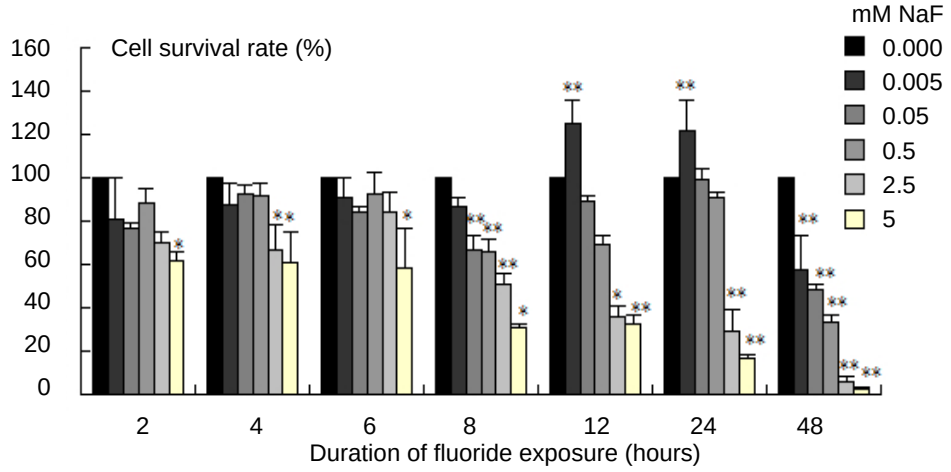


Figure 2. Dose- and time-dependent effects of NaF on the survival rate of PC12 cells. The cells were treated with 0.000, 0.005, 0.05, 0.5, 2.5, and 5 mM NaF for 2–48 hr. Each bar denotes mean±SD. Compared with the control group: *p<0.05, **p<0.01.

Observation of PC12 cell morphology: Figure 3 shows the morphology of the cells after 24 hr. While normal cells were fusiform with more than two protrusions, after treatment with F for 24 hr, the cell density gradually decreased as the F concentration increased. Some cells became round and the number of apoptotic or dead cells increased. Apoptotic vesicles (Figure 3E) and cell debris (Figure 3F) were also observed.

Cell apoptosis: The effect of F on apoptosis in the PC12 cells at the time point of 8 hr is shown in Figure 4 and Table 2. The cell nuclei, stained with Hoechst 33342, were normally oblong or ovoid (Figure 4A). After cell apoptosis occurred, the cell nuclei became round and pyknotic (Figures 4D, 4E, and 4F), the stain became darker, and the fluorescence intensity was enhanced. Compared with the control group, the fluorescence intensity of each F group was significantly enhanced ($p<0.01$), indicating that the number of apoptotic cells was significantly increased (Table 2).

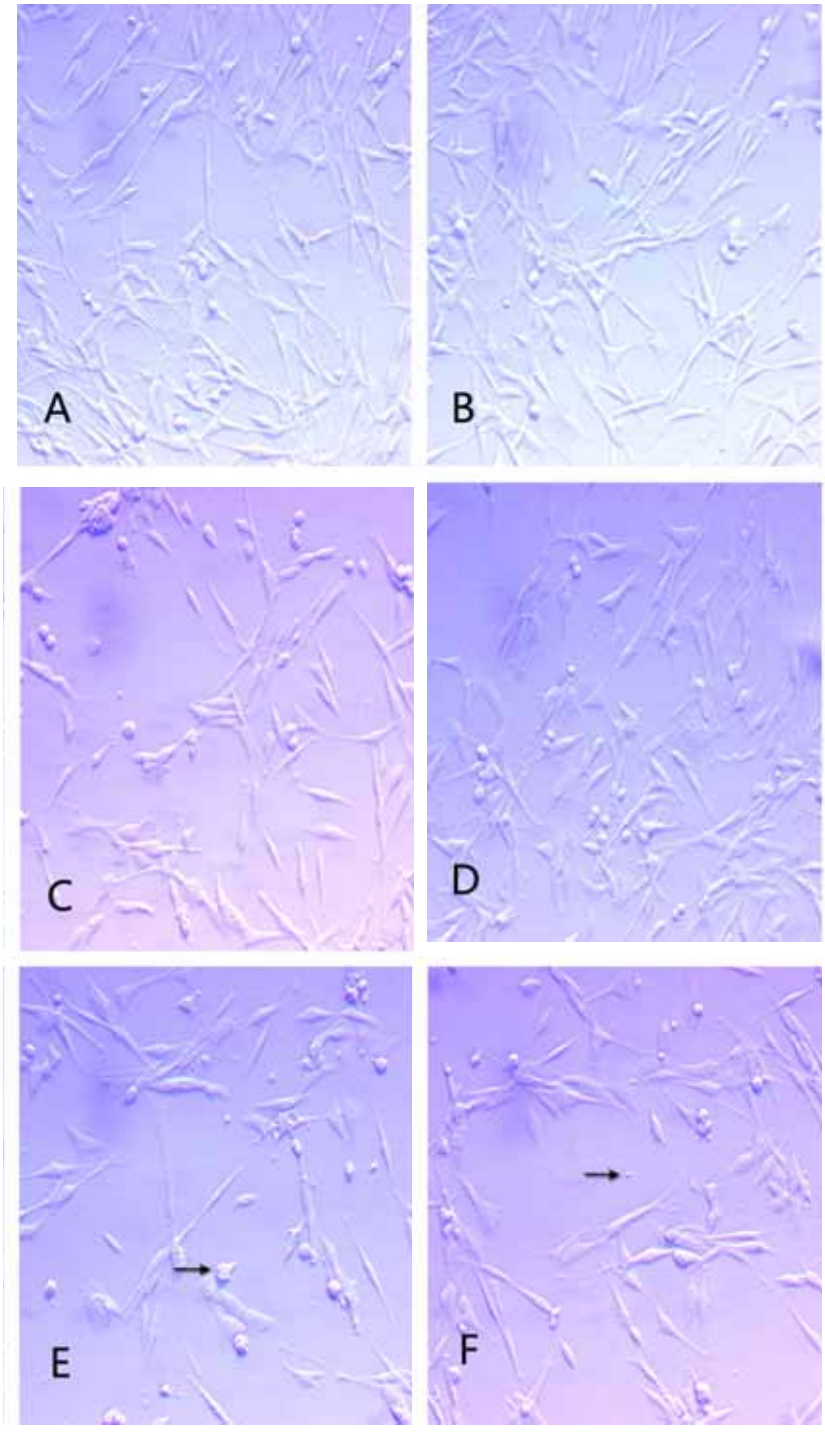


Figure 3. Observation of the cell morphology at the time point of 24 hr. A: control group; B: 0.005 mM group; C: 0.05 mM group; D: 0.5 mM group; E: 2.5 mM group; F: 5.0 mM group. The arrows in E and F point to apoptotic vesicles and cell debris, respectively.

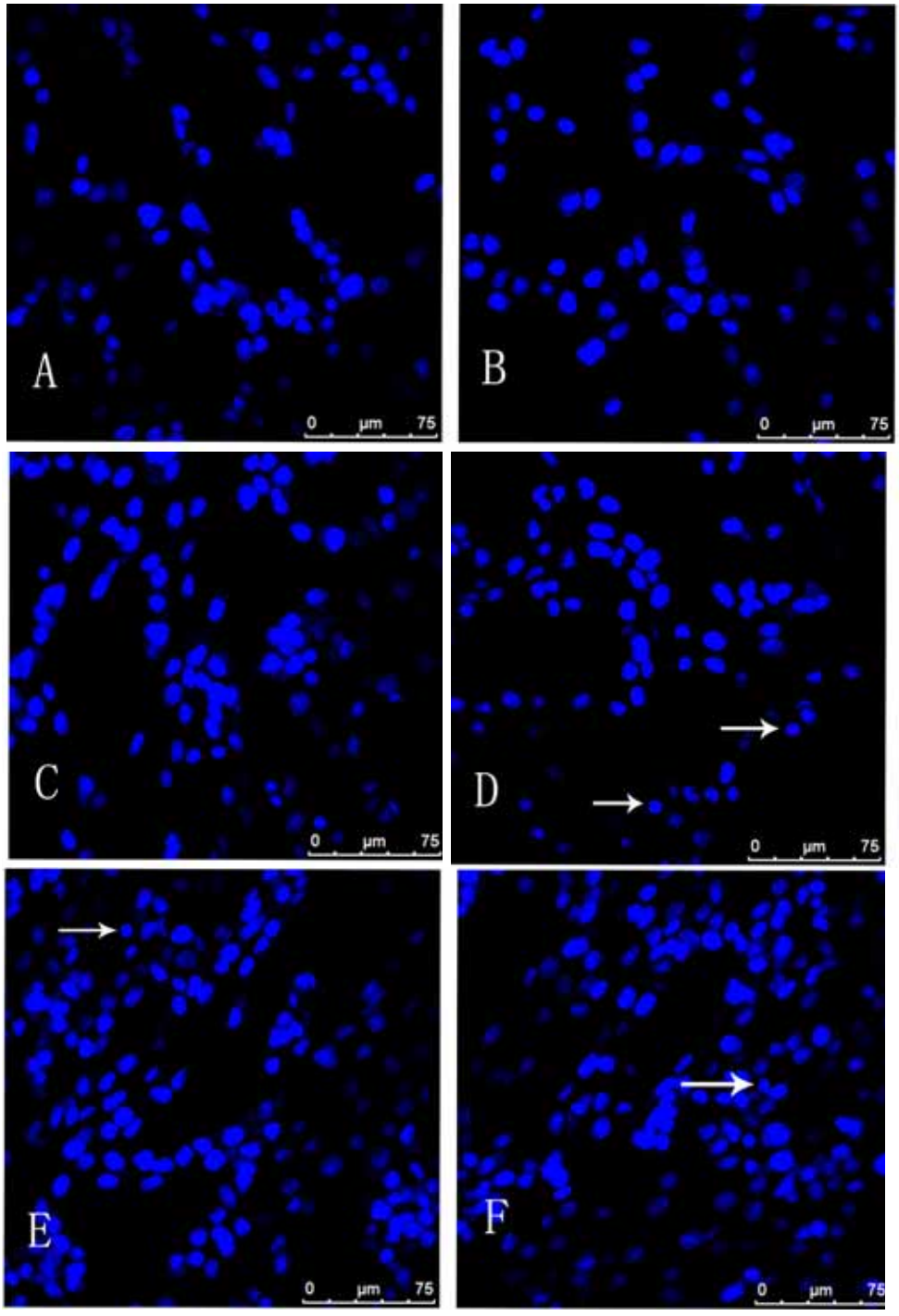


Figure 4. Effect of fluoride on apoptosis in the PC12 cells at the time point of 8hr. A: control group; B: 0.005 mM group; C: 0.05 mM group; D: 0.5 mM group; E: 2.5 mM group; F: 5.0 mM group. The arrows in D, E, and F point to cell nuclei that have become round and pyknotic.

Table 2. Detection of apoptotic cells at the time point of 8 hr

Group (F concentration in mM)	Fluorescence intensity
0 (control)	8.25±0.89
0.005	33.23±1.85**
0.05	36.44±1.95**
0.5	32.05±1.81**
2.5	41.49±1.67**
5.0	37.53±1.88**

The values were expressed as means±SD, n=20. Compared with the control group: **p<0.01.

DISCUSSION

The free radical damage theory,[10-13] involving oxidative stress, is generally acknowledged to be an important mechanism for F-induced damage to cells. Oxidative stress is a type of intracellular or extracellular state induced by chemical and metabolic sources of ROS. Under normal physiological conditions, ROS is beneficial to the organism. However, upon exposure to ionising radiation, ultraviolet radiation, or a lower antioxidant capacity, ROS generation increases and the level of oxidation exceeds the antioxidant defences, thereby causing an imbalance between the oxidant and antioxidant systems in organisms. This imbalance leads to a series of ROS reactions, which result in cell toxicity of different degrees and induce oxidative damage to macromolecules (e.g., membrane lipids, proteins, and DNA), thereby leading to the occurrence of aging, neurodegenerative diseases, cancer, and other diseases.[14,15]

In our study, the morphology of PC12 cells was observed, and the intracellular ROS concentration, cell viability, and apoptosis were assayed. Compared to the control group, after the cells were exposed to NaF for 2 hr, the intracellular ROS increased progressively to a significant degree as the F content of the media increased. This finding of a positive relationship between the levels of oxidative stress and F exposure showed that ROS generation was related to F exposure.

The results of cell viability showed that F exposure decreased cell viability, which was consistent with the research of He et al.,[16] who observed F-induced injury in primary cultured rat hippocampal cells. Moreover, a linear relationship was found between the reduction of cell activity and exposure time, which was consistent with the results of Kubota et al.[17] An intriguing point is that at 12 and 24 hr of F exposure, cell viability showed a dose-effect response with “promotion in low concentration and inhibition in high concentration.” Low F concentrations promoted cell viability while high concentrations of F inhibited cell viability. This

discovery indicated that, as occurs with many poisons,[18] low concentrations of F can result in hormesis.

Apoptosis is a normal physiological activity of the human body, and has an extremely important function in the maintenance of homeostasis. However, extremely high or low levels of apoptosis can adversely influence the body. The experimental results showed that a certain dose of NaF can induce cell apoptosis. Moreover, an approximate positive correlation was observed between the levels of apoptosis and oxidative stress, that is, ROS had a function in F-induced apoptosis of PC12 cells, which was consistent with some previous reports.[2,19] The accurate correlation of increased ROS levels in PC12 cells with F-induced cell damage, reduced proliferation, and apoptosis will be investigated in a further study.

CONCLUSION

In summary, we found a positive effect of F on the generation of intracellular ROS and a negative effect of F on the survival of nerve cells, which was associated with morphological changes of PC12 cells and induced cell apoptosis. All these phenomena indicated that the mechanism of F-induced nerve cell damage might be that excess F led to the overproduction of ROS, induced excessive oxidative stress, aggravated apoptosis of nerve cells, and eventually damaged nerve cells. This provides further support for the theory of free radical damage as an important mechanism for F toxicity and indicates that cell apoptosis might be one of the key steps in the pathophysiology of F-induced nerve cell damage.

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