

## EFFECTS OF FLUORIDE ON EXPRESSION OF CYTOKINES IN THE HIPPOCAMPUS OF ADULT RATS

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**SUMMARY:** To investigate the effects of fluoride on the expression levels of cytokines in the rat hippocampus, 60 adult Wistar albino rats were randomly divided into four groups of 15 rats each, whose drinking water contained: (1) double distilled water (the control group); (2) 25 mg sodium fluoride (NaF)/L, (11.3 mg F/L); (3) 50 mg NaF/L, (22.6 mg F/L); and (4) 100 mg NaF/L, (45.2 mg F/L). After five months treatment, the expression levels of the cytokines TGF- $\beta$ 1, IL-4, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  gene were determined by real-time PCR, and the TGF- $\beta$ 1 protein levels were detected by ELISA. A significant decrease of TGF- $\beta$ 1 was found, in both the gene and protein levels, while no significant change occurred in the levels of IL-4, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  gene. Fluoride may damage the hippocampus by significantly decreasing the expression of TGF- $\beta$ 1 gene and protein, possibly by an unknown post-transcriptional mechanism. The study provides a new perspective for evaluating the neurotoxicity of fluoride.

Keywords: Cytokine; Fluoride; Hippocampus; TGF- $\beta$ 1; IL-4; IL-1 $\beta$ ; IL-6; TNF- $\alpha$ .

### INTRODUCTION

Fluoride, which is distributed worldwide, may have adverse effects on the skeleton,<sup>1</sup> thyroid,<sup>2,3</sup> testis,<sup>4</sup> spleen,<sup>5</sup> cardiovascular system,<sup>6-7</sup> and central nervous system.<sup>8-9</sup> Furthermore, epidemiological investigations show that fluoride may have detrimental effects on learning ability and cognitive capacity.<sup>9-11</sup> Fluoride may induce structural brain changes such as altering neuronal and cerebrovascular integrity,<sup>12</sup> and reducing synaptic membrane fluidity.<sup>13</sup> The biochemical environment in the brain, such as the enzymes associated with anti-oxidants and neurotransmitters, may also be changed by fluoride.<sup>14</sup> Previous studies reveal that fluoride induces apoptosis and oxidative stress in the brain.<sup>15</sup> Nevertheless, it is still not clear whether fluoride can influence the expression levels of cytokines in the hippocampus, which play an important role in a series of physiological and pathological processes. The purpose of this study was to detect the effects of fluoride on the expression levels of cytokines in the hippocampus of rats.

### MATERIALS AND METHODS

*Establishment of animal model:* Sixty adult male Wistar albino rats were obtained from the Experimental Animal Center of Shanxi Medical University. After acclimatization for a week, they were randomly divided into four groups of fifteen rats with differences in their drinking water: (1) the control group: administered double distilled water; (2) the 25 mg/L group: administered sodium fluoride (NaF) at a concentration of 25 mg/L, (11.3 mg F/L); (3) the 50 mg/L

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group: administered NaF at a concentration of 50 mg/L, (22.6 mg F/L); (4) the 100 mg/L group: administered NaF at a concentration of 100 mg/L, (45.2 mg F/L). They were given free access to food and water under standard temperature (22–25°C), and a 12/12-hr light/dark cycle. All animals were kept for five months to ensure that they were exposed in a long-term environment. The study design was approved by the Institutional Animal Care and Use Committee of China.

*Tissue preparation:* All the rats were anesthetized with 20% urethane solution. The hippocampi were removed quickly and stored at –80°C.

*Total RNA extraction and QRT-PCR:* Total RNA was isolated from the rat hippocampus using Trizol reagent (Invitrogen, USA) according to the instructions. Specific primers of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), interleukin-4 (IL-4), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and  $\beta$ -actin (Table 1) were designed with Primer 3.0 plus according to the alignments

**Table 1.** Primer sequences with their corresponding PCR product size and position

Gene	Primers (5'→3')	Primer locations	Product (bp)	Gene bank accession no.
$\beta$ -actin	TACCCAGGCATTGCTGACAG AGCCACCAATCCACACAGAG	997-1111	115	NM_031144.2
TGF- $\beta$ 1	GGCGGTGCTCGCTTTGTA GGGTGACTTCTTTGGCGTAG	370-473	104	NM_021578.2
IL-4	GGAACACCACGGAGAACG GCACGGAGGTACATCACG	209-291	83	NM_201270.1
IL-1 $\beta$	GGCAGTGTCACTCATTGTGG AGGTGCTTGGGTCCTCAT	242-330	89	NM_031512.2
IL-6	GCCTTCTTGGGACTGATG TGGTCTGTTGTGGGTGGT	101-196	96	NM_012589.1
TNF- $\alpha$	ATTGTGGCTCTGGGTCCAAC TCCAGTGAGTCCGAAAGCC	979-1128	150	NM_012675.3

The expression levels of TGF- $\beta$ 1, IL-4, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  genes were determined by QRT-PCR using Mx3000P<sup>TM</sup> (Stratagene, USA) and the Two-Step SYBR<sup>®</sup> QRT-PCR kit (Takara, China). The protocol of PCR under thermocycling condition as follow: after initial denaturation at 95°C for 15 sec, 50 PCR cycles were started with thermocycling conditions at 95°C for 5 sec, 61°C for 20 sec, and 72°C for 6 sec, and then followed by the reaction melting curve analysis to verify the specificity of the amplified products.  $\beta$ -actin, an endogenous control, was used to reflect the relative expression levels of target genes.

*TGF- $\beta$ 1 Protein level detected by ELISA:* To extract the total protein in the hippocampus, tissues were homogenized by a tissue grinder (KIMBLE, USA)

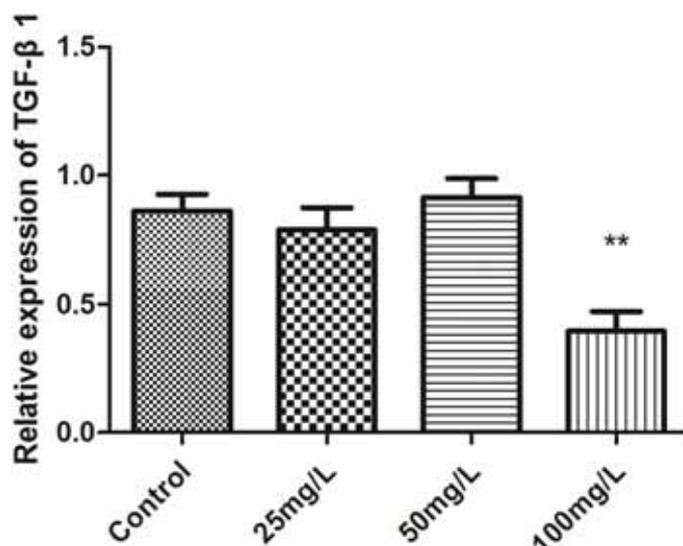
with RIPA lysis buffer to which was added with 1 mM PMSF. The supernatants containing total protein were acquired after the homogenate was centrifuged at 12,000 g for 30 min at 4°C. The total protein of supernatants were measured using the BCA Protein Assay kit (Westang Bio-Tech Co. Ltd., Shanghai, China). The protein levels of TGF- $\beta$ 1 in the hippocampus were detected using ELISA kit (Westang Bio-Tech Co. Ltd., Shanghai, China) following the manufacturer's instruction. 100 $\mu$ L homogenate of samples and TGF- $\beta$ 1 standard were added to the microplate wells which were coated with mouse monoclonal antibody against rat TGF- $\beta$ 1. These wells were then incubated for 40 min at 37°C. The microplate wells were washed 6 times with wash buffer. 50  $\mu$ L of distilled water and 50  $\mu$ L biotinylated antibody were added to the wells and incubated for 20 min at 37°C. The microplate wells were washed 6 more times to remove unbound biotinylated antibody. Then 100  $\mu$ L of enzyme conjugate was added to the wells for 10 min at 37°C. After washing 6 times with wash buffer, 100  $\mu$ L of TMB solution was added to the wells for 15 min at 37°C. Then a 100 $\mu$ L stop solution was added to the wells. The absorbance was read at 450 nm using a microwell-plate reader (Thermo, America). TGF- $\beta$ 1 levels were expressed as picogram (pg) per mg protein and calculated using a standard curve.

*Data analysis and management:* All data are expressed as mean  $\pm$  SEM and analyzed using one-way ANOVA. The significance between pairs was determined by Tukey's Multiple Comparison Test. Levels with  $p < 0.05$  were considered statistically significant.

## RESULTS

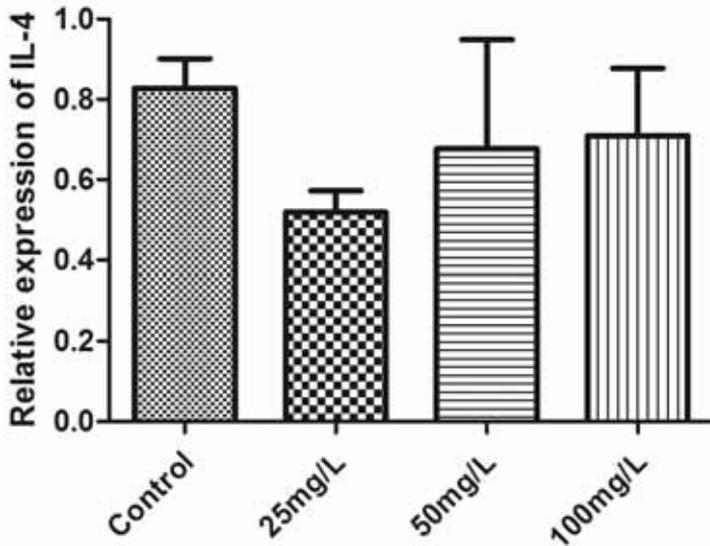
### *Quantification of gene expression:*

Compared to the control group, NaF significantly decreased the expression levels of the TGF- $\beta$ 1 gene in the 100 mg/L group ( $p < 0.01$ ) while levels in the 25



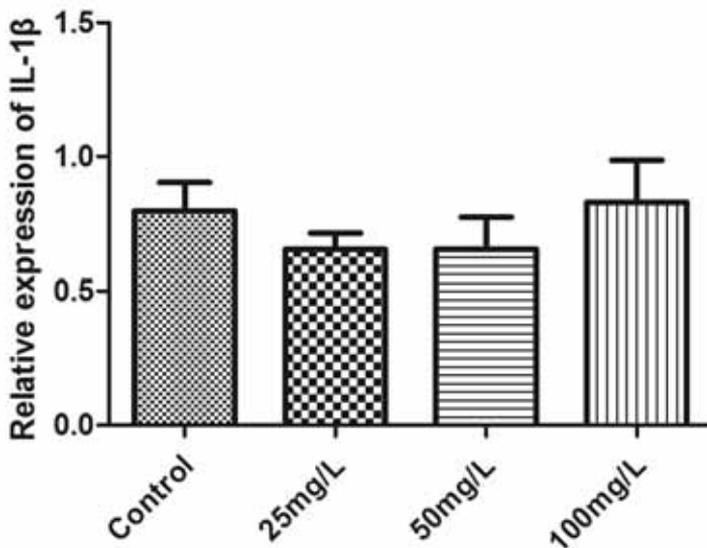
**Figure 1.** Effect of F on expression levels of TGF- $\beta$ 1 gene in hippocampus of male rats. Adult male Wistar albino rats were exposed to NaF at concentrations of 0, 25, 50 and 100 mg/L respectively for 5 months. The gene expression levels of TGF- $\beta$ 1 were analyzed by QRT-PCR. Results are expressed as mean  $\pm$  SEM. Compared with the control, \*\* $p < 0.01$ .

The expression levels of the IL-4 gene in the 25, 50, and 100 mg/L groups were reduced by NaF but not at a statistically significant level (Figure 2).



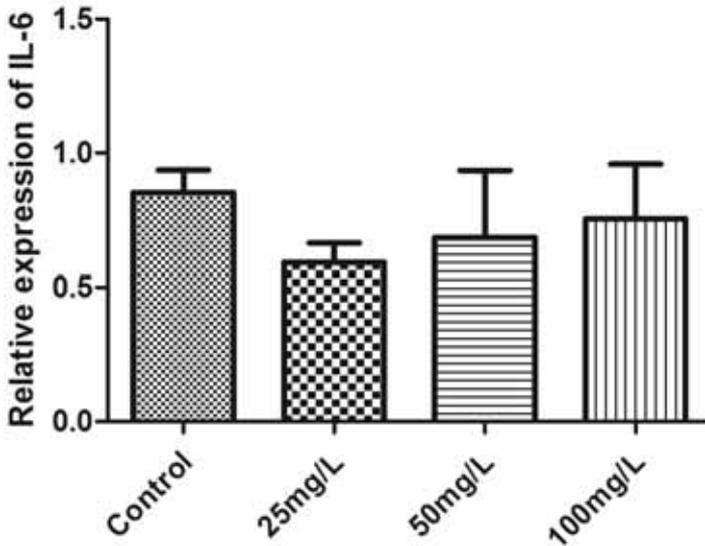
**Figure 2.** Effect of NaF on expression levels of the IL-4 gene in hippocampus of male rats of the control and treatment groups were detected by QRT-PCR. Results are expressed as mean  $\pm$  SEM.

The expression levels of the IL-1 $\beta$  gene were reduced by NaF in the 25 and 50 mg/L groups and increased in the 100 mg/L group but the changes were not statistically significant compared to the control (Figure 3).



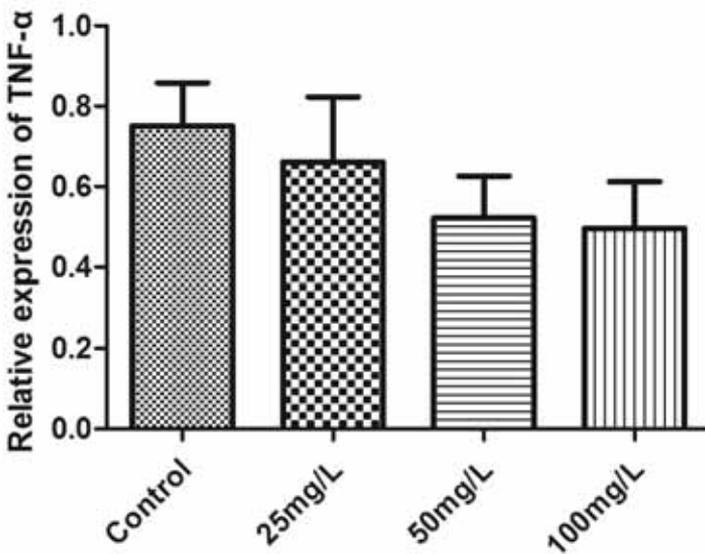
**Figure 3.** Expression of IL-1 $\beta$  gene in hippocampus of male rats. The gene expression levels of IL-1 $\beta$  were examined by QRT-PCR. Results are expressed as mean  $\pm$  SEM.

The expression levels of the IL-6 gene were decreased by NaF but not at statistically significantly level compared with the control (Figure 4).



**Figure 4.** Expression of IL-6 gene in hippocampus of male rat of four groups was measured by QRT-PCR. Results are expressed as mean ± SEM.

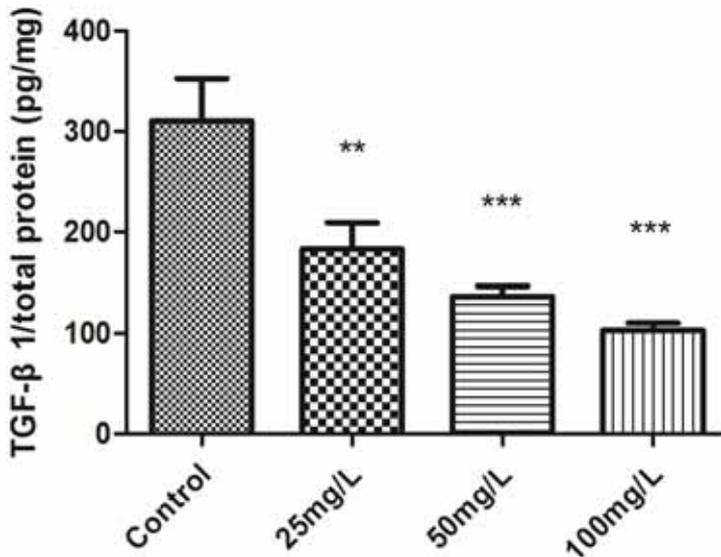
The expression levels of the TNF- $\alpha$  gene were reduced by NaF in the 25, 50, and 100 mg/L groups compared with the control but not at a statistically significant level (Figure 5).



**Figure 5.** Expression levels of the TNF- $\alpha$  gene in hippocampus of male rats of the four groups. The gene expression levels of TNF- $\alpha$  were measured by QRT-PCR. Results are showed as mean ± SEM.

### Protein levels of TGF- $\beta$ 1:

There was a significant decrease of TGF- $\beta$ 1 in the hippocampus of rats after exposure to NaF for five months in all three treatment groups compared with the control group: 25 mg/L group ( $p < 0.01$ ), 50 mg/L group ( $p < 0.001$ ), and 100 mg/L group ( $p < 0.001$ ) (Figure 6).



**Figure 6.** The levels of TGF- $\beta$ 1 in the hippocampus homogenate of the four groups of rats exposed to fluoride were determined by ELISA. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  as compared with the control.

## DISCUSSION

Epidemiological investigations and experimental studies have demonstrated the neurotoxic effects of fluoride. Our previous studies showed that fluoride had adverse effects on learning ability and memory by altering glutamate metabolism and cholinesterase activity.<sup>10,16</sup> The present study was conducted to explore the influence of fluoride on cytokine expression in hippocampus. This is a new perspective for evaluating the neurotoxicity of fluoride.

TGF- $\beta$ 1, one of the TGF- $\beta$  superfamily, is a crucial modulatory factor of various biological processes such as proliferation, differentiation, and apoptosis in different organs and cells.<sup>17-19</sup> A number of studies demonstrate that TGF- $\beta$ 1 plays an important role in neuroprotection.<sup>20, 21</sup> Krishnan et al. reported that TGF- $\beta$ 1 decreased the death of neuronal cells by altering the proportion of apoptotic and antiapoptotic proteins.<sup>20</sup> In addition, it is suggested that the loss of TGF- $\beta$ 1 leads to an extensive degeneration of neurons accompanied by decreased expression of laminin and synaptophysin and by a remarkable microgliosis.<sup>21</sup> In this study, the data indicated that NaF induced a prominent decrease in the expression of TGF- $\beta$ 1 at both the gene and the protein levels. The reduced TGF- $\beta$ 1 may change neuronal cell apoptosis and alter the degeneration of neurons in hippocampus. Consequently, it may influence both learning ability and memory. Another

interesting finding is that the protein levels of TGF- $\beta$ 1 have been altered in the 25 and 50 mg/L groups accompanied by steady-state mRNA expression. This suggests that an unknown regulation mechanism may participate at the post-transcriptional level. However the trigger is pulled, the cascade reactions downstream require further investigation.

A growing amount of evidence shows that the cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have a neuroprotective function and participate in the complicated process of cognition by regulating long-term potentiation, neurogenesis, synaptic plasticity, and synaptic scaling.<sup>22-25</sup> Meanwhile, an increasing number of studies indicate that IL-4 plays a vital role in the processes of learning and memory.<sup>26</sup> It has also been reported that decreased IL-4 is associated with impairment of long-term potentiation.<sup>27</sup> Nevertheless, in this study, the expression of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-4 at gene level were not significantly changed after exposure to NaF for five months. This demonstrates that the adverse effects on cognitive and learning ability of NaF, in this animal model, may not be the result of altering the expression of the cytokines IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-4.

#### ACKNOWLEDGEMENTS

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