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# EFFECT OF FLUORIDE ON THE MICROGLIAL MORPHOLOGY AND THE EXPRESSION OF INFLAMMATORY CYTOKINES IN THE CEREBRAL CORTEX OF MICE

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**ABSTRACT: To investigate the effects of fluoride exposure on the microglial morphology and the expression of inflammatory cytokines in the cerebral cortex of mice, thirty-six ICR male mice were randomly divided into groups and given different doses of sodium fluoride (0, 25, and 50 mg/L NaF). After 50 days, the microglial morphology and the expression of interleukin-6 (IL-6), interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) were detected using immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA). In our results, the degeneration of pyramidal cells and glial cells was one of the most obvious pathological changes in the fluoride-exposed brains. Compared to the control group, the number of ramified, intermediate, and amoeboid microglia was significantly elevated in the NaF treatment groups. Additionally, the ELISA results showed that 50 mg/L NaF dramatically increased the expression of IL-6, IL-1β, TGF-β, and TNF-α when compared to the control group. These findings suggest that NaF can promote morphological changes of activated microglia and the release of inflammatory factors in the cortex, which may be one of the mechanisms of fluoride-induced nerve damage.**

Key words: Fluoride; Inflammatory cytokines; Microglial activation; Microglial morphology;.

## INTRODUCTION

Fluoride exists widely in the environment and is critical for large-scale chemical pesticide applications, pharmaceuticals, and material chemistry.[1,2] The brain is highly sensitive to fluoride. Fluoride can accumulate in the brains of experimental animals exposed to high doses of fluoride.[3,4] Ultrastructural alterations of neuron synapses have been reported, including an indistinct and short synaptic cleft, and thickened postsynaptic density (PSD).[5] Psychiatric symptoms have occurred in workers living in a high fluoride area including lethargy, memory and concentration impairment, and thinking difficulties.[6] In an active avoidance task, fluoride decreased the number of avoidance responses in rats.[7] In the brain, a number of fluoride-induced histopathologies have been observed, including a reduction in the number of pyramidal cells and the thickening and disappearance of dendrites.[8] Fluoride can attack microglia following the release of reactive oxygen species (ROS) and nitrogen oxide ($NO_x$). $NO_x$ is a common term for mono-nitrogen oxides, viz., nitric oxide (NO) and nitrogen dioxide ($NO_2$). $NO_x$ is the major and highly expressing enzyme for the production of extracellular superoxide in activated microglia.[3]

Microglia have strong morphological plasticity and are the key immune cells of the nervous system.[9] Their morphology is obviously changed with harmful brain microenvironments.[9] Microglial processes are constantly motile in the physiological brain and found in close proximity to synapses in both the postnatal and adult cortex.[10] Microglial have been imaged in real time in the intact brain using two-

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photon *in vivo* imaging and found to have extreme motility of their fine processes leading to the concept that "resting" microglia are constantly surveying the brain parenchyma in normal physiological conditions.[11] Microglia continuously detect the state of the brain parenchyma and maintain central nervous system (CNS) homeostasis by pruning synapses and phagocytosing cell debris and harmful substances.[11] Neuronal damage around chronic microglial activation is seemingly caused by the production of potentially neurotoxic substances such as proinflammatory cytokines, ROS, proteases, and complementary proteins.[12] Yan et al. found fluoride treatment increased the level of ROS and the release of inflammatory cytokines by inducing the activation of BV-2 microglia cells.[13]

Microglia are involved in the injury repair response, and the expression of cytokines and chemokines in the healthy brain.[14] The activation of microglia is considered to be a marker of neural inflammation in the brain.[15] Fluoride can induce the activation of microglia and the occurrence of CNS inflammation.[11] To further shed more light on the toxic mechanisms of fluoride on the nervous system, the present study investigated fluoride-induced changes in microglial phenotypes and the expression of inflammatory cytokines.

## MATERIALS AND METHODS

*Establishment of the animal model:* Thirty-six healthy male ICR mice weighing about 20 to 25g were provided by the China Institute of Radiation Protection. During a one-week adaptive period, all the mice were given deionized water and standard pellet feed. After this period, they were randomly divided into groups and received different doses of NaF (0, 25, and 50 mg/L). Throughout the 50 day-experiment, the animals had free access to water and food under light 12 hr/day at 20–25ºC. Standard pellet feed was provided by the Experimental Animal Center of Shanxi Medical University. The study was approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University.

*Tissue preparation:* After the 50 days of NaF treatment, we randomly selected four mice from each group from which we obtained a fixed sample. These mice were anaesthetized with ethyl ether and their hearts were rapidly perfused with 0.9% NaCl (37ºC) followed by 4% paraformaldehyde (37ºC). After perfusion, the brain tissues were removed in 4% paraformaldehyde (4ºC). The remaining eight brain tissues were stored in 1.5 mL centrifuge tubes in a refrigerator at –80ºC.

*Histopathological examination (HE):* The fixed brain tissues were embedded in paraffin and lengthways brain sections were cut at a thickness of 5 μm (routine paraffin sections). The brain tissue sections were stained with HE. The stained slides were observed using a light microscope.

*Immunohistochemistry (IHC):* The brain tissue sections were incubated with primary antibody diluted by rabbit anti-mouse Iba-1 (ionized calcium-binding adaptor molecule 1, Abcam, ab178847) for 12 hr at 4ºC, and secondary antibody Tritc-conjugated goat anti-rabbit IgG was added for 2 hr at 37ºC (Boster, SA1052). The IHC of Iba-1 was examined using a microscope.

*ELISA detection:* Proteins were extracted from eight mice cortices randomly in each experimental group. About 20 to 30 mg tissues were cut and quickly placed into 5 mL centrifuge tubes containing 400 uL PBS (PMSF was added 1:100 in advance).

After manually homogenizing, we poured the supernatant into a 1.5 mL centrifuge tube. This whole process took place on the ice. The supernatants were then allowed to stand for 30 min (4ºC) before being centrifuged at 12,000 revolutions/min for 10 min. Finally, we moved the liquid to 0.2 mL centrifuge tubes for storage. ELISA (Elabscience, E-EL-M0044c, E-EL-M0037c, E-EL-M0051c, E-EL-M0049c), following the manufacturer's instructions, was used for the detection of IL-6, IL-1β, TGF-β, and TNF-α.

*Data analysis:* All the results were expressed as mean±SEM. Statistical analyses between the groups were performed by one-way ANOVA with Dunnett as the posttest (GraphPad Software Inc., San Diego, USA). Differences were considered to be statistically significant when p values were less than 0.05.

## RESULTS

During the whole experimental period, there was no deaths of mice. After NaF exposure for 50 days, no significant differences were found between the control and the NaF treatment groups in the body weight, brain weight, and brain coefficient (brain coefficient = weight of brain in mg ÷ weight of mouse in g) (Table).

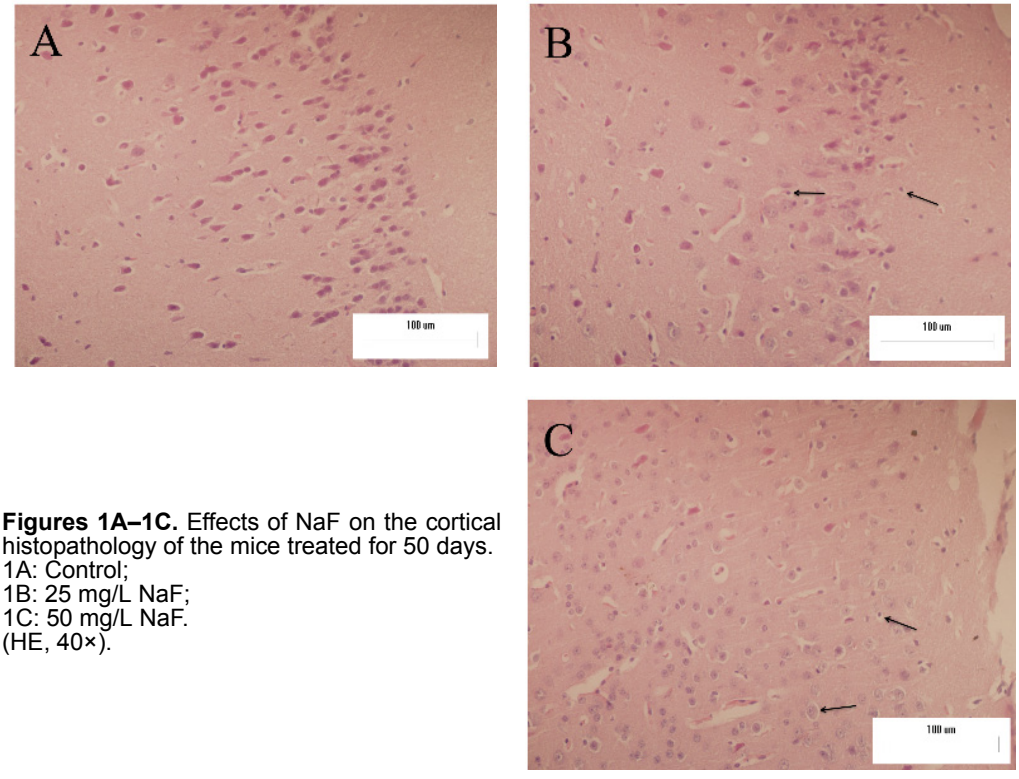
**Table.** Effects of NaF exposure for 50 days on the body weight, brain weight, and brain coefficient in mice (n = 12)

| Parameter | Group | | |
|---|---|---|---|
| | Control | 25 mg/L NaF | 50 mg/L NaF |
| Body weight (g) | 42.18±1.2190 | 43.05±0.9132 | 44.38±1.320 |
| Brain weight (mg) | 46.70±1.3640 | 47.46±0.6407 | 46.42±0.5996 |
| Brain coefficient (mg/g) | 1.111±0.04515 | 1.105±0.02571 | 1.049±0.02089 |

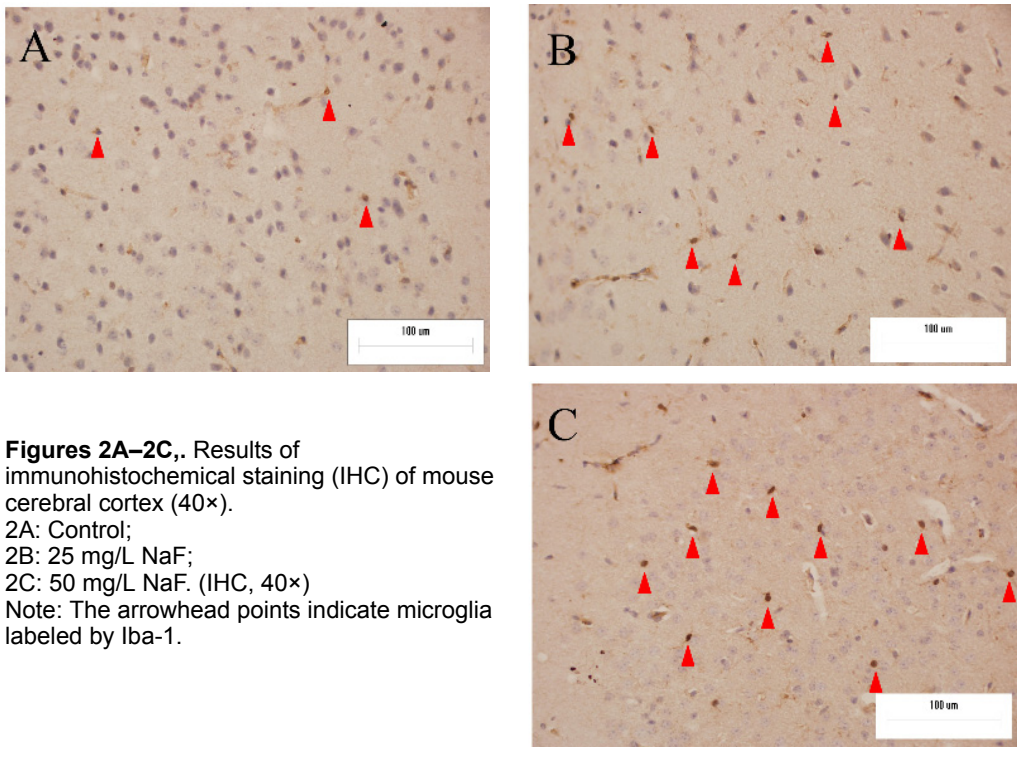
Compared to the control group: *p<0.05, †p<0.01. No significant differences were present between the groups with respect to the parameters.

Histological analysis of the cerebral cortices of the control group and the fluoride-treated mice is shown in Figure 1. There were many cortical cells in the control group, the tissue structure was clear, and the number of pyramidal cells and glial cells were within the normal ranges. These cells were arranged in an orderly manner within the cerebral cortices of the brains (Figure 1A). However, in the NaF treatment groups, shrinkage and fragmentation of the glial cells were observed, the pyramidal cells decreased in number, the external granular layer became thin, the nuclei shrunk, and the pyramidal cells fragmented (Figures 1B and 1C).

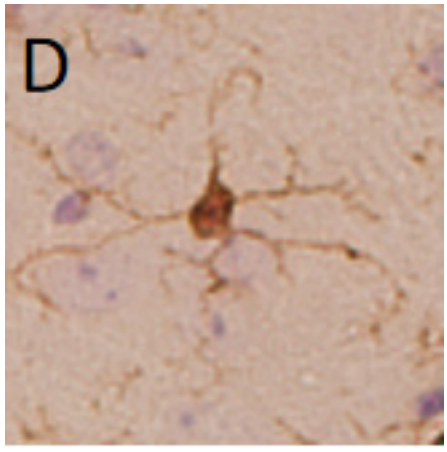
Iba-1 is the marker of microglia. In this experiment, the activation of mice cortical microglia was evaluated by using Iba-1 labeled brain tissue sections. As shown in Figures 2A–2F, the IHC results of Iba-1 confirmed the activation of microglia in the cortices of mice treated with fluoride.

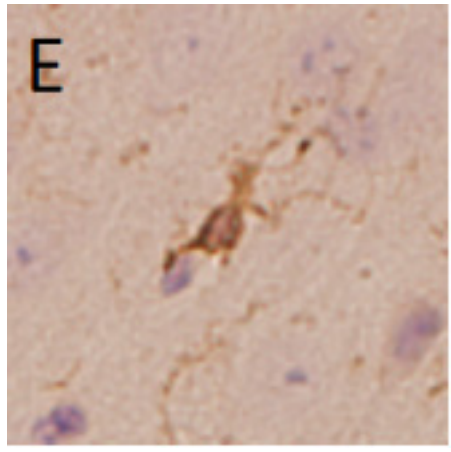


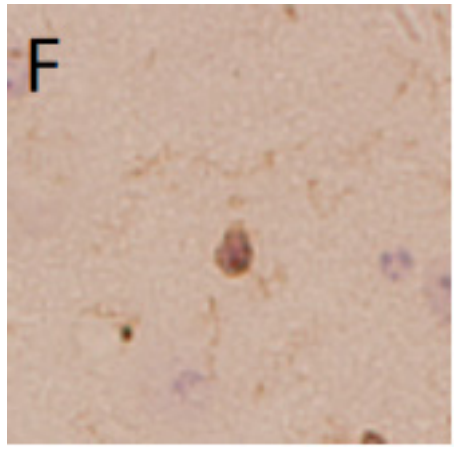

**Figures 1A–1C.** Effects of NaF on the cortical histopathology of the mice treated for 50 days.
1A: Control;
1B: 25 mg/L NaF;
1C: 50 mg/L NaF.
(HE, 40×).




**Figures 2A–2C,.** Results of immunohistochemical staining (IHC) of mouse cerebral cortex (40×).
2A: Control;
2B: 25 mg/L NaF;
2C: 50 mg/L NaF. (IHC, 40×)
Note: The arrowhead points indicate microglia labeled by Iba-1.



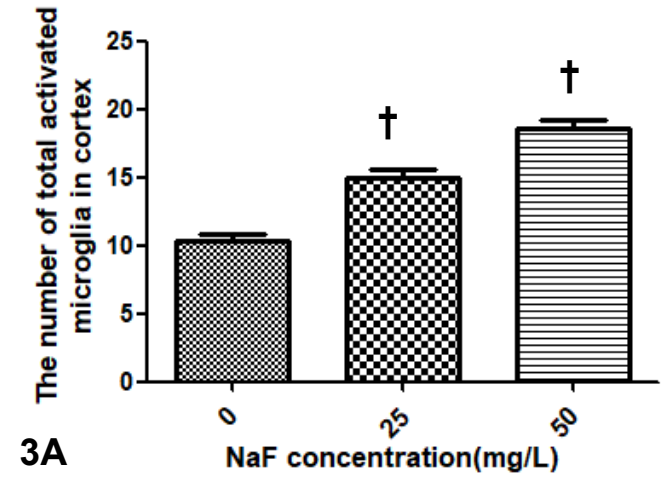



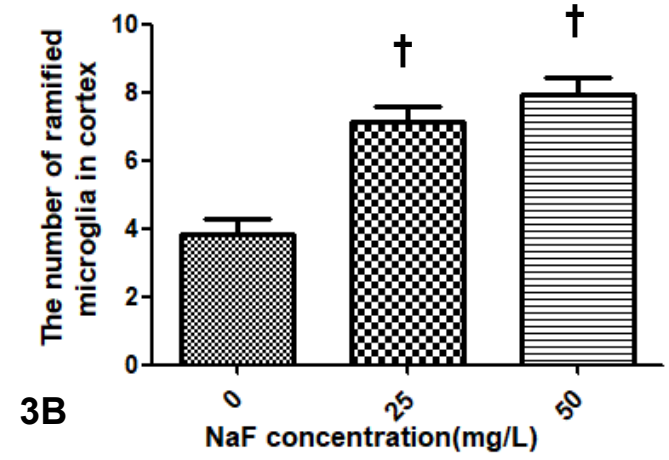

**Figures 2D–2F.** Results of IHC used to quantify the changes in the microglial cell response in mice cerebral cortex to fluoride exposure.(40×).
2D: Ramified microglia cells are characterized by high degree of branching to monitor changes in the microenvironment;
2E: The dense microglia cells in the middle are enlarged with fewer or shorter branching processes;
2F: The activated microglia cells are in the shape of round or amoeboid-like deformation, and the phagocytic function enhanced accordingly.

When compared to the control group, the immunoreactivity of Iba-1 in the cortex of mice treated with fluoride was greatly increased ($p<0.01$). After the quantification of the microglia, the results showed that the ramified microglia dramatically increased in the NaF treatment groups, compared to the control group ($p<0.01$). The number of microglia in the intermediate state ($p<0.01$) and the amoeboid-like state ($p<0.05$) were statistically significant in the group given 50 mg/L NaF as compared to the control group (Figures 3A–3D).

The expressions of inflammatory factors in mice cortices are shown in Figures 4A–4D. Fifty mg/L NaF treatment significantly increased the expression of IL-6 ($p<0.01$), IL-1β ($p<0.05$), TNF-α ($p<0.05$), and TGF-β ($p<0.05$), when compared to the control group.




3A

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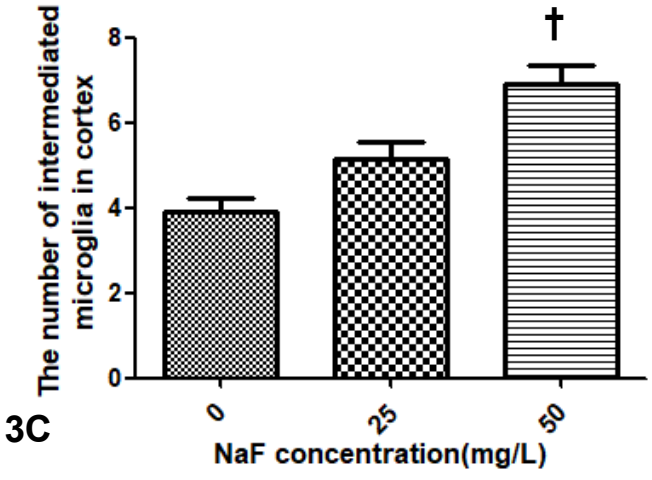



3C

**Figures 3A–3C.** Effect of NaF on microglial morphology of the cortex in mice (n=4). Compared to the control group: *p<0.05, †p<0.01.

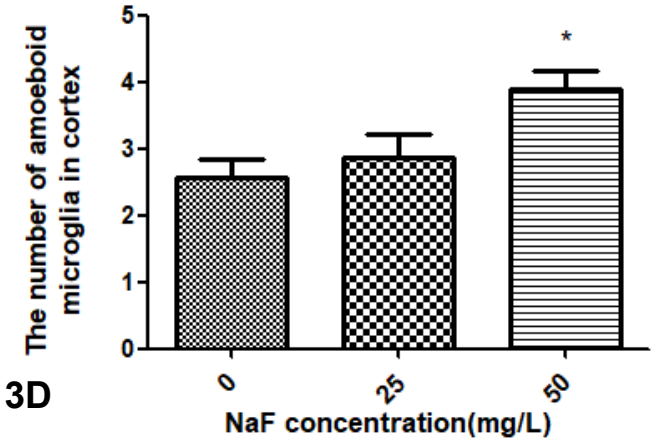
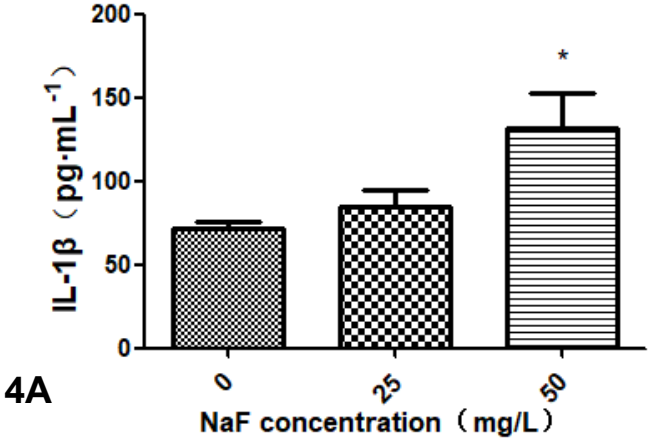



**Figure 3D.** Effect of NaF on microglial morphology of the cortex in mice (n=4). Compared to the control group: *p<0.05, †p<0.01.



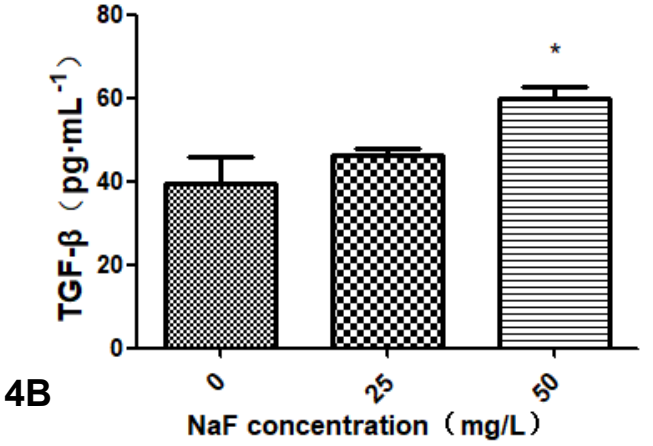
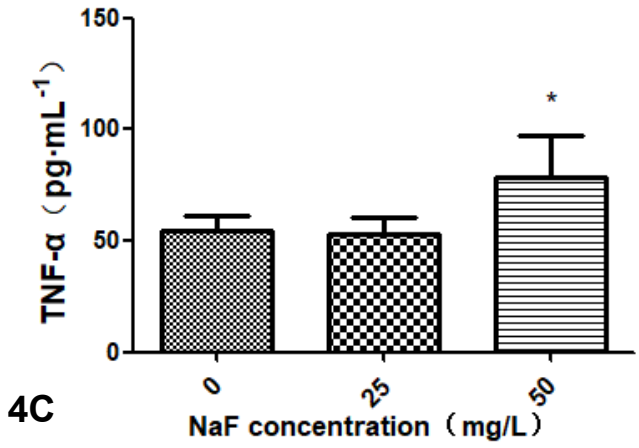
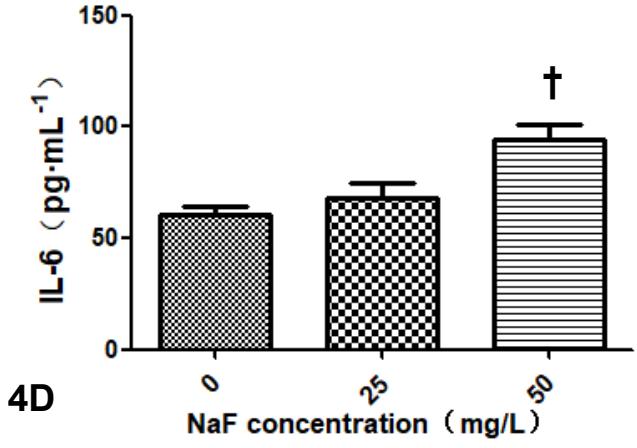




**Figure 4A–4B.** The expression of related inflammatory cytokines in mice cortices (n=8). Compared to the control group: *p<0.05, †p<0.01.




4C




4D

**Figure 4C–4D.** The expression of related inflammatory cytokines in mice cortices (n=8). Compared to the control group: *p<0.05, †p<0.01.

## DISCUSSION

Geographically, chronic fluorosis is widespread in more than 50 countries in Asia, Africa, Europe, North America, South America, and Oceania.[16] Neurological symptoms of humans have been reported in fluoride-contaminated areas, including decreased IQ in children, cognitive and memory impairment, and impaired learning ability.[17,18] Mullenix et al.[19] first demonstrated that the CNS functional output was vulnerable to fluoride. Animal experiments have proven that fluoride accumulates in the cortex, cerebellum, hippocampus, and medulla oblongata.[20] Studies have repeatedly revealed that cerebellar Purkinje neurons and glial cells in the rat cerebellum degenerate to an obvious extent after exposure to fluoride.[21] It has been reported that fluoride causes cell membrane degradation and the magnification of the pyramidal cells of the prefrontal cortex.[8] In agreement with Ge et al.,[22] our histopathologic analysis revealed shrinkage and fragmentation of glial cells in the NaF-treated groups. Compared to the control group, in the NaF-treated groups the pyramidal cells in the cerebral cortices became fewer, thinner, and fragmented with

shrunken nuclei. This may provide a histopathological explanation for the changes found in the fluoride-exposed animals.

Microglia, as mononuclear phagocytes of the CNS, play key immunological roles in maintaining normal brain functions in the CNS.[10] It is well known that Iba-1 is the marker for microglia. In this study, the elevated number of Iba-1 positive cells in the the brains of fluoride-treated mice confirmed the activation of the microglia. The findings of Yan et al. support our results.[23] In CNS development in mice, microglia actively remove the excess of synaptic connections through synaptic pruning.[24] Microglia support neuronal survival by accumulating and secreting trophic factors like IGF-1 around axons.[25] Microglia are morphologically heterogeneous. Once activated by the stimulus of the presence of harmful substances, microglia develop ameboid morphology characterized by cell body enlargement, shortened cell processes, and numerous cytoplasmic vacuoles.[21] In the present experiment, after fluoride exposure, the IHC data indicated that an increase of cortical activated microglia was observed and these cells transformed from a ramified state to an amoeboid state.

In many pathological conditions, microglia can induce the release of cytokines and chemokines from neighboring cells.[26] Microglial activations and inflammatory responses appeared in the brains of rats treated with fluoride-aluminum, which may be closely related to the continuous excessive release of inflammatory cytokines with long-term fluoride exposure.[27] Activated microglia produce several inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are crucial in regulating the physiological immune response of the CNS.[28,29] NaF-treated rats have been found to display neurodegenerative changes.[15] IL-1β is associated with neurodegenerative diseases and can profoundly affect memory.[30] Neurodegeneration mediated by microglia can be driven through TNF-α signals.[31] After injury, TNF-α was mainly produced by microglia.[32] IL-6 can induce brain injury and damage neural cells.[33] TGF-β1 may suppress the activation of microglia in the ischemic brain.[34] Under pathological conditions, activated microglia could secrete excessive pro-inflammatory cytokines, which are involved in tissue damage and neurological dysfunction.[35] In the present study, we observed an increase in the expression of inflammatory cytokines IL-1β, IL-6, TNF-α, and TGF-β in the cortex of fluoride-exposed mice and these may be involved in the formation of the CNS inflammation. However, the deep mechanism of fluoride-induced neural inflammation still needs to be further studied.

## CONCLUSIONS

In conclusion, the degeneration of the pyramidal cells and glial cells in the cerebral cortex was one of the most obvious pathological changes in the brains of the mice administered fluoride. Fluoride intensified the development of microglia from the ramified microglial form to the ameboid state in the mice cortices, which may be one of the mechanisms of fluoride-induced neurological inflammatory response.

## ACKNOWLEDGMENT

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