ALTERATIONS OF APOPTOSIS AND EXPRESSIONS OF Bax AND Bcl-2 IN THE CEREBRAL CORTICES OF RATS WITH CHRONIC FLUOROSIS

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SUMMARY: The aim of the study was to investigate the influence of chronic fluorosis on apoptosis and the expression of Bax and Bcl-2 in the cerebral cortices of rats in an attempt to elucidate molecular mechanisms. Sixty Sprague Dawley (SD) rats were divided randomly into three groups of 20 each: an untreated control group (drinking water naturally containing <0.5 mg fluoride/L), a low-fluoride (F) group (whose drinking water was supplemented with 10 mg F/L by using NaF) and a high-F group (50 mg F/L). The percentage of apoptotic neurons was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, after 6 months of exposure. Moreover, the expressions of Bax and Bcl-2 at both the protein and mRNA levels were detected by Western blotting and real-time PCR, respectively. The results showed that the animal model of chronic fluorosis was successfully established in the study. In the cortices of the rat brains with chronic fluorosis, as compared to controls, the percentage of apoptotic neurons was significantly increased, with a dose-dependent tendency between the rate of apoptosis and the F contents in drinking water. The expression of Bax and Bcl-2, at both the protein and mRNA levels, was clearly elevated in the rat brains with chronic fluorosis. These findings indicate that the raised level of apoptosis in cortical neurons resulting from chronic fluorosis may be regulated by Bax and Bcl-2.

Keywords: Apoptosis; Bax; Bcl-2; Brains; Fluorosis; Rats.

INTRODUCTION

Pathological effects of fluoride (F) intake have been reported for various organs including the brain, kidney, liver, and thyroid of humans living in areas with endemic fluorosis in several developing countries, including China and India.[1,2] Moreover, neuronal dysfunctions of the central nervous system (CNS) have been described in such individuals, as well as in experimental animals subjected to chronic fluorosis, including lethargy, insomnia, and a deterioration of learning and memory, in association with reduced amounts of Nissl substance, swelling of mitochondria, dilation of the endoplasmic reticulum, and frequent apoptosis.[3,4] However the pathophysiology underlying these phenomena remains elusive.[5,6] Interestingly, the apoptosis induced by fluorosis has been recently focused on as a possible toxicological mechanism for the disease.[7,8] Integrating mitochondrial fragmentation, the procedure of apoptosis associated with chronic fluorosis might be involved in the mitochondria-mediated pathway regulated by anti-apoptotic Bcl-2 families.[9] The mitochondrial pathway is initiated by the release of cytochrome c from the mitochondria into the cytosol, which is controlled by the Bcl-2 family.[10]

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The study was designed to examine the involvement of the mitochondrial apoptotic pathways and investigate the roles of the Bcl-2 family, in an attempt to elucidate molecular mechanisms underlying the brain damage of the rat associated with excess accumulation of F.

MATERIALS AND METHODS

Materials:

Mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, USA), mouse monoclonal antibodies to Bcl-2 (Invirogen, USA), mouse monoclonal antibody to Bax (Thermo Scientific, USA), Hyper Performance Chemiluminescence film and ECL (Enhanced Chemiluminescence) plus reagent (Amersham, Sweden), QPCR SYBR Green Mix (Infinigen Biotechnology Inc., USA), and all other chemicals (Sigma-Aldrich, USA) were purchased from the sources indicated.

Experimental animals:

Prior to the treatments, 30 male and 30 female Sprague Dawley (SD) rats, each weighing approximately 90–120 g at the beginning of the experiment, were acclimatized for one week in a well-regulated housing facility (humidity 30–55% and temperature 22–25ºC). The animals, which were housed individually in stainless-steel cages suspended in stainless-steel racks and given access to the appropriate drinking water and standard laboratory chow *ad libitum* for 6 months, were divided randomly into 3 groups of 20 each: (1) an untreated control group (drinking water naturally containing less than 0.5 mg F/L), (2) a group exposed to a low level of F (drinking water supplemented with 10 mg F/L by using NaF), and (3) a high exposure group (50 mg F/L). These experiments were pre-approved by the regional ethical committee in Guizhou, China. The dental fluorosis was diagnosed by the modified method from HT Dean's diagnostic index and the content of F in the urine was measured by the fluorine-ion selective electrode.

Detection of apoptosis:

Apoptotic frontal cortical neurons were detected by using an apoptosis assay kit (Boster Bio-engineering, Wuhan, China) according to the manufacturer's instructions of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The frontal cortical tissue sections were dewaxed, treated with 3% H_20_2 for 10 min to eliminate endogenous peroxidase, and then washed with distilled water. Proteinase K treatment for 15 min was performed, followed by washing with Tris-buffered saline (TBS). Sections were subsequently reacted with the labeling buffer, TdT, and DIG-d-UTP mixture for 2 hours at 37ºC and then washed with TBS. The slides were immersed in the blocking solution for 30 min. After incubation with biotinylated anti-digoxin antibody solution for 30 min at room temperature, the sections were incubated with SABC for 30 min at 37ºC and then stained with DAB after washing with TBS. The sections were visualized under a light microscope, with positive nuclei presenting brown staining.

Detection of the levels of Bcl-2 and Bax proteins by Western blotting:

The frontal cortical tissue of the brain was homogenized in phosphate buffer solution (KH_2PO_4/Na_2HPO_4, pH 7.4) containing 10% Triton X-100 on ice with a Telfon homogenizer,[11] followed by centrifugation at 14,000 × g for 30 min at 4ºC. After determination of the protein content of the supernatant thus obtained by using the BCA protein assay kit, an aliquot at this was subjected to 10% SDS-PAGE and the protein bands then blotted onto polyvinylidene difluoride (PVDF) membranes with a transfer unit (Bio-Rad Inc., USA). Thereafter, for quantification of Bcl-2 and Bax, these membranes were incubated with the appropriate mouse monoclonal antibodies at dilutions of 1:100 and 1:100, respectively, at 4ºC overnight. After washing, the membranes were incubated with the secondary HRP-conjugated anti-rabbit IgG (1:3000 dilution) for 120 min. Finally, following incubation in ECL plus reagent for 5 min, the signals thus produced were visualized by exposure to Hyper Performance Chemiluminescence film.

Determination of the levels of mRNA encoding Bcl-2 and Bax by quantitative Real-time PCR:

Following isolation of total RNA from the frontal cortex by Trizol reagents (Invitrogen, USA), 3 μg was converted into first-strand cDNA employing an appropriate kit (Promega, USA) and oligo-$d(T)_{18}$ primers, in accordance with the protocol recommended by the manufacturer. The primers were designed on the basis of the complete cDNA sequences deposited in GenBank (accession numbers: NM_016993.1 for Bcl-2, NM_017059.1 for Bax) (Table 1).

Table 1. Sequences of the primers employed for amplification of mRNAs encoding Bcl-2 and Bax by quantitative real-time PCR

mRNA	Sequences (5`→3`)	Length	Annealing temperature
Bcl-2	ACGGAGGCTGGGATGCCTTTGTG GCACCCAGAGTGATGCAG	127 bp	61°C
Bax	TTCAACTGGGGCCGCGTGGTT GGAGAGGAGGCCTTCCCAGCCA	177 bp	61°C

Quantitative real-time PCR was carried out using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) in accordance with the manufacturer's protocol and analyzed with GeneAmp7300 SDS software. In brief, 20 μL solution containing 2 μL first-strand cDNA, 2 X QPCR SYBR Green Mix and 1 mM each of forward and reverse primers was subjected to thermal cycling as follows: 10 min at 95ºC, followed by 40 cycles at 95ºC for 15 sec, and annealing for 30 sec (at the temperature listed in Table 1), and at 70ºC for 30 sec. Both RT-PCR and melting curve analysis were routinely performed after amplification to check the specificity of this PCR procedure.

A novel and convenient method for relative quantitation of the results of real-time PCR by fluorescence involves using a serial dilution (here, 625, 125, 25, and 5 fold dilutions) of any one of the unknown cDNA samples tested to determine the standard slope.[12] Moreover, this approach was utilized to calculate median values for the levels of first-strand cDNA, based on the finding that the slope of a real standard curve paralleled the slope obtained in this manner. The ABI7300 software was used to analyze the data and make calculation.

Statistical analysis:

The results were expressed as means±SD or median values for the different groups. These values were examined for statistically significant differences employing one-way ANOVA or a non-parametric test (the Kruskal-Wallis H or Nemenyi test) utilizing the SPSS11.5 software (SPSS Inc., USA).

RESULTS

Dental fluorosis was obvious in the rats exposed to excessive F in their drinking water, in the form of white or pigmented bands (I), gray enamel (II), and even loss of tooth structure (III). Moreover, elevated urinary levels of F and reduced body weight were apparent in these animals (Table 2).

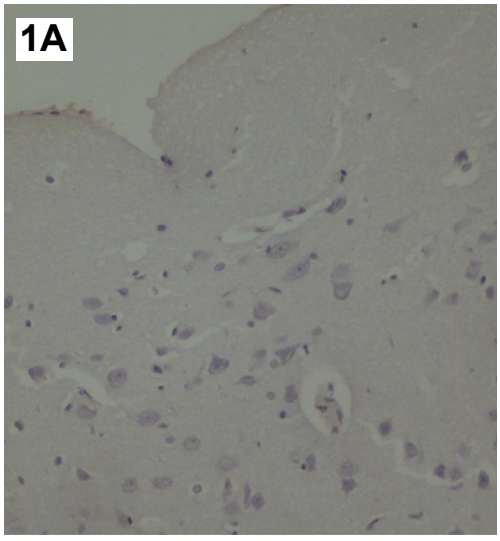
Table 2. Dental fluorosis, fluoride content in urine and the body weight of rats with chronic fluorosis and the control group

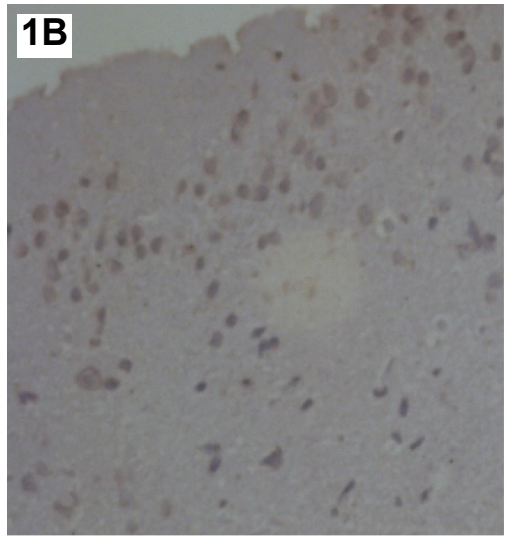
Groups	Cases	Dental fluorosis				Fluoride in the urine (mg/L)	Body weight (g)
		0°	I°	II°	III°		
Control	20	20	0	0	0	0.64 ± 0.37	450±33
Low-F	20	0	14	6	0	4.17 ± 1.39*	431±37*
High-F	20	0	6	13	1	5.70 ± 1.70*,†	424±34*,†

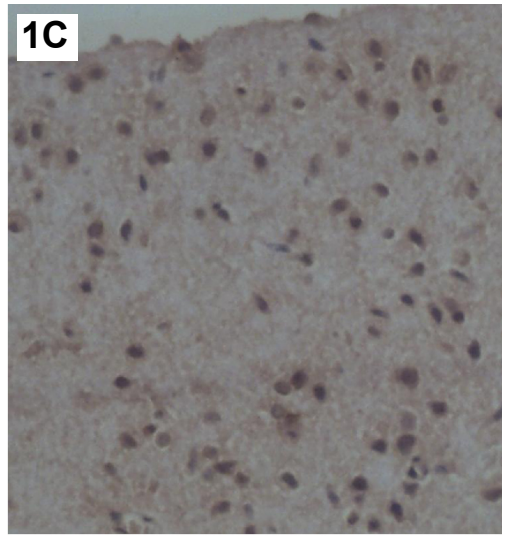
The values given are means±SD. Low-F, the low-fluoride exposed group; High-F, the high-fluoride exposed group. *$p<0.05$ in comparison to the control value; †$p<0.05$ in comparison to the value for the low-fluoride group.

Brown coloration was present in the nuclei of apoptotic neurons under an optical microscope (Figure 1). The mean values for apoptotic neurons in the cerebral cortices of the rats with fluorosis were elevated in both the low-F and high-F groups, with a tendency towards dose-dependency (Table 3).

In comparison to untreated controls, the level of Bcl-2 protein in the cerebral cortices was elevated by 26% in the low-F group and by 16% in the high-F group (Figure 2A). Elevations were also present for the level of Bax, 160% and 94%, respectively (Figure 2B) and the ratio of Bax/Bcl-2, 1.57 and 1.37 fold respectively, (Figure 2C).





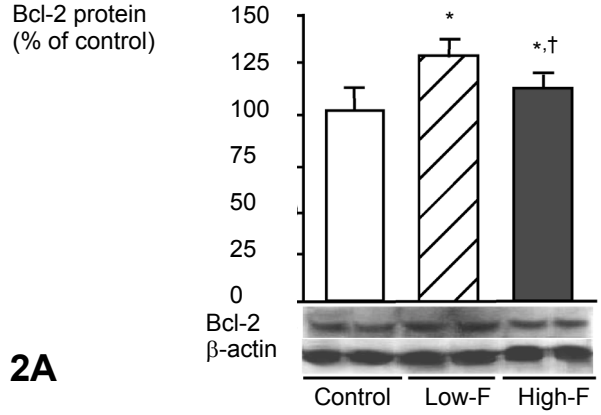


Figures 1A, 1B, and 1C. Apoptotic neurons in the brains of rats with chronic fluorosis and the control group. Brown coloration was present in the nuclei of the apoptotic neurons under an optical microscope (×400). 1A: control group, 1B: low-F group, 1C: high-F group.

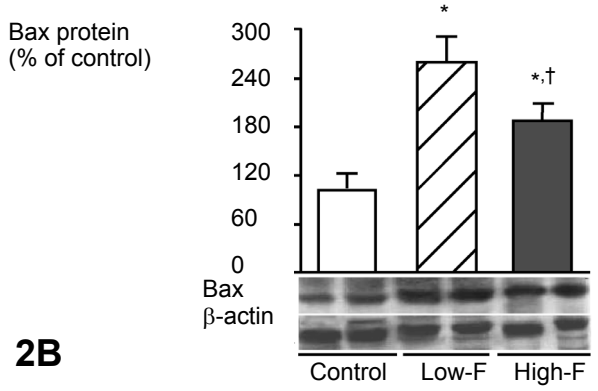
Table 3. Apoptotic neurons in the cortices of the rat brains and the control groups

Groups	Cases	Apoptosis (cells)
Control	20	24.9±3.3
Low-F	20	50.4±4.6*
High-F	20	70.1±10.5*,†

The values given are means±SD. Low-F, the low-fluoride exposed group; High-F, the high-fluoride exposed group. *$p<0.05$ in comparison to the control value; †$p<0.05$ in comparison to the value for the low-fluoride group.



2A



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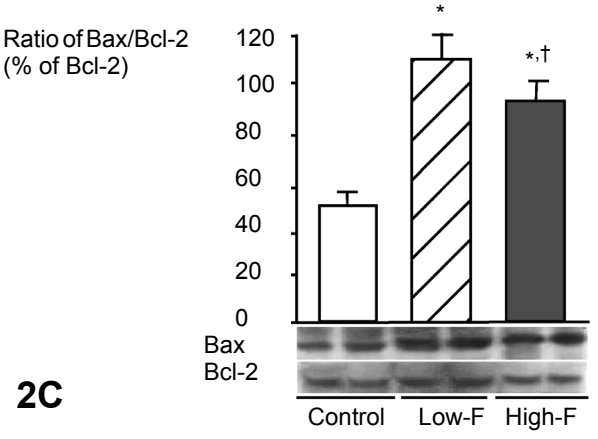

2C

Figures 2A, 2B, and 2C. Protein levels of Bcl-2 and Bax as well as the ratio of Bax/Bcl-2 in the brains of rats with chronic fluorosis and controls (drinking water F <0.5 mg/L). Low-F means the group exposed to a low concentration of fluoride in their drinking water (10 mg F/L) and High-F the group with high drinking water fluoride concentration (50 mg F/L). *p<0.01 as compared to the control group; †p<0.01 as compared to the low-fluoride group.

Levels of Bcl-2 and Bax mRNAs with melting curve analysis by RT-PCR following amplification of cDNA and quantitative real-time PCR confirmed the specificity of the procedure for Bcl-2 and Bax mRNAs. The levels of Bcl-2 and Bax mRNA in the cerebral cortices of the rats with fluorosis were both significantly elevated (Table 4).

Table 4. Median quantity of Bcl-2 and Bax at mRNA level in the cortices of the rat brains with chronic fluorosis and the control group

Groups	Cases	mRNAs (copies)		
		Bcl-2	Bax	Bcl-2 /Bax
Control	20	1.0×10^{3}	1.7×10^{2}	0.17
Low-F	20	6.4×10^{6}*	9.4×10^{6}*	1.47 *
High-F	20	4.3×10^{6}*,†	4.9×10^{6}*,†	1.14 *,†

The values given are medians. Low-F, the low-fluoride exposed group; High-F, the high-fluoride exposed group. *$p<0.05$ in comparison to the control value; †$p<0.05$ in comparison to the value for the low-fluoride group.

DISCUSSION

The rat model of chronic fluorosis was replicated successfully in the study with the development in the rats of dental fluorosis, a high urinary level of F, and a lowering of body weight. [13] In our previous investigations, the attenuated cognition and emotion in patients living in areas with endemic fluorosis and the deteriorated learning and memory in rats with chronic fluorosis were associated with alterations in the cholinergic nervous system, mitochondria, neuronal signal transduction, and the structure of cellular membrane lipids.[3,5,6,11,14,15] However, the molecular mechanisms underlying these phenomena remained elusive.

Recently, apoptosis has been considered to be an important cause of the damage to the brain in fluorosis.[5] Apoptosis is mediated by apoptotic molecules in the body, including the Bcl multi-gene family.[16-18] Bcl-2 is a widely studied modulator to inhibit programmed cell death, as a membrane protein localized to the outer mitochondrial membrane, perinuclear membrane, and the smooth endoplasmic reticulum.[19-21] However, Bax as a member of the Bcl gene family, can form heterologous dimeric complexes with Bcl-2 and accelerate programmed cell death.[20] Cells easily undergo apoptosis when the ratio of Bax to Bcl-2 is increased with an inducing agent such with fluoride in fluorosis.

The protocol here used the TUNEL assay to determine programmed cell death in cortical neurons of the rat brain with fluorosis. In comparison to untreated control animals, the level of apoptotic neurons with fluorosis was significantly enhanced. Using Western blotting and real-time PCR methods, respectively, for detecting the expression of these two genes in cortical neurons of rats, we found that the neurons of the controls had a low expression of Bcl-2 and Bax, at both protein and mRNA levels, while significant increases in the expression of Bcl-2 and Bax, at both the protein and mRNA levels, were found in the low-F and high-F groups. The increases in both Bcl-2 and Bax expression in the F rats might be a feedback effect following the brain damage

caused in the rats by the chronic fluorosis.[22] Furthermore, the higher expressions of Bax at the protein level and the increased ratio of Bax/Bcl-2 in the two F groups indicated that the proportionately greater increase in the Bax proteins might counteract the effect of the increase in Bcl-2 by forming Bax and Bcl-2 heterologous dimeric complexes. In our previous experiments, alterations in the dynamic behavior of mitochondria (with enhanced fission), along with the fragmentation of this organelle were observed in cortical neurons of the brains of rats subjected to chronic fluorosis.[12] Integrating the mitochondria enhanced fission behavior mentioned above, we suggest that the Bcl-2 protein, which cannot localize the fission area of outer mitochondrial membrane, makes an important contribution to the mitochondrial apoptotic pathway with the increased production of Bcl-2 and Bax. Interestingly, the more highly elevated ratio of Bax/Bcl-2 protein in the low-F group compared to the high-F group might reflect some as yet unknown adaptation to the toxic accumulation of F at certain degree or the function of gene expression.[12]

As is known, the majority of intracellular superoxide radicals are generated in connection with the process of oxidative phosphorylation and subsequently eliminated by antioxidants normally targeted to the mitochondria. Recently, more evidence has emerged for the occurrence of a high level of oxidative stress in chronic fluorosis and this change is believed to play an important part in the pathogenesis of the related damage.[23-25] Thus the imbalance between mitochondrial fusion and fission, together with the fragmentation and redistribution of mitochondria in the neurons of the brains of rats subjected to chronic fluorosis, as demonstrated,[12] may disturb the respiratory electron transport chain in such a way that more superoxide radicals are produced, resulting in an increase in the activity of the mitochondrial apoptotic pathway with altered Bcl-2 and Bax expression and related brain injuries.

In conclusion, the rates of apoptosis in the cortical neurons of rat brains were significantly increased in the groups with chronic fluorosis with a dose-dependent tendency. In addition, the expression of Bax and Bcl-2, at both the protein and mRNA levels, was clearly elevated in the cerebral cortices of the rats with chronic fluorosis. These findings indicate that Bax and Bcl-2 may regulate the increased level of apoptosis in the cortical neurons of rat brains resulting from chronic fluorosis.

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