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Potential Effects of Omega-3 (ω-3) Fatty Acids on Neurobehavior in a Murine Model of Acute Fluoride Toxicity

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Gladys ORTIZ-BARROSO[1], Denisse CALDERÓN-VALLEJO[2,3], David MASUOKA-ITO[4], Mayra MACEDO-MENDOZA[5], Tonatiuh BARRIOS-GARCÍA[5], Nayeli Amalinalli PULIDO-HORNEDO[3], Ricardo E RAMÍREZ-OROZCO[5*]

[1] Master Degree in Biomedical Research; Health Sciences Center, Autonomous University of Aguascalientes, México.

[2] Department of Physiology and Pharmacology, Center for Basic Sciences, Autonomous University of Aguascalientes, México.

[3] Department of Morphology, Center for Basic Sciences, Autonomous University of Aguascalientes, México.

[4] Research and Development Laboratory in Molecular Diagnosis and Biomaterials, Health Sciences Center, Autonomous University of Aguascalientes, México.

[5] Department of Nutrition, Nutrition Research Laboratory, Health Sciences Center, Autonomous University of Aguascalientes, México.

*** Corresponding author:**

Dr. Ricardo E Ramirez-Orozco
Department of Nutrition, Autonomous University of Aguascalientes, Av. Universidad #940, 20100, Aguascalientes, México
Phone: +52 (449) 434 3784
E-mail: dcmrero@gmail.com

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ABSTRACT

Purpose: Fluorosis is a toxicity generally associated with oxidative stress, and fluoride can affect central nervous system function. Given the antioxidant properties of omega-3 (ω-3), a protective effect may be possible.

Methods: A fluoride model was implemented in male BALB/c mice (n=10/group). Four groups were formed: Control, NaF (exposed to sodium fluoride at 12 mg/kg/day for 30 days), NaF + ω-3 (NaF: 12 mg/kg/day for 30 days + ω-3: 50 mg/kg/day for 45 days), and ω-3 (administration of 50 mg/kg/day for 45 days of ω-3). After the experimental period, behavioral tests were conducted to assess anxiety-like and depressive-like behaviors. Serotonin (5-HT) and brain-derived neurotrophic factor (BDNF) levels in the hippocampus were measured by ELISA (Enzyme-Linked Immunosorbent Assay).

Results: In behavioral tests, the ω-3 group spent more time in the closed arm of the maze (p=0.04) and had fewer entries into the elevated plus maze compared to the NaF group (p=0.01). In the forced swim test, the ω-3 group exhibited a lower percentage of time with high mobility compared to the NaF group (p=0.001) and the NaF + ω-3 group (p=0.03). The NaF groups had lower BDNF levels in the hippocampus compared to the control and ω-3 groups (p<0.0001).

Conclusions: Mice with fluorosis exhibited behavioral changes and decreased BDNF levels. ω-3 treatment did not restore BDNF levels but showed protective effects in some behaviors compared to NaF.

Keywords: *Fluorosis; Neurodegeneration; Depression; Anxiety*

INTRODUCTION

Fluoride (F) is essential for physiological functions, such as bone and dental health and metabolic processes.[1] Fluoride toxicity is caused by increasing plasmatic levels, usually by ingesting water, food, and dental products.[2] According to the World Health Organization (WHO), the allowable limit of fluoride in groundwater is 1.5 mg/L. Described clinical manifestations are in dental, skeletal, and non-skeletal tissues.[4] The alterations caused by dental and skeletal fluorosis have been described previously; however, the damage caused by non-skeletal fluorosis in soft tissues has yet to be thoroughly studied and clarified.

Non-skeletal fluorosis affects soft tissues, organs, and body systems such as renal, hepatic, gonadal, endothelial, endocrine, and neurological, causing multiple alterations and manifestations.[5] The pathological mechanisms of non-skeletal fluorosis are not fully known; however, it is attributed to cellular oxidation caused by high levels of reactive oxygen species (ROS), ending with cell death in different tissues. Additionally, the ability of fluoride to cross the blood-brain barrier may lead to nervous system alterations such as impaired cognitive processes, memory, and learning, psychiatric disorders (anxiety and depression), as well as neurodegenerative

diseases of great importance like Alzheimer's disease and multiple sclerosis.[2,5-9]

One of the most vulnerable brain structures to fluoride toxicity is the hippocampus. The neuroplasticity capacity of this tissue makes it more sensitive to internal and external stimuli, exposing it to damage that can affect the synaptic plasticity, neuronal survival, and dysfunction in these processes, which have been related to neurological and psychiatric disorders and diseases.[10] Neurological imbalanced functions may affect the secretion of some neurological function markers, such as serotonin (5-HT) and brain-derived neurotrophic factor (BDNF), leading to the appearance of behavioral disorders.[11-13]

The state of fluorosis is not usually identified and diagnosed in the population because its manifestations may need to be recognized correctly and promptly. Currently, there is no cure for fluorosis. Instead, treatments are implemented to reduce its harmful effects and complications; some therapies are based on calcium, vitamin C, and vitamin D supplementation.[2] In this context, diverse diet components have been studied to counter the side effects of this condition. In recent decades, Omega-3 (ω-3) fatty acids have attracted interest among clinicians because of their anti-inflammatory and neuroprotective benefits. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the two primary fatty acids in ω-3, are associated with neuroprotective mechanisms through their ability to modify the composition of lipid rafts by binding to the membrane. They also participate in neurogenesis and play a role in signaling pathways related to the innate immune response, thereby reducing apoptosis in the central nervous system (CNS). [14,15] In other hand, Al Badawi et al. demonstrated the potential therapeutic effects of omega-3 in mitigating fluoride-induced toxicity in the cerebellum on granular cells, Purkinje cells and neuroglia cells.[16]

In the same context, in neurological disorders like depression and anxiety, a deficiency of DHA and EPA is present, affecting the dopaminergic and serotonergic systems mainly; this is provoked by the change in the structure of the lipid membrane and its receptor functions.[17] Therefore, the study of nutraceuticals and their application could display the possible therapeutic effects of DHA and EPA on fluorosis state. The objective of this study was to evaluate the prophylactic effect of DHA and EPA on several physiological processes, such as 5-HT and BDNF levels and the influence in manifestations associated with anxiety and depressive-like behaviors in an acute fluorosis model in BALB/c mice.

MATERIAL AND METHODS

Animals and Ethical Considerations

All experiments were conducted using 10-week-old male BALB/c mice (n=40) and were kept in plastic cages within a pathogen-free environment and controlled temperature, under a 12-hour light cycle, and provided free access to food (LabDiet™ 5001 chow) and filtered water. Animals were handled according to the guidelines established by the Official Mexican Standard NOM-062-ZOO-1999: "Technical specifications for the production, care, and use of laboratory animals." The animals had an acclimation period (2 weeks) and were randomized into four groups: control group (n=10), Sodium fluoride (NaF) group (n=10), ω-3 supplementation (EPA/DHA) group (n=10), and NaF + ω-3 supplementation (EPA/DHA) group (n=10). A timeline of the research methods is depicted in Figure 1.

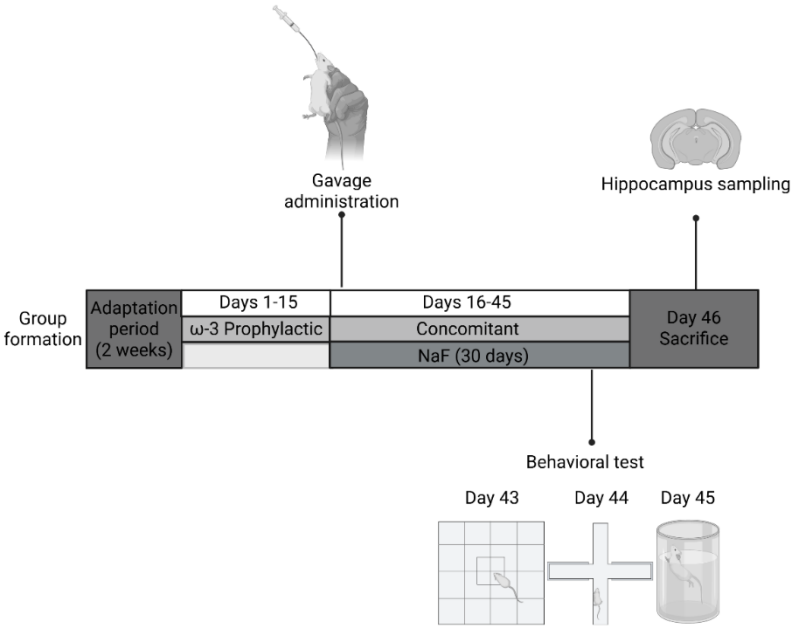

Figure 1. Timeline of the research method

Sodium fluoride and Omega-3 administration

The experimental groups were treated with NaF (12 mg/Kg/day for 30 days); the compound was administered by intragastric gavage for thirty consecutive days, and NaF was administered at the same time each day, between 10:00 and 11:00 a.m., using a volume of 100 µL per mouse and dissolved in distilled water. The fluoride model was corroborated and established in a previous *in vivo* study by examining the morphological and structural changes in kidneys and other tissues.[30] In the same way, ω -3 (Lýsi™ Omega-3 fish oil) supplementation was based on studies that demonstrated therapeutic effects (50mg/Kg/day for 45 days), the proportional EPA/DHA ratio considered was 2:1, respectively, ensuring the daily recommended intake in experimental groups[16]; supplementation began fifteen days before NaF exposure, completing forty-five consecutive days by the same route of administration.

Behavior assessment: Open Field Test (OFT), Elevated Plus Maze (EPM), and Forced Swim Test (FST)

In this study, anxiety-like behavior was evaluated, as well as those associated with depressive-like symptoms, since their relationship has been seen with alterations in brain morphology and biochemistry in states of fluoride toxicity.

Open Field Test

The OFT evaluated anxiety-like behavior using the PanLab™ Infrared Actimeter open-field (45 x 45 cm). Each mouse was placed in the center of the open field. Their activity was registered for 5 minutes using the PanLab™ software Actitrack to get the total distance traveled (cm), center distance traveled (%), periphery distance traveled (%), locomotion (cm3/s), stereotyped movements (cm3/s), and count of the vertical rearing events; between each trial, the surface was wiped with 70% ethanol to remove odors.

Elevated Plus Maze Test

The EPM was used for measuring anxiety-like behavior as well in a maze with two open arms (35 x 5 cm), two closed arms (35 x 5 cm) and a central platform (5 x 5 cm) on which the mice were placed one by one, to evaluate for 5 minutes the percentage of time they spent in the open arms, the closed arms and the center, just like the number of vertical rearing and head dips; between each trial, the surface was wiped with 70% ethanol to remove odors.

Forced Swim Test

The depressive-like behavior was assessed with the FST; in this test, each mouse was placed in a large graduated transparent cylinder tank with a 45.4 cm circumference and a 35 cm height, and the volume of water was established at 20.5 cm as a standard measurement in all tests, considering a height at which the mouse did not touch the bottom of the tank with its paws or tail. The water was maintained in a temperature range of 23-25°C and changed between each trial with clean water; the test was carried out for 7 minutes. During the evaluation, only the last five minutes are considered. The variables considered for this test were total activity (cm3/s), the percentage of time they spent immobile (%), low activity (%), regarded as the minimum movement to keep floating, and struggling (%), defined as high mobility to exit the cylinder. For evaluation, the PanLab™ Smart 3.0 software was used.

Markers of neurological function: 5-HT and BDNF

After the behavioral evaluation, the mice from the four groups were euthanized by an anesthetic overdose of sodium pentobarbital by intraperitoneal administration. To measure 5-HT and BDNF levels, the hippocampus was carefully removed from the brain and stored at −80°C. The tissue was homogenized in lysis buffer using a tissue homogenizer and then incubated at 4°C for 1 minute while shaking. The homogenate was centrifuged, and the supernatants were collected. Total concentrations of 5-HT and BDNF were measured by an ELISA (Enzyme-Linked Immunosorbent Assay) kit (Serotonin Research ELISA ab133053, Abcam™) and BDNF Research ELISA (LS-F2404, Biocompare™), respectively, according to the manufacturer's protocol.

Statistical Analysis

A descriptive and inferential analysis was carried out. The normality and homoscedasticity of the data distribution was analyzed with the Kolmogorov-Smirnov and Levene test, respectively; all data are presented as percentages, mean, and standard deviation (±SD). One-way ANOVA and the post hoc Tukey test were employed, and Kruskal-Wallis with post hoc Dunn's test was also applied for non-parametric data. The statistical software used was R 4.1.2 "Bird Hippie" and Prisma 8.0. A p-value <0.05 was considered statistically significant, denoted as *p<0.05, **p<0.01, ***p<0.001, and **** p<0.0001.

RESULTS

After acclimatizing, the weight was recorded weekly for all groups. The initial weights were not different between groups (Control: 24.6±1.2 g; ω-3: 23.7±2.0 g; NaF: 23.6±1.9 g; NaF + ω-3: 25.9±1.4 g), with no differences observed either at this stage or at the final weight recorded at week 14 of age, which did not represent a substantial change from the initial weight (Control: 27.0±2.4 g; ω-3: 26.2±2.3 g; NaF: 26.9±1.7 g; NaF + ω-3: 28.6±1.4 g). Behavioral tests were conducted at the end of the final week of administration.

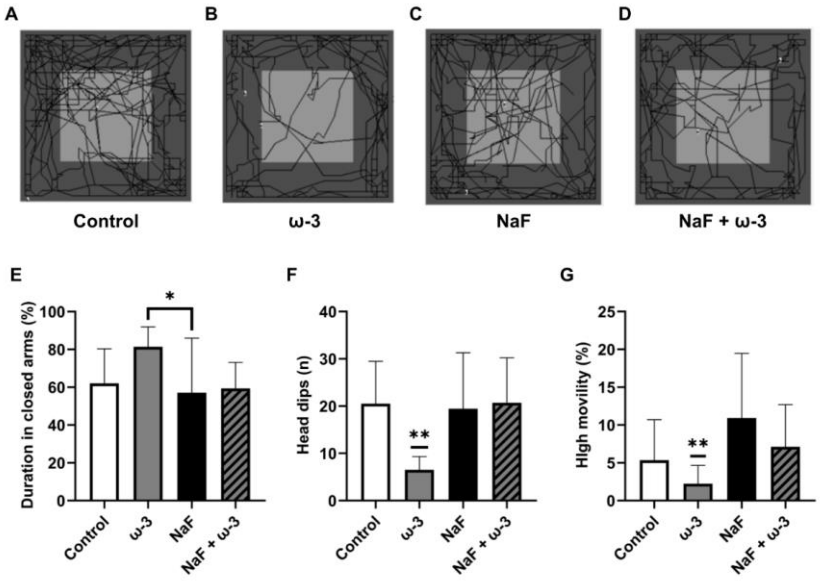

Figure 2. Anxiety-like behavior tests: Open-field test. Travel patterns in the field. A: Control, B: ω-3, C: NaF, D: NaF + ω-3. Elevated plus maze: E: Duration in closed arms (%), *: ω-3 vs NaF; F: Head dips counting (n), **: ω-3 group differed from the rest. Forced Swimming Test. G: High activity time (%), **: ω-3 vs NaF groups. (n= 40); *p<0.05 and **p<0.01

Sodium fluoride (NaF) and Omega-3 (ω-3) effect on exploration and locomotion activity

The first behavioral test applied was OFT, and no differences were observed from those with ω-3 treatment. Figure 2 (A, B, C, and D) shows examples of travel patterns in the field and the division of the inner and peripheral zones.

After 24 hours, the EPM was conducted, revealing differences concerning the percentage of time spent in the closed arms of the maze. The ω-3 group spent 81.4±10.4 of the test time in the closed arms, compared to the NaF group, which spent 57.1±28.8 (p = 0.04) (Figure 2E). Regarding anxiety-like behaviors, the number of head dips was lower in the ω-3 group, with 6.5±2.8 entries, compared to 20.5±8.9 in the control group (p = 0.01), 19.4±11.8 in the NaF group (p = 0.02), and 20.7±9.5 in the NaF + ω-3 group (p = 0.008) (Figure 2F).

The final test was the FST, where differences were observed in the percentage of time spent in high mobility during the test; the ω-3 group exhibited lower high mobility time 1.4±0.9 compared to the NaF group 10.9±8.5 (p = 0.001) and the NaF + ω-3 group 7.1±5.5 (p = 0.03), details can be reviewed in Figure 2G.

NaF acutely decreases BDNF secretion but not 5-HT

After conducting behavioral tests, the animals were sacrificed to extract the hippocampus to quantify the levels of BDNF (pg/ml). The BDNF levels found were 10.65 pg/ml in the NaF group and 18.01 pg/ml in the NaF + ω-3 group, both of which were lower than those in the control group at 49.97 pg/ml (p < 0.0001) and the ω-3 group at 48.91 pg/ml (p < 0.0001) (Figure 3A). The levels of 5-HT were also measured in the hippocampal homogenized; however, the difference between the groups was not significant (Figure 3B).

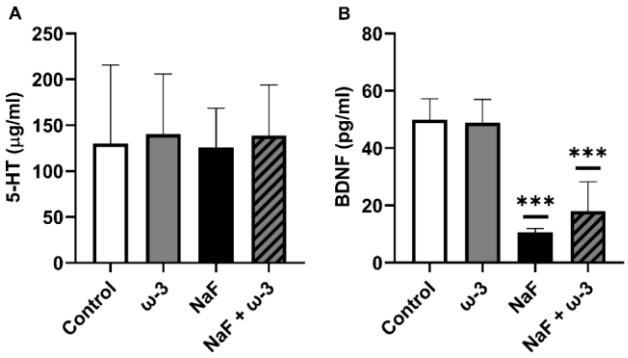

Figure 3. 5-HT and BDNF levels in hippocampus. A: 5-HT (µg/ml) levels no differences between groups. B: BDNF (pg/ml) levels, ***: NaF group differed from the Control and ω-3 groups, ***: NaF + ω-3 group differed from the Control and ω-3 groups. (n=40); *p<0.05, **p<0.01, and *** p<0.001.

DISCUSSION

This study evaluated the effects of NaF administration and the potential protective role of ω-3 supplementation on anxiety-like behavior and stress coping in adult male BALB/c mice. Several articles have demonstrated a central nervous system development with cognitive, behavioral and memory disruption in animals with sodium fluoride exposure.[18]

Despite using three behavioral tests, our results did not yield conclusive evidence regarding either fluoride-induced behavioral changes or the prophylactic effect of ω-3. One possible explanation is the limited duration of exposure; the 30-day administration period may not have been sufficient to elicit significant neurobehavioral alterations. Li et al.[19], for instance, reported anxiety- and depression-like behaviors in mice only after 120 days of exposure to 68 mg/L of fluoride ion, highlighting the relevance of exposure chronicity.

Interestingly, mice supplemented with ω-3 exhibited reduced exploratory behavior, which may indicate enhanced habituation to novel environments. This observation aligns with prior findings suggesting that animals with ω-3-deficient diets show increased locomotor activity, whereas ω-3-enriched diets are associated with calmer behavioral profiles.[20] Chalon et al.[21] demonstrated that ω-3 deficiency affects the frontal cortex, an area involved in motivation and emotional regulation and leads to dopaminergic and serotonergic imbalances. Similarly, Umezawa et al.[22] found increased hyperactivity and exploratory behavior in animals subjected to ω-3-deficient diets. These findings support the hypothesis that ω-3 supplementation modulates behavioral responses through its influence on brain function and structure.

Regarding fluoride exposure, our results are consistent with previous studies reporting NaF-induced hyperactivity and spatial memory deficits in rodents. Such effects have been linked to mitochondrial dysfunctions, including impaired fission and fusion processes, reduced autophagy, and increased apoptosis. Ren et al.[23] showed that fluoride accumulates in brain regions such as the hippocampus, cerebral cortex, and cerebellum areas involved in learning, emotion, and behavior. The absence of strong behavioral differences in our study could be due to the short exposure period, as previous reports of behavioral impairments primarily involve chronic fluoride administration.

Furthermore, we evaluated neurochemical markers associated with emotional regulation and cognitive function. Our results revealed significant differences in BDNF levels: the Control and ω-3 groups exhibited higher BDNF expression than the NaF and NaF + ω-3 groups. This finding aligns with Niu et al.[24], who demonstrated dose-dependent decreases in hippocampal BDNF expression following NaF exposure. Although ω-3 co-administration slightly elevated BDNF levels in fluoride-treated mice, the increase was not statistically significant, indicating limited neuroprotective effects under acute exposure conditions. A meta-analysis demonstrated that ω-3 supplementation significantly increases serum BDNF levels, with greater effectiveness observed in protocols lasting less than 10 weeks and with doses exceeding 2000 mg/day.[25] A study by Molendijk et al.[26] found that individuals carrying the Val66Met polymorphism in the BDNF gene exhibited reduced hippocampal volume; while, research by Cutuli et al.[27] showed that ω-3 supplementation helped counteract the reduction of gray matter volume in the hippocampus.

Hippocampal 5-HT levels did not differ significantly between groups; however, a downward trend was observed in the NaF group. This observation echoes the results of Cao et al.[28] who found significantly reduced cortical serotonin concentrations after eight weeks of fluoride exposure. Although our study's shorter protocol may have precluded such effects from emerging, the trend warrants further investigation. Additionally, existing literature suggests that ω-3 deficiency compromises serotonergic signaling and receptor functionality, contributing to behavioral disturbances and impaired brain plasticity.

Fluoride exerts neurotoxic effects through several interconnected mechanisms that compromise the integrity and function of the nervous system. Firstly, fluoride enhances oxidative stress by increasing the production of ROS, which leads to lipid peroxidation, DNA damage, and neuronal apoptosis. Secondly, fluoride disrupts mitochondrial function, impairing cellular respiration and energy metabolism essential for neuronal survival. Additionally, fluoride interferes with calcium homeostasis and neurotransmitter systems, particularly acetylcholine, altering synaptic transmission and neural communication. These changes are often accompanied by neuroinflammation, mediated by activated microglia and elevated pro-inflammatory cytokines. Furthermore, chronic exposure to fluoride has been shown to disrupt the blood-brain barrier, facilitating the entry of additional neurotoxic agents into brain tissue and amplifying neural damage.[29]

Mitochondria, which play essential roles in energy production and apoptosis regulation, are particularly vulnerable to fluoride-induced oxidative stress, leading to DNA damage and disrupted membrane potential, ultimately resulting in apoptosis. Animal studies show that chronic fluoride exposure results in structural mitochondrial abnormalities, altered fission/fusion processes, and reduced mitochondrial DNA (mtDNA) levels, particularly in the hippocampus. Fluoride also promotes neuroinflammation, evidenced by increased

markers such as COX2, VEGF, and HSP-70 in rat models, in a dose-dependent manner. Additionally, fluoride alters neurotransmitter levels, elevating serotonin, glutamate, and histamine while reducing acetylcholine and dopamine, with concurrent structural damage in neurons.[18]

Key molecular pathways disrupted by fluoride include the PGC-1α/NRF1/TFAM axis involved in mitochondrial biogenesis, and the GSK-3β/β-catenin signaling pathway, which is critical for neurogenesis. Fluoride-induced activation of p53 and inhibition of SIRT1 further exacerbate apoptotic processes. These findings collectively underscore fluoride's potential to impair neuronal integrity through mitochondrial and molecular dysregulation. DHA and EPA are long-chain ω-3 PUFAs that play essential roles in the CNS. DHA is a major structural component of neuronal membranes, crucial for maintaining membrane fluidity, neurogenesis, synaptogenesis, and signal transduction. It supports cognitive function and protects against neurodegenerative diseases. EPA, although present in lower concentrations in the brain, contributes to CNS health primarily through its anti-inflammatory properties, modulating cytokine production and reducing neuroinflammation. Together, DHA and EPA support neurodevelopment, protect neural tissue, and may improve mood and behavior by influencing neurotransmission and inflammatory processes.[25]

Overall, while this study found no clear behavioral differences under acute NaF exposure, it demonstrated alterations in BDNF levels that may precede or underlie future behavioral impairments. The results suggest that ω-3 supplementation alone may not counteract NaF-induced neurotoxicity within a 30-day period. Given the complex interplay of variables influencing behavioral outcomes including exposure duration, dosage, and test sensitivity, future research should incorporate chronic exposure models, broader neurochemical analyses, and complementary behavioral paradigms to more robustly assess neurotoxic effects and potential protective interventions.

CONCLUSIONS

Fluoride toxicity remains a global public health concern, with few available preventive strategies. In this study's acute fluorosis model, mice exhibited behavioral changes such as hyperactivity, possibly linked to anxiety-like behavior. Fluoride-induced neurotoxicity has been associated with such alterations, alongside reduced BDNF levels and hippocampal dysfunction. Although ω-3 treatment did not restore BDNF levels, it demonstrated potential protective effects in certain behaviors. Acute NaF exposure alone did not produce significant behavioral changes, suggesting the need for chronic exposure models and more sensitive behavioral assessments. Future research should also examine learning and memory functions, previously reported as impaired by fluorosis, as ω-3 may have beneficial effects on these cognitive domains.

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DISCLOSURE OF FINANCIAL AND NON-FINANCIAL RELATIONSHIPS AND ACTIVITIES AND CONFLICTS OF INTEREST

The authors declare no conflict of interest related to this study.

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