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The effects of fluoride on neuronal function occurs via cytoskeleton damage and decreased signal transmission

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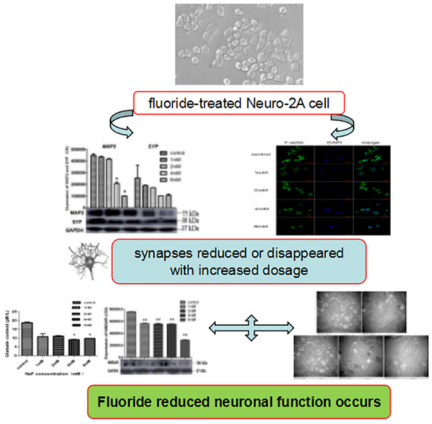
HIGHLIGHTS

- Synapse plasticity and morphology of the Neuro-2A cells changed after treatment with NaF.
- NaF exhibits dose-dependent toxicity in neurons.
- NaF exposure damages cytoskeleton of Neuron-2A cells.
- Expression of glutamate and its receptor (NMDAR) declined during NaF exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

It has been reported that fluoride exposure may cause serious public health problems, particularly neurotoxicity. However, the underlying mechanisms remain unclear. This study used Neuro-2A cells to investigate the effects of fluoride on the cytoskeleton. The Neuro-2A cells were exposed to 0, 1, 2, 4 and 6 mM sodium fluoride (NaF) for 24 h. Cell viability and lactate dehydrogenase (LDH) release were examined. It was observed that exposure to NaF reduced cell viability, disrupted cellular membrane integrity, and high levels of LDH were released. The observed changes occurred in a dose response manner. Morphologic observations showed that cell became rounded and were loosely adherent following exposure to NaF. Axon spines and normal features disappeared with high dose NaF treatment. The expression of MAP2 and synaptophysin decreased, particularly at 4 mM and 6 mM ($P < 0.05$) for MAP2. These results corroborate the morphologic observations. The content of glutamate and NMDAR (glutamate receptor) protein were assessed to help understand the relationship between synapses and neurotransmitter release using ELISA and Western-blot. Compared with the control, glutamate and NMDAR expression declined significantly at 4 mM and 6 mM ($P < 0.05$) group. Finally, the ultrastructural changes observed with increasing doses of NaF were: disappearance of synapses, mitochondrial agglutination, vacuole formation, and cellular edema. Taken together, NaF exposure disrupted cellular integrity

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and suppressed the release of neurotransmitters, thus effecting neuronal function. These findings provide deeper insights into roles of NaF in neuron damage, which could contribute to a better understanding of fluoride-induced neurotoxicity.

1. Introduction

The fluoride ion, derived from the element fluorine, is a very electronegative and reactive, thus it does not occur in nature (Das and Mondal, 2016). Fluoride is used industrially in a fluorine compound, the manufacture of ceramics, pesticides, aerosol propellants, refrigerants, glassware, and Teflon cookware (Peckham and Awofeso, 2014), However, repeated ingestion of fluoride over a prolonged period can result in toxic effects (fluorosis) in humans and animals (Dec et al., 2017). Excessive levels can disrupt many organs and systems. The most well known effects of fluorosis are on the skeleton and teeth, and neurotoxicity of fluoride has been confirmed (Qian et al., 2013; Dong et al., 2015). Chronic exposure to fluoride has been linked to learning disabilities and memory deficits (Jetti et al., 2016; Saxena et al., 2012; Liu et al., 2014). Sebastian reported a strong correlation between fluoride levels in drinking water and intelligence quotients of school children (Sebastian and Sunitha, 2015). On the cellular level, reduction of Nissl substance, swelling of mitochondria, dilation of the endoplasmic reticulum, and increased apoptosis has been observed in the neurons of rats with experimentally induced fluorosis (Liu et al., 2011). Significantly elevated levels of fluoride have been detected in water sources serving millions of people worldwide. The removal of excess fluoride from drinking water is not only difficult, but very costly as well (Lou et al., 2013). This is a troubling finding, as the presence of fluoride in water creates a public health hazard.

The development of mammalian nervous system is one of most complicated *in vivo*. Microtubule-Associated proteins (MAPs) play prominent roles in the development of neuronal cells. Members of the MAP family are best known for their stabilization of microtubules, as well as for their crucial roles in the regulation of microtubule networks in the axons and dendrites of neurons. MAP2/Tau proteins bind along the length and stabilize of microtubules by altering this dynamic behavior (Al-Bassam et al., 2002; Panda et al., 2003). *In vivo*, it has been shown that the MAP2 and Tau projection domains appear to be involved in the regulation of microtubule spacing within neurons (Chen et al., 1992). MAP2 plays important roles in the assembly of microtubules, growth processes, and communication between microtubules and other cellular components and organelles within the neuron (Chen et al., 2015). Such control over microtubule spacing might facilitate efficient organelle transport (Dehmelt and Halpain, 2004).

Synaptogenesis is arguably the most critical events in the development of the central nervous system. The majority of chemical synapses within the mammalian nervous system can be found in the presynaptic apparatus, the synaptic cleft, and the postsynaptic region. Synaptophysin (SYP) was one of the first proteins to be identified in the synaptic vesicles and accounts for about 7–10% of the total synaptic vesicle proteins (Hami et al., 2016). SYP is a 313-amino acid, 38 kDa protein that is found exclusively in presynaptic vesicles (Sinclair et al., 2015), and is commonly used as a biomarker for synaptic vesicles. Synaptic loss was first recognized in 1987 as a major pathological abnormality in Alzheimer's disease (Davies et al., 1987), and it is thought that this loss reflects a combination of not only neuronal loss, but synaptic degeneration as well.

In the present study, we set out to explore the effects of fluoride on neuron signal transmission among synapses. The results of this study provide a theoretical basis from which we can better understand the mechanisms leading to the toxic effects of fluoride.

2. Materials and methods

2.1. Cell culture and treated with NaF

Neuro-2A cells were purchased from IBCB (Insitute of Biochemistry and Cell Biology, China), and were maintained in Minimum Essential Medium (MEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FBS and 100 UmL^{-1} penicillin/streptomycin. The cells were incubated in a humidified, 37 °C, 5% CO_2 atmosphere. These cells were cultured for 72 h prior to exposing them to fluoride. The cells were exposed either 0, 1, 2, 4 and 6 mM final concentrations of NaF for 24 h. Cells were observed by light microscopy for changes in cellular morphology.

2.2. Detection of cell proliferation by MTT

Cells were seeded into 96-well plates at a density of 5×10^4 cells per well, and were cultured for 72 h. Cells were then incubated with fluoride for 24 h, at which point 30 μL MTT (5 mg/mL) was added to each well. The cells were then incubated for 4 h at 37 °C. The culture supernatants were then removed, and 100 μL DMSO was added to each sample to stop reaction, and then lysed for 10 min on a vortex. Optical densities were measured at 570 nm using a Multiscan Spectrum (Thermo Fisher, USA) and were used to calculate the number of viable cells, expressed as the percentage of control cells. The cells were then diluted to normalize the numbers for each sample in order to accurately assess the LDH response following fluoride exposure.

2.3. Cell staining

Stock solutions of phalloidin conjugates (Sigma-Aldrich, USA) were diluted in DMSO to a concentration of 0.5 mg/mL. Working dilutions were prepared in PBS (pH 7.2) yielding a final concentration of 5 μg/mL. DAPI was purchased from Solarbio (Solarbio, Beijing). Cells were washed with PBS, and fixed for 5 min with 4% paraformaldehyde solution, and then washed with PBS. Cells were stained with a phalloidin for 1 h at 37 °C in the dark. The cells were washed three times with PBS to remove unbound phalloidin. Cells were stained with DAPI for 5 min at room temperature. The samples were washed three times with PBS to remove unbound DAPI. Coverslips were mounted to glass slides using floursave mounting medium. Images were collected using the LSM 800 confocal LASER scanning microscope (ZEISS, German), with excitation wave lengths of 488 and 561 nm.

2.4. Western-blot analysis

Cells were seeded at 5×10^4 cells per well in a 6 well plate, and cultured for 72 h prior to treatment with either 0, 1, 2, 4 and 6 mM NaF for 24 h. Cells were harvested and collected by centrifugation at