EFFECTS OF CHRONIC FLUOROSIS ON CAMKIIα, C-FOS , BAX, AND BCL-2 CHANNEL SIGNALLING IN THE HIPPOCAMPUS OF RATS

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SUMMARY: In this study, the neurotoxicity of fluoride (F) in the hippocampus of rats exposed to 15, 30, and 60 mg NaF/L in their drinking water for nine months was investigated. Compared with the control (<0.5 mg F/L), significant increases in the expression of calcium/calmodulin-dependent kinase II alpha (CaMKIIα) (F=5.228, p<0.05) and catus proto-oncogene protein c-fos (cFOS) (F=7.625, p<0.05 or p<0.01) were observed in both the 30 and 60 mg NaF/L groups. In contrast, significant decreases occurred in the two higher NaF groups in the expression of Bcl2-associated X protein (Bax) and B-cell CLL/lymphoma (Bcl-2) (F=13.983, p<0.05 or p<0.01). These results indicate that the signalling pathway of CaMKIIα, c-fos, Bax, and Bcl-2 involves a molecular basis for the effects of fluorosis in the central nervous system.

Keywords: Bax; Bcl-2; c-fos; CaMKIIα; Fluorosis in rats; Hippocampus neurotoxicity.

INTRODUCTION

Well known for its sensitivity to disturbance by fluoride (F), the hippocampus is an important target site for neurotoxicity, and the CA3 area of that part of the brain plays a key role in long-term memory potentiation.[1] Previous studies have shown that fluorosis can impair synaptic interface structure[2] and membrane fluidity[3] in the CA3 area of the hippocampus, thereby affecting the transmission of neural information.[4] Although an increase in the concentration of intracellular Ca^{2+} in postsynaptic nerve cells is associated with mammalian fluorosis,[5-7] it is still unclear what the relationship is between hippocampus damage induced by F and the Ca^{2+} signaling pathway of calcium/calmodulin-dependent kinase II alpha (CaMKIIα), catus proto-oncogene protein cFos (c-fos), Bcl2-associated X protein (Bax), and B-cell CLL/lymphoma (Bcl-2).[8-10] To help shed light on this question, the previously studied rat model of fluorosis[4,6] was chosen to explore the effects of exposure to different concentrations of NaF on the expressions of CaMKIIα, c-fos, Bax, and Bcl-2.

MATERIALS AND METHODS

Twenty-four weanling male Sprague-Dawley rats weighing between 60 and 80 g were obtained from the Experimental Animal Center of Zhejiang Province. Based on the LD50 of sodium fluoride (NaF)[11] and sub-chronic experiments, the rats were divided into four equal groups and treated with NaF in their drinking water as follows:): 15 mg NaF/L (low F group), 30 mg NaF/L (medium F group), 60 mg NaF/L (high F group), and the control group (tap water with <0.5 mg F/L). The rats were housed in air-controlled rooms at a temperature of 23±1ºC and 50–60% relative humidity under a 12-hr light/12-hr dark cycle for 9 months. All procedures

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were performed in accordance with the Animal Care Guidelines of the Institutional Animal Care and Use Committee of China.

At the end of 9 months, each now-adult rat was anaesthetized and decapitated to obtain the whole brain tissues. The hippocampal tissues were isolated rapidly and were perfused with a fixative containing 4% paraformaldehyde in 0.1-mol/L phosphate buffer for immunohistochemistry (IHC). The specimens were cut into 5-μm thick slices, and slides from six slices from six different rats in each group were prepared. The gray values of CA3 area were assessed by 25 consecutive high-power fields (×400 magnification) for each slide (Media Cybernetics Image-Pro V5.1, US).

Data are expressed as mean±standard error (SEM) and analyzed with one-way ANOVA. Statistical differences between the experimental and control groups were estimated with the least significant difference (LSD) test. Statistical significance was set at $p<0.05$ or $p<0.01$. All analyses were performed using SPSS 18.0 software.

RESULTS

A small number of CaMKIIα subunit immunological positive cells in the cytoplasm were observed in the control, while the number of such cells increased with the increasing concentration of NaF in the exposure groups (Figure 1).

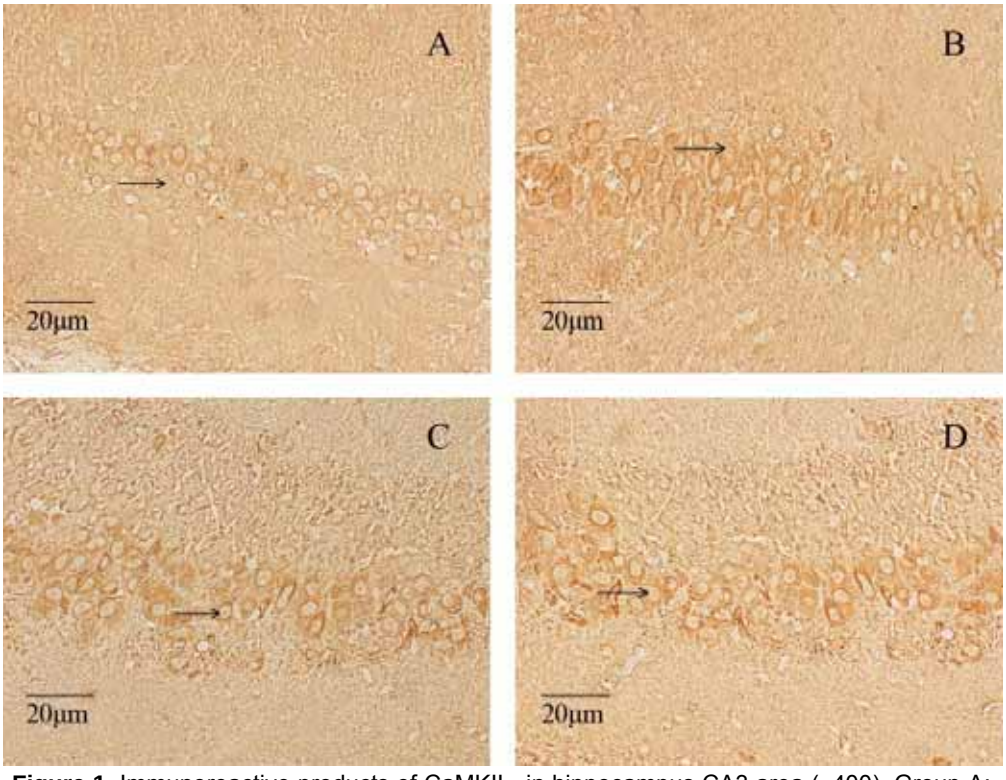

Figure 1. Immunoreactive products of CaMKIIα in hippocampus CA3 area (×400). Group A: control group; Group B: low fluoride group; Group C: medium fluoride group; Group D: high fluoride group.

As shown in Table 1, the integral optical density (IOD) of CaMKIIα increased significantly ($F=5.228$, $p<0.05$) in the 30 mg NaF/L (medium exposure) and 60 mg NaF/L (high exposure) groups compared with the control.

Table 1. Optical density of immunoreactive products of CaMKIIα, c-fos, Bax, and Bcl-2 (Values are mean±SEM)

Group	CaMKIIα IOD	c-fos IOD	Bax IOD	Bcl-2 IOD
Control	0.076 ± 0.008	0.272 ± 0.009	0.430 ± 0.017	0.408 ± 0.010
Low fluoride	0.086 ± 0.011	0.289 ± 0.013	0.411 ± 0.015	0.389 ± 0.011
Medium fluoride	0.121 ± 0.006*	0.345 ± 0.012†	0.325 ± 0.016†	0.303 ± 0.009*
High fluoride	0.117 ± 0.005*	0.307 ± 0.009*	0.304 ± 0.013†	0.276 ± 0.010†

Compared with the control, *$p<0.05$, †$p<0.01$, $n=6$.

Similar changes in the c-fos subunit immunological positive cells in the nucleus are seen in Figure 2. Only a few of these cells occurred in the control, and the number of such cells enhanced significantly as the increasing NaF concentration. Furthermore, the IOD of c-fos, analogous to CaMKIIα, increased significantly ($F=7.625$, $p<0.05$ or $p<0.01$) in the medium and high NaF exposure groups compared with the control.

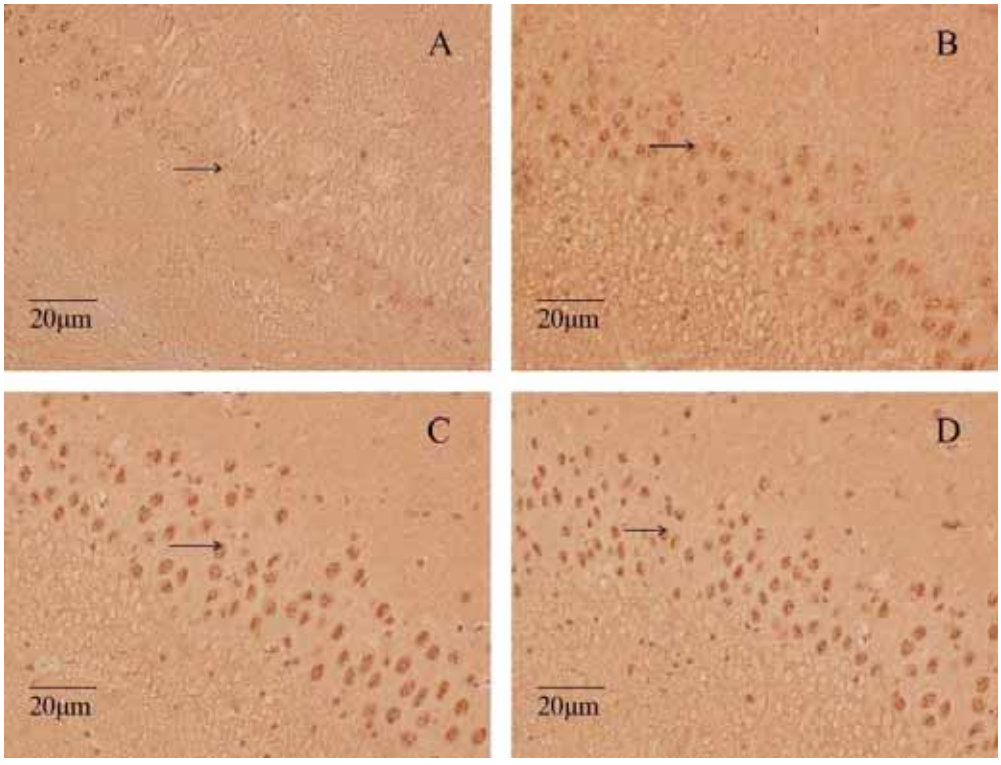

Figure 2. Immunoreactive products of c-fos in hippocampus CA3 area (×400). Group A: control group; Group B: low fluoride group; Group C: medium fluoride group; Group D: high fluoride group.

In contrast to CaMKIIα and c-fos, a fair number of Bax and Bcl-2 subunit immunological positive cells in the cytoplasm were observed in the control, but the expression of these cells was restrained in the NaF exposure groups (Figures 3 and 4).

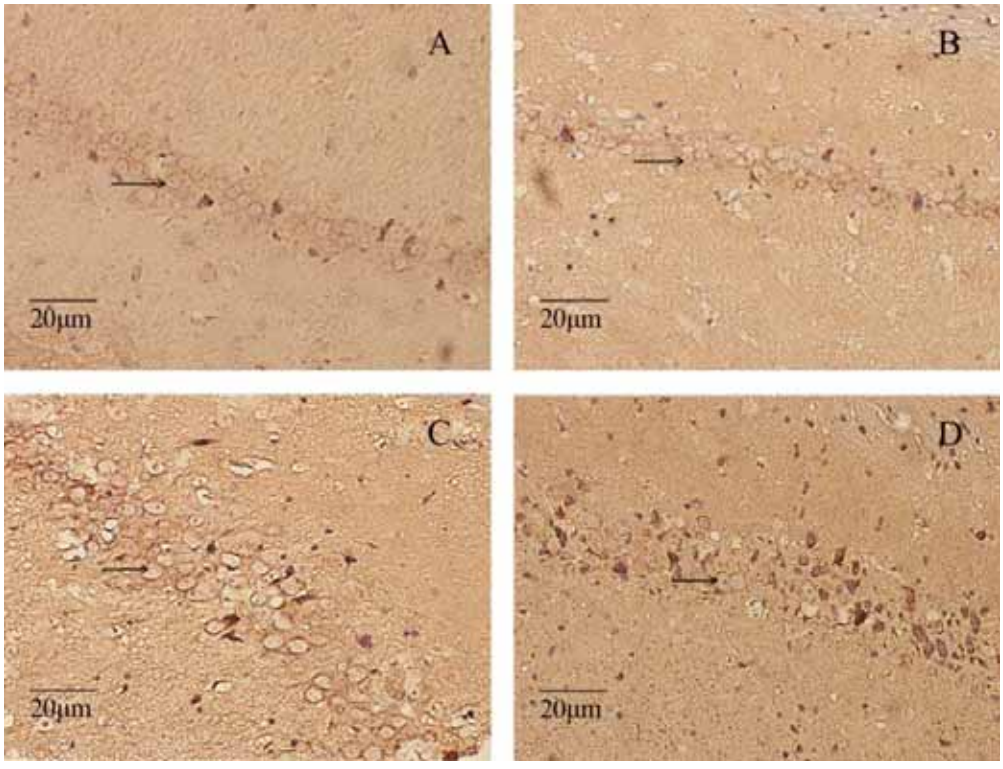

Figure 3. Immunoreactive products of Bax in hippocampus CA3 area (×400). Group A: control group; Group B: low fluoride group; Group C: medium fluoride group; Group D: high fluoride group.

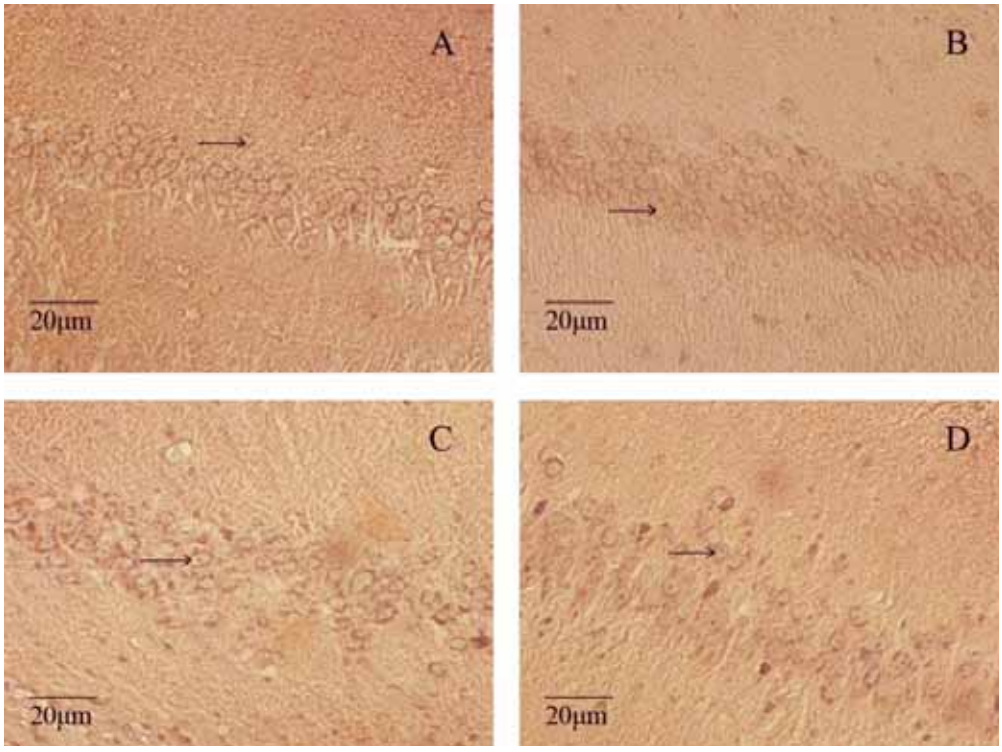

Figure 4. Immunoreactive products of Bcl-2 in hippocampus CA3 area (×400). Group A: control group; Group B: low fluoride group; Group C: medium fluoride group; Group D: high fluoride group.

Moreover, the medium and high NaF exposures significantly reduced the Bax expression ($F=9.764$, $p<0.01$) as well as Bcl-2 expression ($F=13.983$, $p<0.05$ or $p<0.01$). Meanwhile, as seen in Table 2, a slight increase in the Bax:Bcl-2 ratio without statistical significance occurred as the concentration of NaF increased.

Table 2. Optical density of immunoreactive products of Bax: Bcl-2
(Values are mean±SEM)

Group	N	Bax:Bcl-2
Control	6	1.054 ± 0.096
Low fluoride	6	1.057 ± 0.108
Medium fluoride	6	1.073 ± 0.113
High fluoride	6	1.101 ± 0.104

DISCUSSION

Intracellular Ca^{2+} overload is recognized as a common pathway of neuronal damage induced by various stimulations.[12,13] One of the main pathways for such Ca^{2+} overload is combined with the action of CaMKIIα, c-fos, Bax, and Bcl-2.[8-10] Due to an increase in intracellular [Ca^{2+}], Ca^{2+}-sensitive protein kinases such as

CaMKIIα are activated.[14] CaMKIIα is then transited into nuclei and can induce the expression of transcription factors such as c-fos.[15] CaMKIIα is involved in the regulation of neuronal functions such as neurotransmitter release, and long-term potentiation (LTP). c-fos can be regard as a reliable marker of neuronal activation.[16] Furthermore, c-fos is considered to play a role in the initiation of programmed cell death (PCD) or apoptosis in neurons.[17] Our results suggest that c-fos is regulated by Bax and Bcl-2.[18] It is well known that the Bcl-2 family of proteins plays a central role in regulating PCD[19] and various neurodegenerative disorders.[20]

The present study revealed that the expression of CaMKIIα, c-fos, Bax, and Bcl-2 had statistically significant changes induced from F exposure in the CA3 area of the hippocampus of rats. The signalling pathway of CaMKIIα, c-fos, Bax, and Bcl-2 may therefore be one of the related molecular mechanisms of fluorosis affecting the CNS or central nervous system. Interestingly, we found that the expression of CaMKIIα and c-fos in the high F (60 mg NaF/L) group of rats was slightly less than that in medium (30 mg NaF/L) group. Another recent study showed that acute exposure of rats to 150 mg NaF/L in their drinking water caused lower expression patterns of CaMKIIα in the hippocampus.[21] This kind of phenomenon has also occurred in other experiments,[1,5,22] thereby suggesting that toxicity induced by higher concentrations of F or chronic exposure to F are mediated by oxidative stress or regulated by Bax and Bcl-2.[6,23]

However, the mechanisms of fluorosis in the CNS are very complex and require further investigation. Further research is needed to determine the precise molecular mechanism of how F induces changes and apoptosis in hippocampal cells.

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