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Molecular mechanism of brain impairment caused by drinking-acquired fluorosis and selenium intervention

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ABSTRACT

This study investigated the molecular mechanism of brain impairment induced by drinking fluoridated water and selenium intervention. Results showed that the learning and memory of rats in NaF group significantly decreased. Moreover, the number of apoptotic cells, the expression levels of Cytc mRNA and protein, and the expression levels of Caspase-9 and Caspase-3 mRNA significantly increased; by contrast, Caspase-9 and Caspase-3 protein levels significantly decreased. Compared with the NaF group, the mRNA levels of Cytc and Caspase-9, as well as the protein levels of Cytc in NaF + Se group, significantly decreased. Conversely, the protein levels of Caspase-3 and Caspase-9, as well as the mRNA levels of Caspase-3, significantly increased. Thus, the mitochondrial CytC-Caspase-9-Caspase-3 apoptosis pathway in the hippocampus was one of the mechanisms leading to fluorosis-induced brain damage. Furthermore, the Cytc signaling molecules were possibly the key target molecules in fluorosis-induced apoptosis, and selenium could alleviate fluorosis-induced brain injury.

1. Introduction

Fluorine is an essential trace element in the body, but prolonged excessive fluoride intake can produce adverse effects (Luo et al., 2012) called endemic fluorosis. The effects of fluorosis on skeleton and teeth is well documented (Sivasankar et al., 2012), and neurotoxicity of fluoride has also been confirmed (Zhou et al., 2012) wherein fluorosis results in systemic diseases. However, the pathogenesis of fluorosis remains unclear.

Selenium, an element demonstrating antioxidant properties and which exerts curative effect as an anti-fluorine agent, has recently attracted the attention of researchers. The present study further explores subchronic toxicity of fluoride based on our previous experiments (Miao et al., 2013), where we concluded that 1.5 mg/L is the optimum concentration (Qian et al., 2013; Yang et al., 2013) for selenium to antagonize subchronic fluorosis to observe the learning ability and memory of rats, which are indicators of brain function. This study aimed to determine the rate of nerve cell apoptosis in the CA_3 area of the hippocampus and the expression levels of the apoptosis-related factors' genes and proteins in the mitochondria. The molecular mechanism of brain impairment caused by drinking-acquired fluorosis and the intervention of selenium was investigated, and the key target molecules were screened.

2. Materials and methods

2.1. Experimental animal treatment

Eighty newly weaned male Sprague-Dawley rats, each weighing 70–90 g, were procured from the Experimental Animal Center of Zhejiang Province in China. The rats were randomly divided into four groups, each group containing 20 rats. The first group served as control group and received tap water (fluoride and selenium concentrations were <0.2 mg/L and <1 μg/L, respectively). The fluoride-treated group (F) was administered with aqueous sodium fluoride (NaF, 100 mg/L). In the selenium only group (Se), selenium was used as sodium selenite at 1.5 mg/L concentration mixed in drinking water. The last group (F + Se) received NaF (100 mg/L) + Se (1.5 mg/L). All of the rats were fed with a standard pellet diet (containing 0.2 mg/kg fluorine and 0.1 mg/kg selenium) and were provided with water ad libitum for three months.

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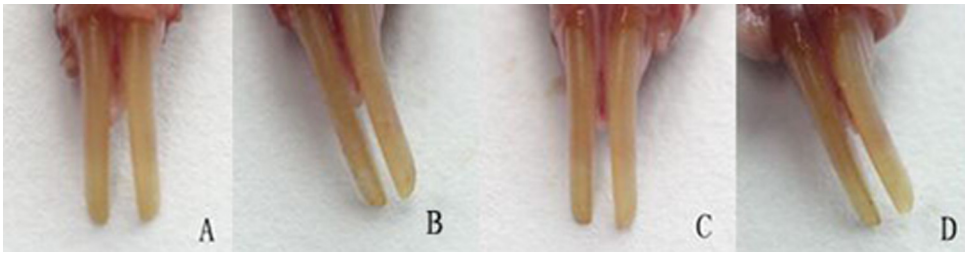

Fig. 1. Comparison of rat teeth after exposure to fluoride: (A) control; (B) F; (C) Se; and (D) F + Se.

2.2. Chemicals and reagents

NaF and sodium selenite solids were obtained from Jinhua Reagent Company (China), whereas rabbit polyclonal antibodies against Cytc, Caspase-9, Caspase-3, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-labeled goat anti-rabbit IgG was obtained from Boyun Biotech (Shanghai, China). The RT-PCR PrimeScript II first-strand cDNA synthesis kit and dNTP mix and tag enzyme used were procured from Takara Biotechnology (Dalian, China). Cytc, Caspase-9, Caspase-3, and β-actin primers were synthesized by Sangon Biotech (Shanghai, China). All other chemicals were obtained from local commercial sources.

2.3. Observation of dental fluorosis

Symptoms of dental fluorosis in rats were diagnosed using the Dean's method (Wang, 2007).

2.4. Determination of blood/urine fluoride

The concentrations of blood and urine fluoride in rats were detected using the fluoride electrode method (Liu et al., 2010).

2.5. Determination of behavior and learning memory

Spatial learning and memory of the rats were evaluated using the open-field (Crawley, 1985) and Morris water maze tests (Gong and Wang, 2005). The open-field test measures the frequency of running, standing, buttresses, and grooming, whereas the Morris water maze test measures the escape latency, as well as the frequency and duration by which the site, where the original platform was located, is crossed.

2.6. Detection of hippocampal apoptosis

Apoptotic cells were detected using an apoptosis assay kit (BOSTER, Wuhan, China) according to the manufacturer's instructions. Apoptosis was detected using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay. The tissue sections were de-waxed, treated with 3% H_2O_2 for 10 min to eliminate the endogenous peroxidase, washed with distilled water, treated with proteinase K for 15 min, and then washed with TBS. The sections were allowed to react with the labeling buffer, mixed with TdT and DIG-d-UTP for 2 h at 37 °C, and then washed with TBS. The slides were subsequently immersed in the blocking solution for 30 min. After incubating the tissues with biotinylated anti-digoxin antibody solution for 30 min at room temperature, the slides were incubated with SABC for 30 min at 37 °C, washed with TBS, and then stained with DAB. The positively staining nuclei had a dark-brown appearance as visualized under a light microscope.

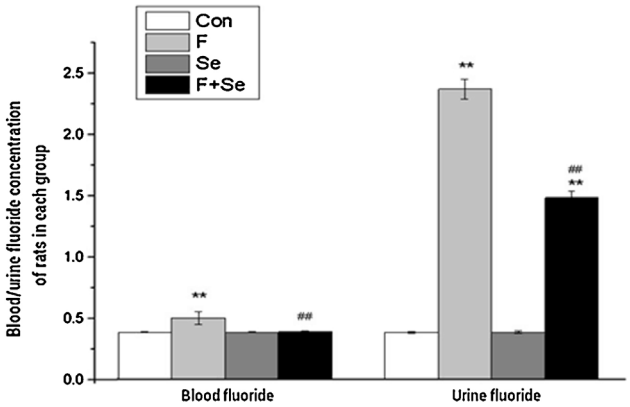

Fig. 2. Trends of blood/urine fluoride. Data are expressed as the mean ± SEM ($n = 20$ animals per group). $^{**}P < 0.01$, compared with the control group. $^{\#\#}P < 0.01$, NaF + Se group compared with the NaF-treated group.

2.7. Semi-quantitative PCR

RNA was extracted from 100 mg of hippocampus tissue by using Redzol reagent (SBS Genetech, Beijing, China) according to the manufacturer's instructions. The RNA was then quantified using Multiskan Spectrum, and RNA purity was tested using agarose gel electrophoresis. The RNA solution was stored at −80 °C for further analysis. The first-strand cDNA was synthesized from 5 μg of total RNA using an RT-PCR PrimeScript II first-strand cDNA synthesis kit. β-actin was used as reference.

The primer pairs used were as follows: β-actin (482 bp): forward: GAGAGGGAAATCGTGCGTGA, reverse: CATCTGCTGGAAGGTGGACA; Caspase-3 (540 bp): forward: CATGGAAGCAAGTCGATGG, reverse: GGGTGCGGTAGAGTAAGCAT; Caspase-9 (527 bp): forward: CTTCTTCGCTTCATCTCCTG, reverse: AGGTCGTTCTTCACCTCCAC; and Cytc(535 bp): forward: GGAGGCAAGCATAAGACTGG, reverse: TCAATAGGTTTGAGGCGACAC. The reaction conditions were as follows: 94 °C preheating for 5 min; 30 cycles of 95 °C for 30 s (denaturation); 59 °C (β-actin), 53 °C (Caspase-3), 53 °C (Caspase-9), 59 °C (Cytc) for 30 s (annealing); and 72 °C for 1 min (elongation) followed by 72 °C for 5 min. The reaction mixture (20 μL) contained 2 μL of 10× PCR buffer, 2 μL of dNTP mixture, 1 μL of Mg^{2+}, 1 μL of template, 1 μL of primer 1, 1 μL of primer 2, 0.3 μL of Taq E, and 11.7 μL of ddH2O. The PCR products were separated on 2% agarose gel, stained with Gold View, scanned, and quantified using a gel imaging system and the Bio-Rad's Quantity One software. Data are reported as expression ratios, which were calculated from the expression of each target gene divided by the geometric mean expression of the housekeeper gene.

2.8. Tissue preparation, gel electrophoresis, and immunoblotting

The hippocampus in the brain was dissected at 4 °C and then stored at −80 °C until use. The tissue was thawed and homogenized in ice-cold 0.32 M sucrose (pH 7.4) containing protease inhibitor