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Cover Page Footnote
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THE EFFECTS OF FLUORIDE IONS ON NEUROMUSCULAR ACTIVITY AND REGENERATION IN *Dugesia tigrina*

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ABSTRACT

Fluoride ions have been implicated in numerous nervous system pathologies, though the association of these ions with these conditions has been controversial. The purpose of this research is to determine the effects that fluoride ions have on the nervous system development and function in planarians as a simple model for fluoride-nervous system interactions. In the first set of experiments, planarians were exposed to one of four concentrations of sodium fluoride: 0 mM, 1 μM, 1 mM, and 5 mM. In the second set of experiments, planarians were bisected and exposed to 1 mM of NaCl and NaF for regeneration purposes. Regenerated planarians were then stained using Luxol staining methods to determine any physical effects of fluoride on nervous system regeneration. The results showed that swimming activity decreased with increasing levels of fluoride ions, suggesting an inhibition of nervous system activity by fluoride. Additionally, planarians that regenerated in the presence of NaF lacked key structural nervous system components, suggesting a negative impact on the system. It was concluded that fluoride ions negatively impact nervous system activity and development in planarians and possibly in similar organisms.

Keywords: planarians, fluoride, halogen, nervous

INTRODUCTION

Fluoride ions have been added to municipal water sources for public consumption since the 1940s. Fluoride was found to have a major beneficial effect by reducing the prevalence of cavities in humans (Mullen 2005). Physiologically, fluoride ions increase the rate of remineralization, or strengthening, of tooth enamel, thus leading to lower prevalence of dental caries. Multiple countries started artificially fluoridating their water after these ions proved to reduce the tooth decay (Mullen 2005), and most literature has shown that fluoride ions can provide beneficial effects to our dental health. For example, it has been noted that after being exposed to fluoridated water, there was a significant reduction of caries in children (McDonagh et al. 2000). The same research also shows a change in the dental health of people with missing, decayed, and filled primary or permanent teeth.

Shortly after the initiation of fluoridation of water, scientists and politicians became concerned about unintended health effects (Fawell 2006). Fluorosis, a relatively cosmetic condition that stains tooth enamel, was one of the first conditions attributed to fluoride supplementation (Mullen 2005). However, since then, more serious conditions have been linked, although controversially, to fluoride consumption, including osteosarcoma (Levy and LeClerc 2012), hormonal impairment (Sharma et al. 2009), and

even memory loss and brain dysfunction (Chase and Isaacson 1992). Therefore, researchers continue to investigate these potential links in order to completely understand the positive and negative effects that fluoride may have in the body. In this paper, the neurological effects are the focus, but testing the effects of fluoride on nervous system tissue can be challenging.

Other mammalian models have been used to investigate the effects of fluoride on biological systems. Mullenix et al. (1995) reported that fluoride ions produced neurotoxicity in a dose dependent manner within Sprague-Dawley rats, and that this toxicity affected multiple portions of the brain's physiology. Additionally, Antonio et al. (2016) determined that fluoride also has renal toxicity in mammalian systems though, admittedly, it occurs in nonphysiological, high concentrations. This is important, because the complexity of the mammalian system may prevent the direct measurements of flouride's effects.

In order to study the direct effects of fluoride ions on the nervous system, it is important to find a model organism that is simple, yet functionally comparable to the human nervous system. Planarian (*Dugesia*) flatworms have many features that make them suitable models for our purposes. These worms have been used historically to study nervous system activity, such as memory and behavior (Hartry et al. 1964; Shomrat and Levin 2013; and others), and modifiers of that activity, such as psychoactive agents (Pagán et al. 2009; Raffa and Valdez 2001). In this study planarians were used as a model to investigate the effects of fluoride ions on the nervous system activity and regeneration. It is hypothesized that fluoride will inhibit nervous system activity, as measured by their innate antiphototropic swimming response, and disrupt the regeneration of nervous system structures in planarians.

MATERIALS AND METHODS

Materials

Planarian worms (*Dugesia tigrina*) were purchased from Carolina Biological Supply Company (Burlington, North Carolina). Petri dishes were purchased from Fisher Scientific (Waltham, Washington). Sodium fluoride salt was purchased from Sigma-Aldrich Corporation (St. Louis, Missouri). Luxol staining kits were purchased from IHC World (Woodstock, Maryland). Other common laboratory supplies (transfer pipettes, water, and measurement grids) were supplied by the Department of Natural and Forensic Sciences at Albany State University.

Acute Toxicity Assay

Four petri dishes were labeled as 0 M (control), 1 μM, 1 mM, and 5 mM. The labeled dishes contained the respective concentrations of NaF solution. Using a transfer pipette, 10 planarian worms were transferred to each petri dish. The worms were then incubated for 24 hours. After incubation the number of live worms was recorded. Death in planarians was determined by disintegration of the head region, which is the standard indicator (Pagán et al. 2009). Assays were conducted in duplicate to ensure reproducibility.

Motility Assay

Worm activity was measured in terms of motility events, or the number of grid units

crossed, within a 5 min period (Pagán et al. 2009). The movement of the planarians (n = 10 per group) was recorded under four different conditions.

1. Control, which did not contain any NaF solution.
2. Immediate exposure in which we placed the worms in 5 mM NaF solution and started observing their motility without an incubation period.
3. Prolonged exposure with incubation of the worms in 5 mM NaF solution for 45 min.
4. Prolonged exposure with 24 hours incubation.

Assays were conducted in duplicate to ensure reproducibility.

Regeneration Assays and Tissue Staining

The planarians were fed boiled egg yolk, and those that consumed food were selected for the study. Twenty planarians were then dissected using transverse sections, and the heads and tails were placed in one of three experimental conditions: spring water (control), 1 mM NaCl, and 1 mM NaF. NaCl was used as a control to account for other halogens and to account for sodium within the results. In each dish, four dissected planarian parts were added, and they were separated based on whether the starting tissue was from the head or the tail region of the worm. The worms were incubated at room temperature (~20°C) for 7 days. On day 7, we observed the planarians to monitor regeneration and swimming under a dissecting microscope. On day 12, the planarians were placed on slides to be observed under a dissecting microscope to ensure regeneration. After observing them under the microscope, the slides were then placed in a freezer to dry out the planarians for staining, and subsequently stained using Luxol staining methods (Klüver and Barrera 1953; Lockhart and Reers 1962). Assays were conducted in duplicate to ensure reproducibility.

RESULTS

Toxicity

The acute toxicity assays showed little effect of 5 mM NaF and less on the viability of the planarians. In each experimental condition, the planarians were able to survive after 24 hour incubation. However, there were some qualitative effects denoted in terms of the motion of swimming, which was intermittent in the presence of NaF, and a visible reduction in the antiphototropic light response (Table I), which was investigated further in the motility assays (see Figure 1).

Table I. Qualitative results of acute toxicity assays. No quantitative results were obtained though, qualitatively, NaF has an effect on the phototropic response of the planarians and the continuity of swimming activity.

	NaF Cytotoxicity Assay (n = 10)		
Concentration	Cephalization	Swim Activity	Antiphototropic Response
0 M (Control)	Yes	Continuous	Normal
1 μm	Yes	Intermittent	Normal
1 mM	Yes*	Intermittent	Reduced
5 mM	Yes	Intermittent	Reduced

*Reduction of cephalization was seen in one animal of this particular sample, but not to the extent of death.

Motility

There was not a significant difference between the control condition and the individuals with no incubation. As seen in Figure 1, the swimming activity of the planarians in both control conditions (no NaF) and no incubation (5 mM NaF) did not show a statistically significant difference at the conclusion of the 5 min time period. The 45 min and 24 hour incubations together showed a statistically significant decrease from the no incubation group, and thus showed a reduced motility. Figure 1 shows that the planarians exhibited reduced motility only after the incubations and not after the immediate exposure to NaF. Figure 1 highlights the difference between the motility after immediate exposure and after the two incubation periods.

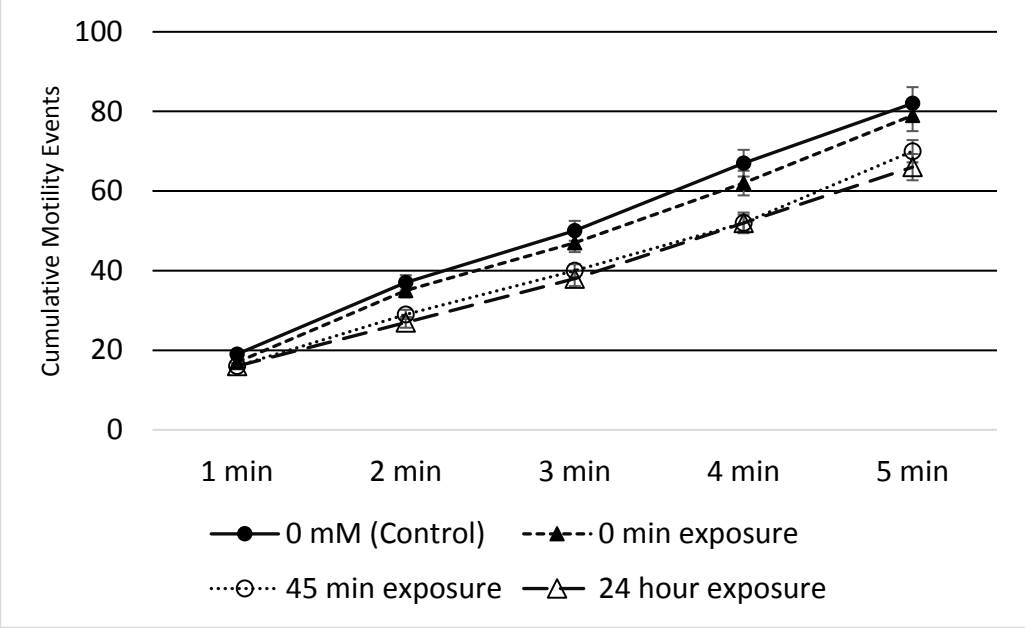

Figure 1. Cumulative motility events for four NaF exposure conditions. The four conditions include a control at 0 M (square), no incubation to 5 mM NaF (diamond), 45 min incubation (triangle), and 24 hour exposure (circle). There is a statistically significant difference between immediate exposure and the incubation samples, but not between the control and the immediate exposure NaF treatment. $P < 0.05$.

Regeneration

In these pictures, it was determined whether the "neural ladder" (shown by an arrow) has regenerated properly or not in the planarians, and this served as an indication of nervous system regeneration.

The control planarian samples regenerated normally. The neural ladder, as seen in Figure 2a, developed completely in both the head and tail regions, signifying normal structural regeneration. Similarly, the planarians exposed to 1 mM NaCl regenerated normally, with recephalization and the nervous ladder visible, though less defined when compared to the control (Figure 2b). In Figure 2c, the development of the neural ladder has been inhibited, indicating that the presence of fluoride has a pronounced negative effect on the development of the regenerated nervous system of planarians compared to the control and NaCl treatment.

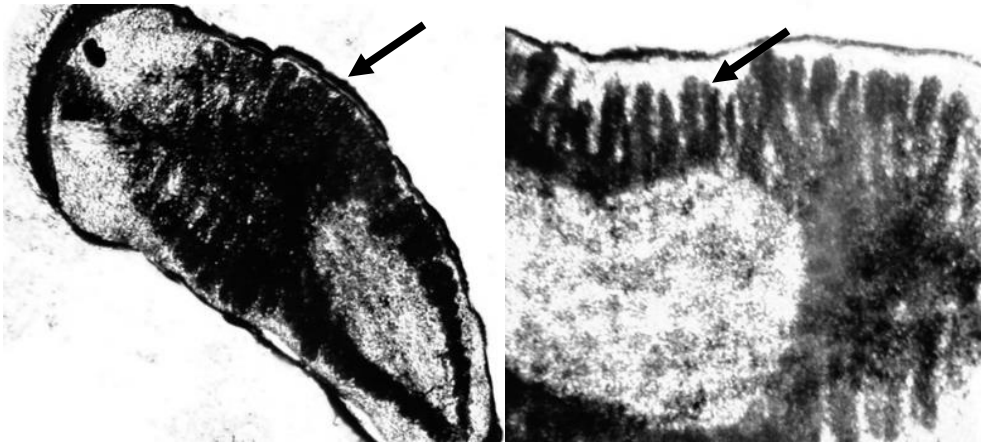

Figure 2a. Regeneration of the planarian nervous system with spring water (control); head (left) and body (right). The arrows indicate that the nervous system has regenerated and developed into the ladder structure indicative of planarians. Additionally, recephalization (head development) indicates normal regeneration.

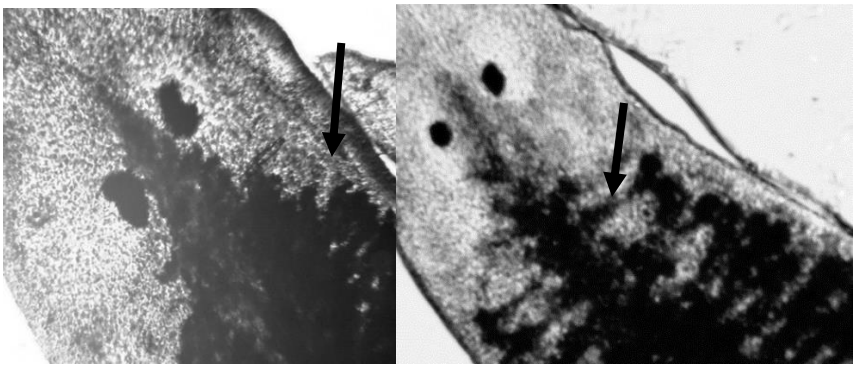

Figure 2b. Regeneration of the planarian nervous system in 1 mM sodium chloride. The arrows show the ladder-like structure of the nervous system after regeneration.

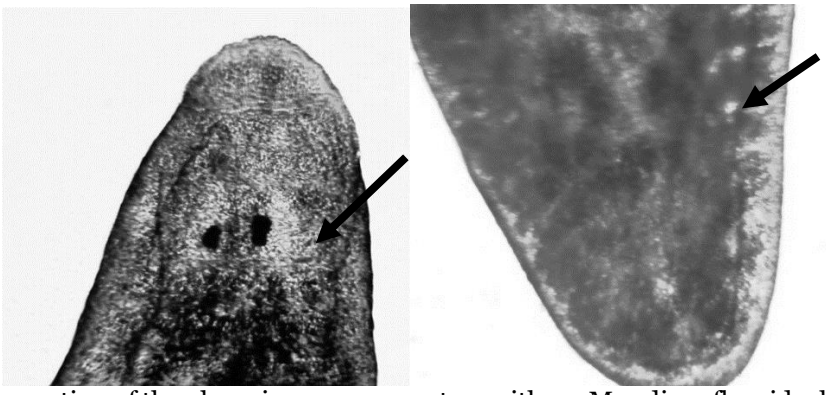

Figure 2c. Regeneration of the planarian nervous system with 1 mM sodium fluoride; head (left) and body (right). The arrows indicate that the nervous system has not regenerated into the ladder structure indicative of normal planarian physiology.

DISCUSSION

The results presented here demonstrate a negative effect of fluoride on both the swimming activity and regeneration of planarians as compared to spring water and sodium chloride. These observations indicate two key things.

First, the swimming activity assays indicate that the inhibitory effect of NaF, and subsequently fluoride ions, on the nervous system may be time dependent. That is, when incubated in a fluoride solution for longer periods of time, the swimming speeds will decrease. There is an approximately 13% decrease in swimming speed after 45 min of incubation, yet this value does not decrease significantly over 24 hours. Therefore, for the purposes of planarians, 45 min appears to be the time period where the maximum effect against the nervous system is achieved.

Secondly, the regeneration assays demonstrate that there is a structural change in the physiology of the planarian nervous system when planarians are allowed to regenerate in the presence of NaF. This does not appear to be due to sodium ions, as sodium chloride incubation did not produce similar structural changes in neural ladder development. Additionally, this suggests that chloride did not cause this effect, which suggests that these effects are likely not indicative of all halogens. While this is not definitive for other halogen ions, it does suggest that fluoride is toxic to the growth and development of newly formed nervous tissue.

Fluoride consumption is still very controversial, as the complete benefits and potential negative effects are still being investigated. The observations presented in this paper provide a simple view of the direct effects of fluoride on nervous system tissue and planarian behavior. However, to fully examine these effects in human beings, researchers have to account for other physiological properties of the human body that are absent from the planarian system, such as skeletal deposition of fluoride, renal filtration, and other mechanisms for processing these ions. On the other hand, these results may speak to the larger problem of long term consumption of fluoride and the effects that can occur if fluoride is allowed to bioaccumulate within an organism. Bioaccumulation has been documented in multiple classes of animals, including invertebrates (Buse 1986; Carmago 2003), small mammals (Vani and Reddy 2000; Boulton et al. 1994), and humans (Jha et al. 2011; Luke 2001; and others). In many of these reports, fluoride accumulation can be detected in muscle and nervous system tissue, which may negatively impact the activity of these two physiologically linked systems over time. More research is needed in both simple animal models and in human clinical tests to determine the exact nature of fluoride's effects in the human body, and to characterize the effects of fluoride within humans in both long-term and short-term exposures.

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